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Dynamique des principales maladies transmises par les moustiques et de leurs vecteurs en Indonésie

Dynamic of the main mosquito-borne diseases and their vectors in Indonesia

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List of abbreviations

ACT	Artemisinin combination therapy
ALF	Alfuy virus
ARDS	Acute respiratory distress syndrome
AROA	Aroa (a name of virus)
APOI	Apoi (a name of virus)
AURAV	Aura virus
BAG	Bagaza (a name of virus)
BANV	Banzi virus
BB	Bukalasa bat (a name of virus)
BC	Batu cave (a name of virus)
BEBV	Bebaru virus
BFV	Barmah forest virus
BI	Breteau index
BOUV	Bouboui virus
BSQ	Bussuquara (a name of virus)
CABV	Cabassou virus
CFR	Case-fatality rate
CHIKV	Chikungunya virus
CI	Container index
CIV	Carey islan virus
CM	convoluted membrane
CoxI	Cytochrome oxidase subunit I
CPC	Cacipacore (a name of virus)
CR	Cowbone ridge (a name of virus)
CRP	C-reactive protein
CSA	Chondroitin sulfate
DB	Dakar bat (a name of virus)
DDT	Dichlorodiphenyltrichloroethane
DENV	Dengue virus
DHF	Dengue Haemorrhagic Fever
dsRNA	double-stranded RNA
DSS	Dengue shock syndrome
EB	Entebbe bat (a name of virus)
ECSA	East Central and South African
EEEV	Eastern equine encephalitis virus
EHV	Edge Hill virus
ELISA	Enzyme-linked immunosurbance assays
EVEV	Everglades virus
FETBE	Far eastern tick borne encephalitis (a name of virus)
FMV	Fort morgan virus
GGY	Gadgets gully (a name of virus)
HBI	Human Blood Index
HI	Hemagglutination inhibition
HI	House index
HJV	Highlands J virus
HVR	hypervariable region
ICAM-1	Intercellular adhesion molecule-1

IFA	Indirect immunofluorescence assays
IFN-g	Interferon-g
IGU	Iguape (a name of virus)
IL-10	Interleukin-10
ILH	Ilheus (a name of virus)
IOL	Indian ocean lineage
IR	Incidence rate
IRS	Indoor residual spraying
ITV	Israel Turkey meningoencephalomyelitis virus
JE	Japanese encephalitis
JEV	Japanese encephalitis virus
JUGV	Jugra virus
JUT	Jutiapa (a name of virus)
KAD	Kadam (a name of virus)
KEDV	Kedougou virus
KFD	Kyasanur forest disease (a name of virus)
KOK	Kokobera (a name of virus)
KOU	Koutango (a name of virus)
KUNV	Kunjin virus
LI	Louping ill (a name of virus)
LF	Lymphatic Filariasis
LGT	Langat (a name of virus)
LLINs	Long-lasting insecticide-treated betnets
MAYV	Mayaro virus (a name of virus)
MDPV	Mosso das pedras virus
MICA	major histocompatibility complex class I chain-related protein A
MICB	major histocompatibility complex class I chain-related protein B
MIDV	Middleburg virus
MML	Montana Myotis Leucoencephalitis (a name of virus)
MNt	Microneutralization
MOD	Modoc (a name of virus)
MoH	Ministry of Health
mtDNA	mitochondrial DNA
MUCV	Mucambo virus (a name of virus)
MVE	Murray Valley Encephalitis
NDUV	Ndumu virus (a name of virus)
NEG	Negishi (a name of virus)
NHP	Non-human primate
NJL	Naranjal (a name of virus)
NTA	Ntaya (a name of virus)
NTD	neglected tropical diseases
OHF	Omsk haemorrhage fever (a name of virus)
ONNV	O'nyong-nyong virus
ORF	Open reading frame
PBB	Phnom Penh bat (a name of virus)
Pf	*Plasmodium falciparum*
*Pf*API	*Plasmodium falciparum* Annual Parasite Incidence
*Pf*EMP-1	*Plasmodium falciparum* erythrocyte protein-1
*Pf*PR	*Plasmodium falciparum* parasite rate
PIXV	Pixuna virus

POTV	Potiskum virus
POW	Powassan (a name of virus)
Pv	*Plasmodium vivax*
PvPR	*Plasmodium vivax* parasite rate
RAPD	Random amplification of polymorphic DNA
RB	Rio bravo (a name of virus)
RdRp	RNA-dependent RNA polymerase
RDT	Rapid diagnostic tests
RF	Royal farm (a name of virus)
RFLP	Restriction fragment length polymorphism
RIG-I	retinoic acid-inducible gene
RLR	RIG-I like receptors
RNV	Rio negro virus
ROC	Rocio (a name of virus)
RR	Ross River
RSSE	Russian spring and summer encephalitis (a name of virus)
RVE	Rift Valley Encephalitis
SABV	Saboya virus
SEPV	Sepik virus
SFV	Semliki forest virus
SIN	Sinbis virus
SLEV	St Louis encephalitis virus
SOF	Sofjin (a name of virus)
SOK	Sokoluk (a name of virus)
SP	San perlita (a name of virus)
SPDV	Salmon pancreas disease virus
SPO	Spondweni (a name of virus)
ss+RNA	single-stranded positive-sense RNA
ss-RNA	single-stranded negative sense RNA
SRE	Saumarez reed (a name of virus)
STR	Stratford (a name of virus)
SV	Sal vieja (a name of virus)
TGF-β	transforming growth factor β
TLR	Toll-like receptor
TMU	Tembusu (a name of virus)
TNF-α	tumor necrosis factor-α
TONV	Tonate virus
TROV	Trocara virus
TYU	Tyuleniy (a name of virus)
UGSV	Uganda S virus
UNAV	Una virus
USU	Usutu (a name of virus)
VCAM-1	Vascular cell adhesion molecule-1
Ve	Vesicle membrane
VEEV	Venezuelan equine encephalitis virus
WEE	Western wquine encephalitis (a name of virus)
WESSV	Wesselsborn virus
WHAV	Whataroa virus
WHO	World Health Organization
WNV	West Nile Virus

Vp	Vesicle packets
YAO	Yaounde (a name of virus)
YF	Yellow fever (a name of virus)
ZIKV	Zika virus

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Résumé

L'Indonésie est un grand archipel situé entre les océans Indien et Pacifique. Il est le rendez-vous des deux zones biogéographiques que sont les régions asiatiques et australasiennes. En raison de sa géographie distincte et variée, ce pays est reconnu comme l'un des pays ayant la plus grande biodiversité dans le monde avec de nombreuses espèces endémiques, y compris parmi les moustiques. Au total, 457 des 3 567 espèces de moustiques Culicidae, répertoriées dans le monde, ont été identifiées en Indonésie. Certaines espèces sont responsables de la transmission de maladies, dont au moins 46 espèces de moustiques signalées comme étant des vecteurs d'agents pathogènes humains en Indonésie. Jusqu'à présent, 13 maladies transmissibles par les moustiques ont été signalées dans la population humaine de ce pays, dont le paludisme, la filariose lymphatique, la dengue (DEN), l'encéphalite japonaise (JE), l'encéphalite de la vallée de Murray (MVE), Zika (ZIK), Kunjin, le virus du Nil occidental (WNV), la Edge Hill, le chikungunya (CHIK), le Sinbis, le Getah et la Ross River. De toutes ces maladies transmises par les moustiques, la dengue, le paludisme, l'encéphalite japonaise et le chikungunya sont les plus importantes en Indonésie car leur impact sur la santé publique au cours des deux dernières décennies a été majeur. Les moustiques vecteurs des agents pathogènes concernés appartiennent principalement à trois genres, *Anopheles, Aedes* et *Culex.*

Dans le cadre de la réponse nationale visant à prévenir et à contrôler la propagation des principales maladies transmises par les moustiques, il est essentiel de comprendre la dynamique de transmission de ces maladies, qui doit être fondée sur des données mises à jour et précises. L'objectif de notre étude était de comprendre la dynamique des principales maladies transmises par les moustiques afin de renforcer et optimiser le système de surveillance en Indonésie. Nous avons également étudié la diversité et la phylogénie de certains vecteurs du paludisme et leur rôle dans la transmission de cette maladie. Les objectifs spécifiques de recherche étaient les suivants : 1) Mieux comprendre l'épidémiologie de l'encéphalite japonaise et sa transmission ; 2) Identifier les caractéristiques génétiques des flavivirus, en particulier ceux de l'encéphalite japonaise provenant de moustiques capturés sur le terrain ; 3) Décrire la diversité, la phylogénie et l'importance de certains vecteurs du paludisme ;

4) Evaluer la pertinence des indices entomologiques officiels pour et le risque de transmission de la dengue ; 5) Analyser l'efficacité des méthodes actuelles de surveillance vectorielle de la dengue en Indonésie ; 6) Décrire la variabilité génétique d'*Aedes aegypti* (Diptera : Culicinae), vecteur des virus de la dengue, du chikungunya et de la fièvre jaune ; et 7) Analyser l'épidémiologie moléculaire de la transmission du virus du chikungunya en Indonésie. Un certain nombre d'enquêtes sur le terrain et d'examens systématiques ont été utilisés pour atteindre ces objectifs.

Les études réalisées dans le cadre de cette thèse concernent la dynamique des principales maladies transmises par les moustiques en Indonésie, en mettant l'accent sur l'épidémiologie de l'encéphalite japonaise (JE) et la distribution de ses vecteurs (Chapitre 2). Le Chapitre 3 porte sur la diversité des espèces d'anophèles et les implications pour le contrôle du paludisme. Le Chapitre 4 décrit la diversité génétique des vecteurs de la dengue, les méthodes de surveillance vectorielle en Indonésie, et les indices entomologiques pour évaluer le risque de transmission.

L'encéphalite japonaise a été signalée comme une maladie importante transmise par les moustiques depuis qu'elle a été identifiée pour la première fois en Indonésie en 1960. Bien que JE puisse représenter une menace majeure pour la santé publique depuis longtemps en Indonésie, étonnamment, les études pour comprendre les facteurs qui jouent un rôle dans la transmission du virus et ses facteurs de risque, sont encore très limitées. Dans ce Chapitre 2, notre étude de JE fournit des informations sur l'épidémiologie de cette maladie en Indonésie. Notre étude a révélé que la JE a été détectée dans tout l'archipel indonésien avec des cas humains identifiés dans au moins 29 des 34 provinces. Des cas de JE chez des voyageurs venus en Indonésie ont également été signalés. Les facteurs de risque d'infection par le virus de JE (JEV) chez les voyageurs varient selon la destination, la durée du séjour, l'itinéraire, l'activité et l'hébergement. Une surveillance sentinelle et des activités de recherche ont été menées en Indonésie, mais des rapports réguliers sur la JE n'ont pas faits dans toutes les provinces. L'une des raisons de l'absence de rapports nationaux de surveillance de routine de la JE est la difficulté à effectuer un diagnostic des cas de JE au niveau hospitalier en raison du coût élevé de la logistique opérationnelle. Par conséquent, les données sur le nombre de cas et la charge de la maladie sur la population, comme base de mise en œuvre du programme de vaccination, ne peuvent pas être déterminées avec

précision au niveau national. Parmi les 9 espèces de moustiques qui ont été confirmées comme étant vecteurs de JEV, *Culex tritaeniorhynchus* est considéré comme le vecteur le plus important en Indonésie. Bien que les porcs aient servi d'amplificateur principal de JEV en Indonésie, d'autres vertébrés, tels que le bétail, les chèvres, les chevaux et les chiens étaient également positifs aux anticorps de JE par ELISA compétitive. Ainsi, le réservoir de JE être plus étudié de façon plus approfondie et le rôle des animaux d'élevage, autres que le porc, doit aussi être exploré afin de mieux comprendre la transmission de JEV et de mettre en place un contrôle approprié.

La seconde partie de notre étude sur JE montre la présence en Indonésie du génotype-1 de JEV chez une espèce de moustiques collectée sur le terrain (*Culex gelidus*). Des études antérieures ont révélé que trois génotypes de JEV, le génotype II (GII), le génotype III (GIII) et le génotype IV (GIV) ont été trouvés dans tout l'archipel indonésien de 1974 à 1987. Cependant, le génotype I (GI) et le génotype V (GV) n'ont jamais été signalés en Indonésie. Dans notre étude, le génotype I-a (GI-a) a été isolé pour la première fois en 2017 à partir d'un moustique de l'espèce *Culex gelidus* dans la province de Jambi, en Indonésie. L'analyse phylogénétique du gène E a indiqué que ce virus est étroitement lié à un isolat de GI de 1983 provenant de Thaïlande. GI remplace actuellement GIII en Asie. Ce génotype de virus n'a pas été trouvé dans le liquide céphalo-rachidien due à l'absence d'anticorps IgM spécifiques de JEV synthétisés avec du GIII-JEV. Cela peut causer un risque de faux négatif et de diagnostic erroné en présence de GI. Par ailleurs les vaccins actuels sont basés sur le GIII. D'autres études et le renforcement de la surveillance de JE devraient être mis en œuvre pour pouvoir déterminer la distribution précise du GI-JEV en Indonésie afin de faire face aux risques potentiels de transmission. Deux publications sur JE dont une revue de référence et un article de recherche original ont été publiés dans des revues internationales à comité de lecture.

Le Chapitre 3 porte sur l'importance de mieux comprendre la diversité des espèces d'*Anopheles* et leurs implications dans la transmission du paludisme pour la mise en place de méthodes de lutte antivectorielle appropriées. En Indonésie, l'étude des anophèles et les mesures de lutte, en particulier ciblant les espèces qui agissent comme d'importants vecteurs du paludisme, sont d'une grande importance dans l'optique de l'élimination du paludisme d'ici 2030. Cependant, les espèces d'*Anopheles*

en Indonésie s'avèrent être très complexes avec de nombreux vecteurs difficilement différentiables des espèces non-vectrices, associées à divers contextes épidémiologiques. Au moins 90 taxa d'*Anopheles* ont été identifiés en Indonésie dont 25 confirmés comme étant des vecteurs du paludisme. En outre, une compréhension globale de la dynamique de la transmission accompagnée d'efforts appropriés de lutte antivectorielle est assez compliquée à appréhender en raison de plusieurs facteurs, y compris les variations intraspécifiques des espèces liées aux changements écologiques et le statut vectoriel des espèces en fonction de leur distribution. La complexité et la diversité des espèces d'*Anopheles* pourraient être attribuées à la sélection naturelle, aux processus historiques, aux variations écologiques et aux flux génétiques. Cela a conduit à des divergences et à l'homogénéisation des variations à l'intérieur ou entre les espèces et pourrait être la clé pour comprendre la dynamique de la transmission du paludisme et la base d'une lutte antivectorielle appropriée. Ce chapitre présente l'homogénéité génétique d'*Anopheles maculatus,* l'un des vecteurs du paludisme les plus importants d'Indonésie. Ce taxon a été signalé comme étant un vecteur majeur du paludisme dans la région des Collines de Menoreh, à la frontière de la province centrale de Java et de la province de Jogjakarta. Il a également été confirmé en tant que vecteur important du paludisme dans le sud de Sumatra. Bien qu'*An. maculatus* soit largement réparti dans les principales îles de l'archipel indonésien, à l'exclusion des Moluques et de Papouasie, cette espèce n'a jamais été signalée comme vecteur du paludisme dans les îles de Bornéo, Célèbes, Bali et petites îles de la Sonde. Auparavant, cette espèce était considérée comme le seul membre du groupe Maculatus présent en Indonésie. Nous avons analysé la diversité et la phylogénie d'échantillons d'*An. maculatus* prélevés à plusieurs endroits à Java, aux petites îles de la Sonde, à Sumatra et à Kulon Progo (région des Collines de Menoreh). Des échantillons provenant d'une colonie maintenue en laboratoire depuis 30 ans et provenant de Kulon Progo ont également été inclus dans cette étude. Grâce aux outils d'identification moléculaire utilisant les marqueurs ITS2 (nucléaire) et *cox1* (mitochondrial), deux espèces du groupe Maculatus ont pu être identifiées en Indonésie dont une nouvelle espèce provenant de Kulon Progo. Cette nouvelle espèce, génétiquement proche d'*An. dispar* présente uniquement aux Philippines, diffère de tous les autres membres connus du groupe Maculatus, y compris d'*An. maculatus* (s.s.). La population de Kulon Progo a été temporairement nommée *An. maculatus* var. menoreh. Ce résultat est important pour identifier et mettre en œuvre des stratégies ciblées et plus efficaces de lutte antivectorielle contre le paludisme. Dans

cette perspective, une meilleure connaissance de cette nouvelle espèce est maintenant nécessaire pour mieux définir sa distribution géographique et son rôle de vecteur du paludisme. Cette étude a été publiée dans une revues international à comité de lecture.

La dengue est un problème environnemental lié à plusieurs facteurs, tels que la croissance démographique, le mouvement de la population, le transport, l'approvisionnement en eau des ménages, les services d'assainissement et le comportement communautaire qui contribuent à créer les conditions optimales pour la reproduction des moustiques *Aedes* et pour la circulation du virus de la dengue (DENV). Des stratégies essentielles doivent être appliquées pour la prévention et la lutte contre la dengue, en particulier pour les cas de dengue (DF) et dengue hémorragique (DHF), la surveillance des vecteurs, la gestion des maladies, mais aussi le renforcement à la fois de la participation communautaire à la lutte contre la dengue et du réseau intersectoriel du gouvernement local et central. Le Chapitre 4 porte sur l'étude de la diversité génétique des vecteurs de la dengue, des méthodes de surveillance vectorielle et des indices entomologiques pour évaluer le risque de transmission de la dengue, afin de pouvoir mettre en place en Indonésie une lutte antivectorielle plus efficace.

Considérant le rôle d'*Ae. aegypti* en tant que vecteur majeur de la dengue dans les régions hyperendémiques d'Indonésie, l'étude des caractéristiques génétiques des populations d'*Ae. aegypti* est essentielle pour mieux comprendre leur variabilité génétique et les relations entre elles. Ces informations sont importantes pour identifier l'origine et la distribution de cette espèce qui peuvent être utilisées pour identifier la relation entre les populations *d'Ae aegypti,* et étudier leur compétence et capacité vectorielles, leur adaptation écologique, et leur résistance aux insecticides. L'étude des caractéristiques génétiques d'*Ae. aegypti* et *Ae. albopictus* a révélé un potentiel remplacement rapide des populations de ces deux espèces en Indonésie. Cette dynamique de remplacement représente une menace pour les stratégies massives de lutte antivectorielle contre la dengue. Une conséquence est que la lutte antivectorielle ne doit pas être basée sur la population. Que ce soient des populations d'*Ae. aegypti* et *Ae. albopictus* déjà établies ou invasives, elles devront se reproduire dans l'environnement humain et la meilleure façon d'empêcher toute population de vecteurs de prospérer est certainement de mettre en œuvre la lutte antivectorielle au niveau local,

au maximum au niveau communautaire, afin d'éliminer le plus possible les gîtes de reproduction en utilisant des moyens de contrôle très simples et abordables tels que l'élimination des conteneurs et des ordures. La stratégie de prévention de la transmission de la dengue par la participation communautaire est actuellement recommandée en Indonésie et la plus susceptible d'être le moyen le plus efficace.

Une analyse comparative des méthodes de collecte de moustiques a été effectuée dans le cadre de la thèse visant à évaluer l'efficacité relative de plusieurs méthodes, dont la collecte matinale des adultes à l'aide d'un aspirateur, la collection des nymphes, la capture sur animaux, la collecte sur appât humain durant une nuit entière (la loi indonésienne n'autorise pas cette activité dans la journée) et la collecte des larves pour la surveillance de la dengue, sont les thèmes discutés dans le Chapitre 4. Une étude a été menée dans 39 sites correspondant à 39 districts/municipalités de 15 provinces d'Indonésie, endémiques pour la dengue, d'Aceh aux Moluques du nord. En ce qui concerne le nombre d'échantillons prélevés, le plus grand nombre d'individus capturés a été obtenu lors des collectes de larves. Parmi les méthodes de collectes larvaires, celle dites des larves simples était la plus efficace en termes de nombre d'individus recueillis par rapport à la méthode d'élevage, aux collectes sur animaux, aux collectes sur appât humain durant la nuit et aux captures de faune résiduelle le matin. En ce qui concerne le nombre d'échantillons positifs pour la dengue, les résultats ont révélé que les larves de moustiques étaient la source presque exclusive du virus de la dengue (93,3 %), 70,8 % ayant été trouvé par la méthode des larves uniques et 22,5 % par la méthode d'élevage. Seulement 7,6 % des échantillons totaux prélevés sur les moustiques adultes étaient positifs au virus de la dengue. Parmi les collectes de moustiques adultes, 2,3 % des échantillons obtenus par captures nocturnes sur appât humain ont été trouvés positifs, comparativement à 4,4 % avec la méthode résiduelle du matin. En conclusion, il n'y avait pas de cohérence dans l'efficacité d'une méthode donnée de détection de la dengue. Par conséquent, des méthodes de surveillance vectorielle plus efficaces et plus appropriées sont nécessaires pour déterminer la distribution des vecteurs, leur densité, les habitats larvaires et les facteurs de risque liés à la transmission et à l'évaluation de des efforts de lutte antivectorielle. En outre, l'élaboration d'un nouvel ensemble d'indices est nécessaire comme outils efficaces pour gérer et anticiper le risque d'épidémies de dengue.

Une étude portant sur les indices *Stegomyia* et leur utilisation a également été réalisée pour analyser la relation entre les indices *Stegomyia* et le risque de transmission de la dengue sur une très grande zone couvrant 78 sites d'échantillonnage dans toute l'Indonésie, de Sumatra à la Papouasie. Les indices *Stegomyia* ont été élaborés en tant qu'indicateurs quantitatifs du risque de transmission et d'épidémie de dengue. Cette étude a été menée sur la base du fait que, conformément aux recommandations de l'OMS, l'Indonésie utilise ces indices *Stegomyia* pour l'analyse du risque de transmission de la dengue depuis plus de trois décennies. Les résultats de cette étude ont révélé qu'aucune corrélation n'existe entre l'incidence de la dengue et les indices *Stegomyia*. D'autres indices plus précis et plus sensibles, de nature sociétale et non entomologique, doivent être développés pour surveiller et prévoir plus efficacement et plus précisément le risque de transmission de la dengue en Indonésie.

Le Chapitre 5 traite de la dynamique des virus du Chikungunya (CHIKV) isolés de moustiques *Ae. aegypti*, *Ae. albopictus* et *Ae. butleri* capturé sur le terrain. L'étude a révélé que tous les CHIKV identifiés sur toute l'Indonésie dans cette étude étaient similaires à ceux isolés en Indonésie depuis 2000. Ces CHIKV appartiennent tous au génotype Asie-Pacifique, le nom du nouveau génotype CHIKV proposé dans cette étude, qui est différent du génotype Asiatique. Si tous les spécimens collectés d'*Ae. aegypti* appartiennent à la même population, ce n'est pas le cas pour les échantillons d'*Ae. albopictus*. Les individus positifs pour CHIKV et ceux négatifs pour CHIKV appartiennent à des groupes distincts. Cependant, la taille de l'échantillon est trop petite pour aboutir à une conclusion définitive et une étude plus large est nécessaire pour analyser correctement la structure de la population d'*Ae. albopictus* en relation avec la compétence vectorielle pour CHIKV. Les preuves du remplacement de la population de CHIKV et la faible diversité des *Aedes* vecteurs en Indonésie méritent une attention toute particulière afin de mettre en œuvre une gestion plus appropriée et efficace de prévention des épidémies potentielles par des actions locales de lutte contre les moustiques.

Cette thèse donne un aperçu de la dynamique actuelle et du risque de transmission des principales maladies transmises par les moustiques en Indonésie. En outre, l'évaluation des méthodes de collecte des moustiques pour la surveillance vectorielle est analysée dans cette thèse afin de soutenir la mise en œuvre de

programmes de surveillance et de contrôle des principales maladies transmises par les moustiques en Indonésie. Enfin, les conclusions de cette étude aideront le public et les autorités concernées à mettre en œuvre des programmes nationaux plus efficaces pour lutter contre les maladies à transmission vectorielle, en particulier le paludisme, l'encéphalite japonaise et la dengue.

General Introduction

Indonesia, the largest archipelagic country in the world, located between the Indian and Pacific oceans, has became the rendez-vous of two biogeographical zones: western Indonesia, which is more influenced by Asian organisms, and the east part, more influenced by Australian organisms. Due to its distinct and varied geography, this country contains many endemic and unique species of animals with various habitats and ecosystems, including mosquitoes. O'Connor and Sopa recorded a total of 457 species of mosquitoes from Indonesia out of 3,567 species of Culicidae listed worldwide (1,2). Certain species are responsible for important disease transmission, of which at least 46 species of mosquitoes have been reported as vectors of human pathogens in Indonesia (3–5).

Mosquito-borne diseases are illnesses caused by viruses or parasites transmitted by mosquitoes in human populations. In Indonesia, so far, 13 mosquito-borne diseases have been reported to infect humans, i.e. Malaria, Lympathic filariasis, Dengue (DEN), Japanese encephalitis (JE), Murray valley encephalitis (MVE), Zika, Kunjin, West Nile virus (WNV), Edge hill, Chikungunya (CHIK), Sinbis, Getah and Ross river (4,6–9). Of all these, Dengue, Malaria, Japanese encephalitis, and Chikungunya, are the most important mosquito-borne diseases, for which a major impact on public health in the country has been recorded during the last two decades (3,4,9,10).

Dengue or Dengue Haemorrhagic Fever (DHF) is a benign to severe and even fatal syndrome caused by dengue viruses (DENV). Benign means asymptomatic or mild form with symptoms of undifferentiated fever, aches, pains, nausea, vomiting and rash. Meanwhile, severe dengue is a more serious form of clinical symptoms that can result in shock, internal bleeding complications, such as gingival bleeding, epistaxis, hematuria, gastrointestinal bleeding, menorrhagia, and even death (11). This disease is known as the most rapidly spreading mosquito-borne viral disease in the world. The World Health Organization (WHO) estimated that more than 2.5 billion people (over 40% of the world population) live in endemic countries in which more than 100 million dengue infections occur with 20,000 deaths worldwide every year. Indonesia is recognized as one of the highest dengue endemic countries in the world. *Aedes aegypti*

and *Ae. albopictus* are respectively the principal and secondary dengue vectors and breed extensively in all regions from western to eastern Indonesia (12). All four dengue serotypes (DENV1 to DENV4) are endemic in almost of the big cities of the country (13). In the past 45 years, annual DHF incidence increased significantly from 0.05/100,000 population (58 cases) in 1968 to 78.85/100,000 (204,171 cases) in 2016. By contrast, the fatality rate of DHF decreased considerably from 41% (24 deaths) in 1968 to 0.78% (1,598 deaths) in 2016. The areas affected by the disease in 2016 included 90.08% of the total 463 of districts/municipalities (14). To deal with this disease transmission, dengue control programs have been conducted since 1968 at the national level by the Ministry of Health (MoH) Indonesia that issued in 1992 a strategy concerning the national DF/DHF program. The critical strategies for the DF/DHF prevention and control include vector and human cases surveillance system, disease management, strengthening community participation in DF/DHF prevention and control activities, and cross-sectoral parthnership. The implementation of dengue control programs has also included health education at the community level, appropriate case management and vector control with focus on source reduction. Based on the community participation and intersectorial coordination, selected fogging (two cycles with weekly interval) of adult *Aedes* mosquitoes within 100 metres radius of reported DHF case house and mass larviciding were implemented (9). However, although dengue control efforts have been carried out continuously, the results is still not as expected. Dengue has spread in almost all regions of Indonesia with multiple co-circulating DENV serotypes (9,15,16). Moreover, major dengue outbreaks have been reported in the country over the past years (17–22).

The Japanese encephalitis virus (JEV) is another mosquito-borne *flavivirus* that has also became a public health threat in Indonesia. JEV is transmitted to humans through mosquito bites of *Culex* species from amplifier animals, especially pigs, as well as other vertebrate animals. This viral infection can cause severe central nervous system disorder with an estimated 68,000 cases every year and a case fatality rate (CFR) among patients ranging from 20% to 30% (23,24). The development of permanent neurological symptoms or psychiatric sequelae is estimated to occur in 30 to 50% of surviving patients (25–29). JEV was first isolated in the country from field-collected mosquitoes in Bekasi district, West Java and Kapuk sub-district, West Jakarta around 1972 (30). Since then, encephalitis cases have been reported in several big hospitals of Indonesia.

A total of 7,933 encephalitis cases were reported during 1979 to 1986 with 36.3% fatality (31). Further studies were then conducted in North Sumatera, West Kalimantan, North Sulawesi, South Sulawesi, East Nusa Tenggara, Papua, and Bali during 1993 to 2000. A total of 1,830 samples were collected among which 1,137 samples (62.13%) were reported as JE positive cases (32). Since then, several small-scale JEV studies have been conducted in Indonesia. In Bali, active surveillance of JEV was conducted in 10 hospitals during 2000 to 2002. A number of 33 positive cases of JEV infection were found among which 8.5% died (33). Although presumed to be endemic contrywide, the comprehensive national data on the current situation that describes the epidemiology of JE and its transmission patterns are still not available.

Beside dengue and Japanese encephalitis, Chikungunya is also an important arbovirus, which is a nationalwide public health problem in Indonesia. Chikungunya is caused by the chikungunya virus (CHIKV), a member of the genus *Alphavirus* belonging to the family *Togaviridae* and transmitted mainly by *Ae. aegypti* and *Ae. albopictus* mosquito species. The disease is a febrile illness characterized by high fever, arthralgia, myalgia, headache, skin rash and intense asthenia (34). Chikungunya was formally reported for the first time in Samarinda, East Kalimantan in 1973 (35,36). In the last 16 years, Indonesia has frequently experienced outbreaks of chikungunya fever caused by both the Asian and the East Central and South African (ECSA) lineages. Prior to these outbreaks, the incidence of Chikungunya was less than 10,000 cases/year. The massive nationwide outbreaks with 137,655 cases were reported during 2009 - 2010. Subsequent a smaller outbreak was also noted in 2013 with 15,324 cases. No death from chikungunya cases was reported during this outbreak. In 2009 and 2010, the incidence increased significantly to reach 83,756 and 53,899 cases, respectively (36– 38). In spite of many outbreaks that occurred in Indonesia, the data regarding the epidemiology, the magnitude of the disease, the role and capacities of *Ae. aegypti* and *Ae. albopictus* to transmit the virus and the dynamic of chikungunya transmission are still insufficient.

Malaria is still a prominent public health problem along the tropical belt, including Indonesia. According to a WHO report, there were about 228 million cases of malaria and an estimated 405,000 deaths in the world in 2018 (39). Indonesia is also an endemic malaria country and home to about 25 *Anopheles* species, which transmit

all five *Plasmodium* species that infect humans. In 2019, as many as 222,085 confirmed malaria cases with prevalence of 0.93 per 1,000 population, and 49 confirmed malaria deaths were reported (40). Malaria transmission occurs in 267 districts/municipalities in all of the provinces with highest risk of acquiring malaria in the eastern part of Indonesia. At present, Indonesia is heading towards the goal of malaria elimination. Comprehensive malaria control efforts continue to be made through strengthening the surveillance system, upscaling diagnostic and treatment interventions, and vector control not only in high-transmission districts, but also in low-transmission areas. Monitoring and evaluation efforts have been carried out to support the achievement of the target of malaria elimination by 2030 with the support of the national and local governments, national technical components (Directorate general of disease prevention and control-MoH and National Institute of Health Research and Development-MoH), donor agencies (Global Fund for Malaria, WHO, UNICEF), other governmental components, and private sectors. Several activities carried out include monitoring anti-malaria drug resistance; montoring the accuracy of diagnosis, both of rapid diagnostic test (RDT) and microscopy, monitoring the resistance of mosquito vectors to long lasting insecticide bednets (LLINs), mapping malaria receptivity, especially those areas that have been and will be eliminated, and monitoring behavior changes of malaria vectors (41).

As part of the national response to prevent and control the spread of main mosquito-borne disease in Indonesia, understanding epidemiology and transmission dynamics of these diseases is essential to provide up-to-date and accurate information on transmission pattern of these main mosquito-borne diseases in Indonesia.

Our objective was to understand the dynamics of the main mosquito-borne diseases to strengthen the surveillance system in Indonesia. We also investigated the diversity and phylogeny of malaria vectors and its roles in malaria transmission.
The specific objectives of the research aims were:

1) To better understand the epidemiology of Japanese encephalitis and its transmission ecology in Indonesia;
2) To identify genetic characteristics of flaviviruses, especially Japanese encephalitis from field-caught mosquitoes in study areas;

3) To describe the diversity and phylogeny of malaria vectors and their roles in malaria transmission in Indonesia;

4) To identify the relationship between entomological indexes and the risk of arboviruses transmission in Indonesia;

5) To analyze the effectiveness of current dengue vector surveillance methods in Indonesia;

6) To describe the genetic variability of *Aedes aegypti* (Diptera: Culicinae), vector of dengue, chikungunya, and zika viruses in Indonesia;

7) To identify the dynamic of chikungunya virus in Indonesia.

A number of field surveys and systematic reviews were used to achieved these aims. All studies were published in international peer-reviewed journals, and submitted or in preparation. These articles are presented within chapters of this thesis.

Chapter 1. Background

Mosquito-borne diseases

     Mosquitoes are insects belonging to the order of Diptera within the family of Culicidae. Currently, a total of 3,568 species of mosquitoes have been identified and classified into subfamilies and 113 genera (42). Mosquitoes are the most deadliest animals in the word. Females of many mosquito species are bloodsucking insects that have the ability to carry and spread pathogens (viruses, helminths, and protozoa) that causes mortality and morbidity within human population every year (42,43). Mosquito-borne diseases are the largest contributors of the vector-borne disease burden and important emerging diseases to human. According to the World Health Organization (WHO) report, malaria is the most important mosquito-borne parasite disease that caused in 2018 a total of 228 million human cases with 405,000 deaths worldwide (44). In addition, Dengue is the most important mosquito-borne virus that caused 4.2 million human cases in 2019. An estimated 500,000 cases annually had severe dengue requiring hospitalization, of which about 1 to 2.5% mortality (45,46). Mosquitoes also carry many other important human pathogens, such as viruses responsible for Japanese Encephalitis (JE), Yellow fever (YF), West Nile (WN), Muray Valley Encephalitis (MVE), Kunjin (KUN), Edge Hill (EH), Zika (ZIKV), Chikungunya (CHIKV), Getah (GET), Ross River (RR), Sinbis (SIN), Rift Valley Encephalitis (RVE) and parasites responsible for Lymphatic Filariasis (LF) (4,47,48). Mosquito vectors mostly belong to three genera, *Anopheles, Aedes,* and *Culex.*

     Malaria parasites are transmitted to humans through the bite of female *Anopheles* mosquitoes. A total of 478 species as part of subfamily *Anophelinae* have been identified worldwide (1). At least 58 unknown member of the species complexes are also recognized based on biological and morphology of the *Anopheles* genus (1). The *Anopheles* species are distributed into eight subgenera, *Anopheles* (187 species), *Baimaia* (1 species), *Cellia* (233 species), *Christya* (2 species), *Kerteszia* (12 species), *Lophopodomyia* (6 species), *Nyssorhynchus* (42 species), and *Stethomyia* (3 species) (49). At least 70 species are showing vectorial capacity to transmit human malaria parasites and 41 species among them being considered as dominant malaria vector

species (50,51). Some *Anopheles* species are also known as important vectors of lymphatic filariae (48,52–54). Recent studies have revealed that at least 51 viruses have been identified and associated with *Anopheles* species. Many of these viruses have the potential to cause febrile disease and encephalitis in humans (55). *Anopheles* are nocturnal mosquitoes biting from sundown to sunset (6 pm to 6 am). They breed in a large variety of aquatic habitats, mostly natural, sometimes human made, with stagnant or slow running freshwater or brackish water, shaded or sunny, temporary or permanent, associated with sunlight or shade, water salinity, presence of floating or emergent vegetation, and turbidity (56–59). *Anopheles* mosquitoes colonize a large variety of environments from coastal to montainous areas, even caves. They are distributed worldwide, except the majority of the pacific islands (49).

Aedini (subfamily Culicinae) is the largest tribe of mosquitoes in the world. A total of 1,260 species within 10 genera have been recorded in this tribe. According to Wilkerson *et al.* (60)*,* the genera of *Aedini* are as follows *Zeugnomyia* (4 species), *Verallina* (95 species), *Udaya* (3 species), *Psorophora* (49 species), *Opifex* (2 species), *Heizmannia* (39 species), *Haemagogus* (28 species), *Eretmapodites* (48 species), *Armigeres* (58 species), and traditional *Aedes sensu* (934 species). Several species of the genus *Aedes*, particularly *Aedes* (*Stegomyia*) *aegypti* and *Ae.* (*Stegomyia*) *albopictus* are known as principal vectors of several important arboviruses in the world, including dengue, yellow fever, chikungunya, and zika (12,61). Historically, *Ae. aegypti* originated from Egypt, Africa, as shown by its species name. Currently, the species has spread to all tropical and subtropical continents, and some temperate regions throughout the world. There are two different forms of *Ae. aegypti* according to geographic variations, behaviour, ecology and susceptibility to dengue virus*,* i.e. *Ae. aegypti formosus* and *Ae. aegypti aegypti* (62,63)*.* Although morphologically difficult to distinguish, gene flow between them is restricted. *Ae. aegypti formosus* is involved in the dengue forest transmission in West Africa, while *Ae. aegypti aegypti* is the main dengue vector worldwide (64). *Aedes aegypti formosus* is also known as a less anthropophilic form. This mosquito is mostly reported to colonize natural breeding sites, whereas *Ae. aegypti aegypti* prefers to breeds in man-made water containers (62).

Aedes albopictus, also known as the Asian tiger mosquito, is native from temperate and tropical regions of Southeast Asia. *Aedes albopictus* is a diurnal outdoor

mosquito with a very broad range of hosts, including humans, reptiles, livestock, dogs and other mammals, ampibians and birds (65). The tiger mosquito is a competent vector for at least 22 arboviruses of human and animal related diseases that led to serious arboviruses outbreaks (66,67). This species is living at the edges of forests and breeds in various natural habitats such as bamboo stumps, tree holes, decaying leaves, and other small, restricted, shaded bodies of water surrounded by vegetation (68). However, *Ae. albopictus* has ecological flexibility to adapt well to many types of man-made sites, even very small water bodies (water storage containers, tires, bottles, cemetery urns, opened coconuts, etc) in suburban and urban environments. The flight range of adult *Ae. albopictus* is quite short (300 meters). Hence, the spread of this species has occurred due to passive transportation facilitated by human activities (boats, cars, planes, etc). The introduction of *Ae. albopictus* is mostly due to the easy transportation of dormant eggs that resist to dessiccation, especially through the trade of used tyres and lucky bamboos. As a consequence, the asian tiger mosquito has undergone a dramatic global expansion and colonized rapidly almost all continents (except Antartica) in the past 40 years. Competition between *Ae. albopictus* and *Ae. aegypti* has been recorded. In many places, distribution of both species partially overlap despite their occurrence in different biotopes. Currently, *Ae. albopictus* has been proven to have a competitive advantage over a number of other mosquito species, including *Ae. aegypti* (69,70).

The genus *Culex* is known as the most widespread mosquito across the globe (71,72). *Culex* contains 769 species belonging 26 subgenera distributed worldwide (73). Adult *Culex* species have high preference for biting humans and animals (74). Therefore, several species of *Culex* are vectors of many relevant animal and human pathogens including arboviruses responsible for Japanese encephalitis, Murray Valley Encephalitis, West Nile, Rift valley fever, St Louis Encephalitis and parasites such as Bancrofti lympatic filariae. The *Culex pipiens* complex is the most widely distributed and important one, which comprises 6 members, *Cx. pipiens, Cx. molestus, Cx. globocoxitus, Cx. pallens, Cx. australicus,* and *C. quinquefasciatus* (75,76). *Cx. quinquefasciatus* is known to have the major role in the transmission of several important vector-borne diseases. *Cx. quinquefasciatus* is a peridometic mosquito that breeds in various types of natural and man-made water containers, including permanent and temporary stagnant water bodies such as organic polluted breeding sites, septic tanks, drains, wet pit latrines, puddles, concrete tanks, vases, bottles, cans, skillet,

earthen plates, sewage drains, cesspools, etc (77,78). *Culex quinquefasciatus* has unique adaptation to various environments. Although the flight range of this mosquito is short (less than 600 meters), this species has spread throughout the world by commercial ships and aircraft (70,79,80).

Malaria

1. Malaria parasites

Malaria is an important vector-borne disease that causes death and illness in children, particularly those <5 years old, and adults in tropical and sub-tropical regions (81). Malaria is caused by single-cell protozoan parasites of the genus *Plasmodium* and transmitted to humans through bites from *Anopheles* species. There are five parasite species that infect and cause the disease in humans: *Plasmodium falciparum, P. vivax, P. malariae, P. ovale* (consist of 2 subspecies : *P. ovale wallikeri* and *P. ovale curtisi*), and *P. knowlesi* (82–84). *Plasmodium falciparum* is known as the deadliest form of human malaria, while *P. vivax*, *P. malariae* and *P. ovale* are milder forms and fatal cases rarely occur (85). *Plasmodium falciparum* is the most prevalent in sub-Saharan Africa, which represents 93% of all malaria cases worldwide in 2018 (44). Subsequently, *Plasmodium vivax* is predominant and widely distributed species compared to other human *Plasmodium* species (86,87). In the region of the Americas, *P. vivax* represents 64% of malaria cases, whereas in the Southeast Asia and the Eastern Mediterranean regions, 64% and above 30% of human malaria cases are caused by this species respectively (88). In the last decade, the fifth species of human malaria, *P. knowlesi,* has posed a threat to public health, especially in Southeast Asia. *Plasmodium knowlesi* is a simian malaria parasite species (89). It was experimentally transmitted to humans for the first time in the 1930s (90,91). The first case of natural infection in human was reported in Malaysia in 1965 (92). However, natural transmission to humans are considered rare and human are likely the accidental hosts. *Plasmodium knowlesi* received much attention after it was dicovered in the large number in human samples in the Kapit division of Sarawak, Malaysian Borneo (93). After that, subsequent studies showed that knowlesi malaria was also reported to infect humans in almost all regions of Southeast Asia. Currently, *An. knowlesi* is recognized as a cause

of potentially fatal human malaria, particularly in forest areas of Southeast Asia (93–102).

Nearly half of the world population is at risk of malaria. A total of 91 countries are malaria endemic regions. Of which, most of the malaria cases were recorded from the African region (93%), followed by the Southeast Asian region (3.4%) and the Eastern Mediterranean region (2.1%) in 2018 (44). WHO estimated 228 million malaria cases with 405,000 deaths during 2019 (44).

2. Life cycle of malaria

All malaria parasites require two hosts to complete their life cycle (**Fig. 1**), i.e. the definitive host represented by a female *Anopheles* mosquito, in which reproduction occurs (sexual stages allow ookinete formation) and the intermediate host, which is a vertebrate host, including human (developement of asexual stages) (103,104). The cycle of malaria in humans is initiated by the inoculation of sporozoites into human blood vessel through the bite of a female *Anopheles* mosquito. Sporozoites, the infectious *Plasmodium* stage located in mosquito salivary glands, will then be transferred into the blood stream, and then enter the parenchyma cells of the liver. The parasite develops asexually, producing thousands of merozoites in the cell. This phase is known as exoerythrocytic schizogony or pre-erythrocytic schizogony (104).

The second phase is the parasite dispersal and invasion of the host target cells. This phase is called as erithrocytic schizogony (103). Merozoites come out from the liver parenchyma cells, gain the blood stream to invade red blood cells and initiate a series of asexual multiplication cycles from trophozoite, schizont and then produce 8 to 24 new infective merozoites per cell. At this point, the red blood cells burst and the infective cycle begins anew (105). The time length required for completion of the erythrocytic cycles varies, depending on the *Plasmodium* species. The length of the cycles ranges from approximately 24 hours (quotidian periodicity for *P. knowlesi*) to 72 hours (quartan periodicity for *P. malariae*). During this step, not all merozoites produced in the erithrocytic schizogony phase will re-infect red blood cells in the next cycles (106). Some merozoites will develop into immature gametocytes, which are precursors of male and female gametes (104).
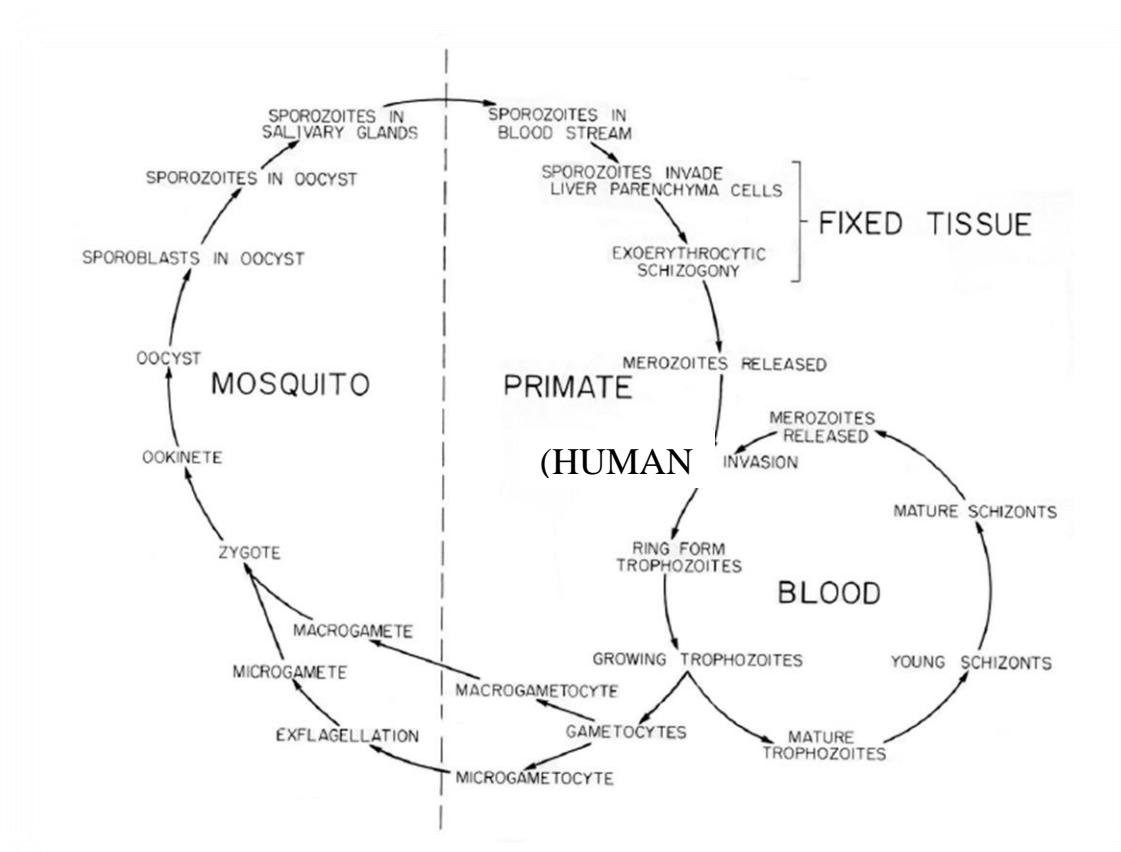
Fig. 1. The life cycle of *Plasmodium,* malaria parasit (104)

During the sexual stage, when a female *Anopheles* bites an infected person, gametocytes are taken up together with the blood and they mature in the mosquito midgut. A mosquito blood meal is approximately 2 to 3 µl, and may contain at least one male (micro) and one female (macro) gametocytes (101). In the midgut or mesenteron, the mature microgametocytes and macrogametocytes shed their red cell envelopes and transform into the mature sexual forms, microgametes and macrogametes. Subsequently, the microgamete enters the macrogamete to form the zygote that develops into active moving ookinete. The ookinete then moves actively and forces its way into an epithelial cell of the *Anopheles* midgut, and becomes oocyst on the outside gut wall. Meiotic division occurs 48 hours after the blood meal during young oocyst phase (107). Afterwards, mitotic divisions take place to produce nuclei with the particular haploid number of chromosomes for the species. The number of nuclei increases when the oocyst grows. The cytoplasm splits into sporoblasts to be vacuoles. Afterwards, the sporozoite is formed. Each sporozoite contains a single nucleus and the number of sporozoites produced per oocyst has been estimated at around 10,000 (108).

The sporozoites then migrate to all parts of the body. In mosquito vectors, some sporozoites will be able to enter the acinal cells of the salivary glands. Sporozoites enter the salivary duct when the mosquito begins to feed and enter to the blood stream of hosts (humans) (104). When mosquito vectors have sporozoites in their salivary glands, they may infect humans every two days for the rest of their life.

3. *Plasmodium*

3.1. Classification

The genus *Plasmodium* is the causative protozoan parasite of malaria, which belongs to the phylum *Apicomplexa*, a single-celled parasite with a unique form of apical secretory organelles that can help penetration into the host cell (109). *Plasmodium* is part of the sub-order Haemosporidae, member of apicomplexans that live in the blood cells. This genus also belongs to the family *Plasmodiidae,* which is characterized by three phases in the life cycle, i.e. 1. exoerythrocytic schizogony without pigmentation in monocytes of viscera and reticuloendothelial cells in hepatic cells of mammals and birds; 2. Schizogony with pigmentation in erythrocytes of the vertebrate hosts where periodic febrile coincide with the release of merozoites; 3. Sexual phase with pigment-producing gametocytes that emerge in the vertebrate host erythrocytes (110).

So far, fourtheen subgenera and nearly 200 species of *Plasmodium* have been identified on the basis of their morphology in blood smears of the infected hosts and their host range (111). The great majority of malarial parasites are transmitted by mosquitoes, for instance the parasites of humans are exclusively transmitted by species of the genus *Anopheles*. Human parasites are divided into two subgenera, *Laverania* and *Plasmodium.* The subgenus *Laverania* includes *P. falciparum,* the most pathogenic form of malaria, and the closely related species, *P. reichenowi,* a highly pathogenic form of primate parasites. The subgenus *Plasmodium* includes the remaining human parasites: *P. vivax, P. malariae, P. ovale,* and *P. knowlesi,* the fifth cause of human malaria. Parasites of the other mammals are included in two subgenera, namely *Plasmodium* and *Vinckeia,* whereas parasites in birds and reptiles are classified in the

subgenus *Plasmodium* (103). The complete classification of the human *Plasmodium* is showed in Table 1.

Table 1. Classification of human *Plasmodium* species (84,112)

Domain	Eukaryota
Kingdom	Chromalveolata
Superphylum	Alveolata
Phylum	Apicomplexa
Classis	Aconoidasida
Order	Haemosporida
Sub-order	Haemosporidiidea
Family	*Plasmodiidae*
Genus	*Plasmodium*
Sub-genus	*Plasmodium; Laverania*
Species	*P. falciparum* *P. malariae* *P. ovale (*subgenus *wallikeri* and subgenus *curtisi)* *P. vivax* *P. knowlesi*

Recently, Non-human primate (NHP) *Plasmodium* species have been a real concern since reports of *P. knowlesi* infecting humans. *Plasmodium knowlesi* is known as a zoonotic malaria parasite, which is normally residing in long-tailed macaques (*Macaca fascicularis),* pig-tailed macaques (*Macaca nemestrina*) and leaf monkeys (*Presbytis melalophos*) (100)*.* Thirteen genes in *P. falciparum* and *P. vivax* are not found in *P. knowlesi* that could be the cause of a barrier to the parasite success to infect human host, however, this *Plasmodium* species can still be transmitted and become parasites in humans. At least 30 *Plasmodium* species have been identified as the cause of infection in the NHPs in which 53 host species of more than 25 genera can be infected (113,114). Five species of NHP *Plasmodium* have been reported as predominate in Southeast Asia. They are *P. knowlesi, P. cynomologi, P. fieldi, P. inui,* and *P. coatneyi* (84,115)*.* Host-switching from NHP malaria parasites into humans has also been reported from Brazil with *P. simium,* Venezuela and Costa Rica with *P. brasilianum / P. malariae,* Malaysia with *P. cynomolgi,* and the Central African Republic with *P. vivax-like* strain from the great apes (116–122)*. P. brasilianum* genome is 99.7% identical to human *P. malariae* and considered as an anthropozoonosis. Futhermore, *P. simium* is considered genetically similar and indistinguishable from *P. vivax* (123,124)*.*

Table 2. *Plasmodium* of humans, primates and other mammals (103)

Genus : *Plasmodium*		
Subgenus : *Plasmodium* Group vivax		Species *P. vivax, P. cynomolgi, P. eylesi, P. gonderi, P. hylobati, P. jefferyi, P. pitheci, P. schwetzi, P. simium, P. sylvaticum, P. youngi*
Group malariae		*P. malariae, P. brazilianum, P. inui*
Group ovale		*P. ovale, P. fieldi, P. simiovale*
Group uncertain		*P. knowlesi* (quotidin periodicity)*, P. coatneyi, P. fragile* (both with tertian periodicity)
Sub-genus : *Laverania*		
Sub-genus : *Vinckeia*		*P. falciparum, P. reichenowi*
		Large number of species infecting rodents, bats, lemur, and other animals. Some of them of uncertain taxonomy status

3.2. Origin and evolution of *Plasmodium*

Long-standing hypotesis about the origin of *Plasmodium* suggested that chimpanzees and humans inherited *P. falciparum*-like infections from their common ancestors and co-evolved with each of their host species over millions of years. Conversely, *P. vivax* was believed to have appeared several hundred thousand years ago, following the cross-species of *Plasmodium* transmission from macaques in Southeastern Asia (125). However, the recent studies following the characterization of large numbers of additional *Plasmodium* parasites from African apes indicated that *P. falciparum* infection is relatively new for humans and arose after the acquisition of parasites from gorillas, possibly occurring in the last 10,000 years (126,127). This has put an end to the previous hypothesis. The important sign that apes have became harbouring *Plasmodium* infections was the evidence of three morphologically distinct forms of *Plasmodium* parasites in the wild-caught chimpanzees and western gorillas's blood in Cameroon. Microscopic examination for morphological identification of parasites from the blood revealed that *Plasmodium* from apes suggests the existence of different *Plasmodium* spp., which were classified as *P. reichenowi, P. rhodaini,* and *P.*

schwetzi. Interestingly, these three *Plasmodium* resemble to *P. falciparum, P. malariae,* and either *P. ovale* or (the similar) *P. vivax* respectively in humans (128). Furthermore, *P. falciparum* and *P. reichenowi* were found to differ substantially in both life cycle and gametocyte morphology from other *Plasmodium* species. This has led to the placement of these two species separately from the other subgenus, called *Laverania.* Based on the sequence of the rRNA small subunit gene, a study conducted by Escalante and Ayala showed that genetic relationship amongst *P. falciparum* and *P. reichenowi* were very close relatives of each other. In contrast, both of these *Plasmodium* species were only distantly related to other *Plasmodium* spp. (**Fig. 2.**). If it is estimated that the rRNA gene sequence in *Plasmodium* spp. evolved at the same rate as expected for some bacteria, it was concluded that *P. falciparum* and *P. reichnowi* evolutionarily diverged 10 million years ago, close to time of the ancestors of human-chimpanzees. This leads to the conclusion that parasites that infect humans and chimpanzees have co-existed with their respective hosts (125,128).
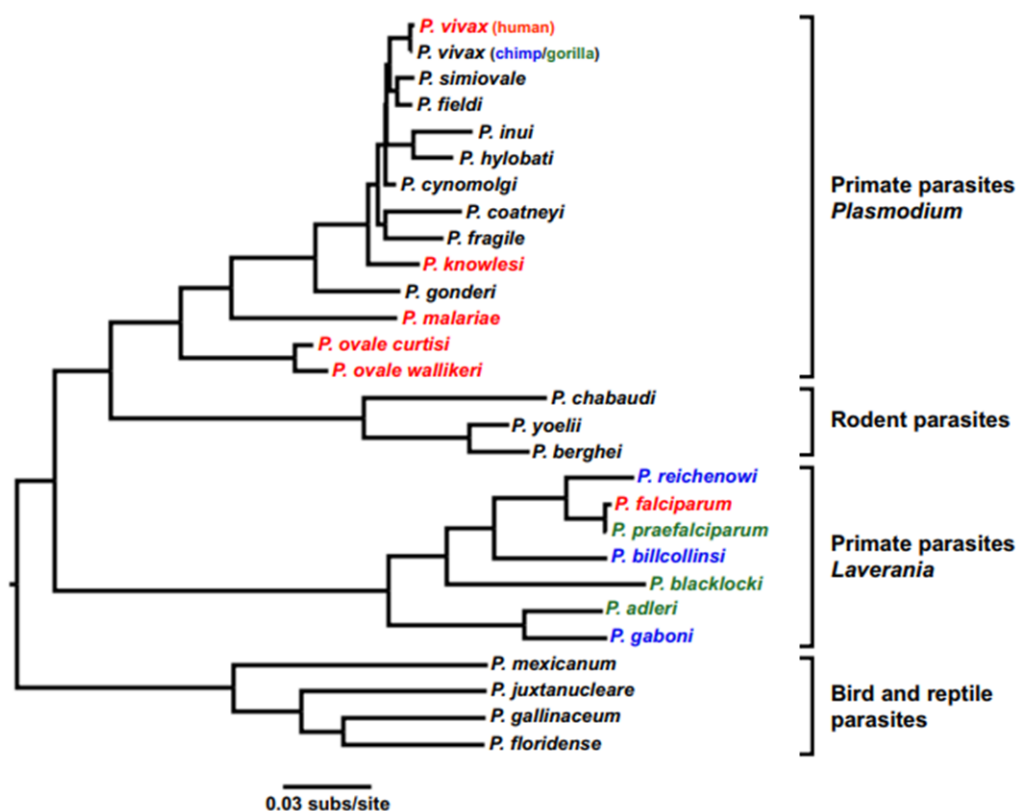
Fig. 2. Evolutionary relationship of *Plasmodium* spp. (*P.*) that infect gorillas (green), chimpanzees (blue), and human (red). Phylogenetic analysis was estimated by using maximum likelihood analysis of 2.4 kb of the mitochondrial genome (128)

For *P. vivax,* two hypotheses have been raised, either the "Out of Africa" (129) or the "Out of Asia" scenario (130). However, very recent findings showed more arguments in favor of the second option due to the highest genetic variability found in *P. vivax* from Asia and the fact this *Plasmodium* species infect mostly wild Asian monkeys, at least 15 species of them (130). *Plasmodium vivax* is known to have closest relatives in macaques in Asia, such as *P. cynomolgi.* Analysis of the evolutionary relationship (**Fig. 2**) showed that *P. vivax* and *P. cynomolgi* are included in a clade of *Plasmodium spp.* which infects primates in Southeast Asia following the cross-species transmission of macaque parasites. However, there are two other different observations and hypothesis. First, the high prevalence of the duffy-negative phenotype in sub-saharan Africans, suggesting the long-term pressure of *P. vivax* infection (129). In addition, modern humans did not arrive in Asia until about 60,000 years ago (131). However, the parasite *P vivax* has tended to diverge from the macaque parasites much earlier than this. The recent discovery of large numbers of *P. vivax* in chimpanzees and gorillas has provided strong evidence of the origin of human *P. vivax*, that is African rather than Asian (128). The precise origin of *P. vivax* is yet not solved and more extensive analyses are needed to obtain a clear answer.

The phylogenetic analysis based nuclear and organelle gene sequences revealed that all current human *P. vivax* strains form a monophyletic clade within the radiation of ape parasites, which means that *P. vivax* originated in humans following a single transmission event (132).

3.3. Geographic distribution of two major human *Plasmodium* species in Indonesia

Plasmodium falciparum is the most important cause of malaria in Indonesia. An estimated 6–21 million clinical cases of *P. falciparum* are reported every year (40). A spatial distribution study of *P. falciparum* has been conducted by Elyazar et al. in 2010 and 2011 to generate a comprehensive map of both the distribution of *P. falciparum* and the malaria transmission risk in Indonesia in 2010 (133). National data of *P. falciparum* Annual Parasite Incidence (*Pf*API) during the period of 2006-2008 were used for this analysis. Of which, a total of 2,516 out of 2,581 community blood surveys

of *P. falciparum* parasite rate (*pf*PR) were included into database of age-standardized 2-10 year old *Pf*PR during the analysis. All data of community blood surveys were identified during the period of 1985-2009. A Bayesian model-based geostatistics approach was applied to generate a predicted surface of *Pf*PR$_{2-10}$ endemicity with uncertainty estimation (**Fig. 3**). Estimated population at risk was referred from the total human population in 2010. The study results showed that an estimated 132.8 million people live in areas at risk of *P. falciparum* transmission in Indonesia in 2010. More than 70% of the population inhabits areas with unstable malaria transmission, and nearly 30% of the population lives in areas with stable malaria transmission (133).

In the western Indonesia, 78.5% of 112.1 million people live in unstable regions and 21.5% among them live in areas with a stable transmission risk of *P. falciparum*. In areas with a stable risk of *P. falciparum* transmission, 97.3% of the population live at low risk and the remaining (2.7%) at moderate levels. The distribution of the population at risk across the islands in western Indonesia is not uniform. In this study, the proportion of unstable risk compared to stable risk in Java, Sumatra, and Kalimantan were 96% vs. 4%, 38% vs. 62%, and 23% vs. 77% respectively. In contrast, more than 73% of 20.7 million people in eastern Indonesia live in stable regions and the remaining people (26.7%) inhabit unstable transmission areas. Within a stable risk of *P. falciparum*, the majority of people (> 87%) live in areas at low risk, while 12.8% among them live at medium risk, and only 0.04% in the risk of high endemicity. Of these, more people in eastern Indonesia live in areas where malaria transmission is stable. Conversely, fewer people in western Indonesia live in unstable transmission areas (133).
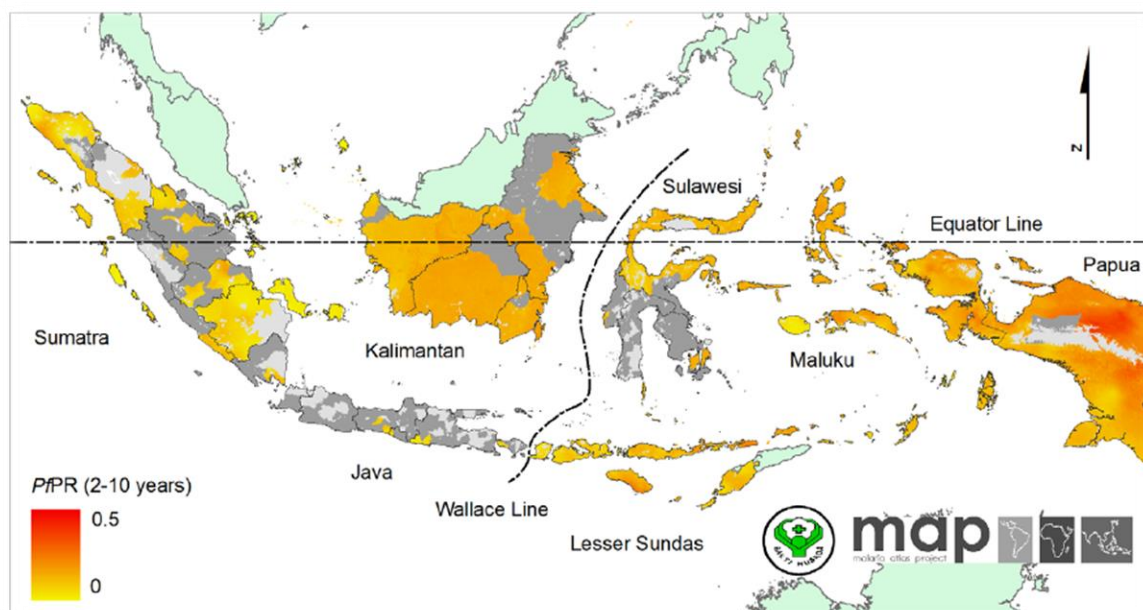
Fig. 3. The map of endemicity of *Plasmodium falciparum* malaria *Pf*PR$_{2-10}$ (doi:10.1371/journal.pone.0021315.g003). A continuum of yellow to read from 0% - 50% was defined as stable limits of *P. falciparum* malaria trasmission. While the rest of the land areas, which displayed as grey (medium risk, where *Pf*API<0.1 per 1,000 pa) and light grey (no risk, where *Pf*API = 0 per 1,000pa), were defined as unstable risk of *P. falciparum* malaria trasmission (133).

Plasmodium vivax is another important species of human malaria in Indonesia. Globally, this species is known as the most widely distributed plasmodium-causing malaria. By using the same method as the previous Elyazar et al. study on *P. falciparum* in 2010 (133). Annual Parasite Incidence data of *P. vivax* during the period of 2006-2008, a national database of *P. vivax* malariometric prevalence, and Indonesia human population data were used for this analysis. A total of 4,457 out of 4,658 community blood surveys of *P. vivax* parasite rate (*PvPR)* were included into a database of age-standardized 1-99 years old *PvPR* during the analysis. All data of community blood surveys were identified during the period of 1985-2010. A Bayesian model-based geostatistics approach was applied to create a predicted surface of *Pv*PR$_{1-99}$ endemicity with uncertainty estimation (**Fig. 4**). Estimated population at risk was referred from the total human population in 2010. This study result showed that an estimated 129.6 million people live in areas at risk of *P. vivax* transmission in Indonesia in 2010. More than 79.3 % of the population inhabits areas with unstable malaria transmission, and nearly 20.7% of the population lives in areas with stable vivax-malaria transmission.

However, most of the population (102.8 million) was living in unstable areas (79.3%), while the rest (26.8 million) inhabited areas of stable transmission (134).

In western Indonesia, 52.8% of 129.6 million people live at risk of *P. vivax* transmission. Of these, almost 77 million people on Java and Bali Islands (7% of the land area of Indonesia), representing 71% of population at risk in western region, live in areas of *P. vivax* transmission. In this study, the proportion of unstable risk of *P. vivax* transmission compared to stable risk in Java, Sumatra, and Kalimantan were 99% vs. 1%, 63% vs. 37%, and 62% vs. 38% respectively. In the eastern region of Indonesia, approximately 21.5 million people (77.7%) live at risk of *P. vivax* transmission. A majority of people lives in stable regions (71.2%) and the remaining people (28.8%) inhabite unstable transmission areas. Within a stable risk of *P. vivax*, the majority of people (10.8 million) live in Sulawesi, while 6.7 million live at risk of *P. vivax* in Lesser Sundas, 1.9 million each in both Maluku and Papua. The proportion of people living in unstable *versus* stable risk in Sulawesi, Maluku, Lesser Sundas and Papua was 49% vs. 51%, 8% vs. 92%, 9% vs. 91% and 3% vs. 97% respectively (134).
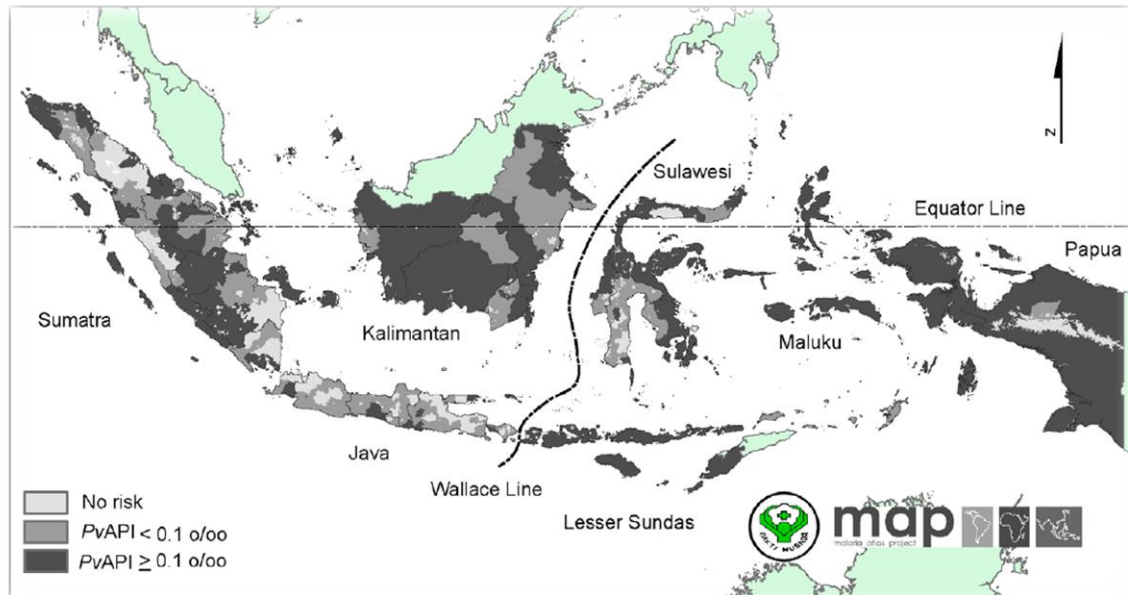
Fig. 4. The map of endemicity of *Plasmodium vivax* malaria $PvPR_{1-99}$ (doi:10.1371/journal.pone.0037325.g001). Dark grey areas, where $PvAPI{\geq}0.1$ per 1,000 pa was defined as stable limits of *P. vivax* malaria trasmission. While medium grey areas, where $PvAPI <0.1$ per 1,000 pa was defined as unstable areas. light grey, where $PvAPI =0$ defined as no risk of *P. vivax* malaria trasmission (134).

3.4. Pathogenesis of malaria and clinical symptoms

Malaria symptoms begin in *Plasmodium*-infected patient after the first liver parenchyma cells with schizont rupture and release of merozoites into the blood stream circulation. Afterwards, parasitic development continues through the asexual life cycle of their merozoite reinvasion, which then develops into trophozoites. Schizont rupture over 24 to 48 hours, the level of parasitemia being parallel to the level of human response (e.g., fever, C-reactive protein [CRP], and tumor necrosis factor a [TNF-a]), the patient crosses the threshold of consciousness and "feel sick" (135).

During the initial infection in human body, macrophage ingestion of merozoites, ruptured schizontes, or antigen-presenting trophozoites in the circulation or spleen leads to release of TNF-α (136,137). This molecule is responsible for the occurrence of fever during infection. Other important molecules, also found during active infection, include interleukin 10 (IL-10) and interferon g (IFN-g) (138–142). The prior macrophage, T cell and B cell, which are the axis of the immune system, will produce several levels of antibodies. This will have an impact on increasing macrophage activity leading to efficient clearance of parasites and the production of new antibodies (140,143,144). Immune system in humans works through the existence of a continuous parasite protein repertoire. The increase in antibodies will act as an additional protection. Malaria with uncomplication is easy to treat during each symptomatic episode with specific antimalarial drugs and the vast majority of patients will recover completely if they are quickly treated with proper compliance (145).

3.5. *Plasmodium falciparum*

In the pathogenesis of *P. falciparum* (Pf), the parasite will modify the surface of the infected red blood cells and make an adhesive phenotype, which removes parasites from the blood circulation for almost half of the asexual life cycle, an unique timeframe among malaria parasites. The binding between the infected erythrocytes can occur with endothelium, platelets, or uninfected red blood cells (146–149). The parasite accomplishes this cytoadherant state through the *P. falciparum* erythrocyte protein-1 (*Pf*EMP-1), which is the product of *var* gene transcription. Within *P. falciparum* parasite, there are 60 copies of the *var* gene, each of which varies and is different from the others. These genes represent some of the most diverse ones in the parasitic genome

and in the total parasitic population. Those expressions are driven by several mechanisms including immune and epigenetic selection pressures. The biological aspects of this parasite (*var* gene expression) occur in all infections including asymptomatic and uncomplicated malaria. In the case of infections in travelers or residents living in areas of malaria with low endemicity, a single vector mosquito bite will be able to introduce a single brood of synchronous parasites to humans. Under this condition, the patient may show a negative *Plasmodium* diagnostic in peripheral blood smears. It can occur in every human infection for half of the sexual life cycle due to the sequestration, temporary removal of the parasite from circulation through red cell surface binding. In the situation of high endemic areas, patients may get repeated infected mosquito bites and can show a persistent fever that is accompanied by consistently positive blood smears. However, certain local hosts will evolve with the emergence of local immunity against *P. falciparum*. Under these conditions, blood smears may again drop to very low levels and even become undetectable in spite of ongoing transmission (145,149).

Furthermore, *P. falciparum* parasite can cause pathological changes in pregnancy due to the parasitic ability to pair with novel placental molecules such as Chondroitin sulfate (CSA)(150,151). The PfEMP1 protein will bind CSA as parasites pass through the placenta, removing them from the circulation, whereas non-CSA binding will continue to circulate. Maternal antibodies that have developed during a previous malaria infection will destroy non-CSA binding parasites, whereas the placenta acts as a protected space for propagation. In addition to the direct effect of placental binding, mononucleate cells will be able to inflitrate and enter in very high numbers. Under these conditions, the placenta at examination will show pigments that are trapped in fibrin (for longer infections) or parasites and / or mononuclear cells (active infection) (145,152,153).

Cerebral malaria with clinicopathological syndrome can occur in children and adults due to the ability of *P. falciparum* to bind with the endothelium. In high malaria endemic areas, children under 5 years old are most at risk for the disease with mortality reaching 10% to 20%. In contrast, all ages are at risk and mortality can be higher in adults in low malaria endemic areas. In non-immune populations, originating from non-endemic areas of malaria, such as tourists and army troops, etc., the potential for life-

threatening cerebral malaria can occur, although they visit areas with low infection rates (1% parasitemia). The clinical symptoms of cerebral malaria begin with general clinical manifestation of malaria, but then quickly experience a decrease in consciousness to a coma. Cerebral malaria can be confirmed through retinal examination showing signs of malaria retinopathy. In fatal cases, confirmation can be done at autopsy with the presence of *P. falciparum* parasites of more than 20% in the brain capillaries through tissue histological smears. Other pathological features that appear include fibrin thrombi, ring hemorrhages, brain discoloration, axonal injury and capillary leakage. The brain vessels will appear congested in all cases with prominent brain swelling, especially in the case of children in Africa (within 48 hours after symptoms develop). Multiorgan failure and acute respiratory disorders with more diffuse alveolar damage are also common in adults (154–159).

3.6. Plasmodium vivax

Unlike *P. falciparum*, *P. vivax* (Pv) does not show a prolonged period of sequestration during a parasite infection. The parasite is probably more frequently seen in peripheral blood smears during infection. One of the *P. vivax* characteristics is the red blood cell preference for reticulocytes and the use of most of Duffy antigens for invasion. This condition leads to a clinical infection with a lower level of parasitemia when compared to *P. falciparum* because the reticulocytes are larger than the mature red blood cells, and the infected blood cells appear larger than the surrounding cells in the peripheral blood smear. Schuffner's characteristic points that represent the caveola-vesicle structure and amoeboid form of *P. vivax* with cytoplasm, which have finger-like projections, are features that are seen in the diagnosis of Pv during infection. Clinically, Pv infected patients are almost identical to other malaria infections with clinical manifestations of fever and a constellation of other possible symptoms. Unlike *P. falciparum* and *P. malariae*, *P. vivax* and *P. ovale* infections can recur without the bite of a malaria vector when hypnozoites, which are inactive forms of a single sporozoite in the liver that can last for months to years, release merozoites. The appearance of symptoms of *P. vivax* infection can occur from several weeks to several years after exposure (145,160–162).

Although severe / fatal cases due to *P. vivax* infection are very rare, these outcomes are reported (163–165). Recurrent or chronic *P. vivax* infections in highly endemic areas contribute to a higher risk of severe anemia, malnutrition, acute respiratory distress syndrome (ARDS) and splenic rupture (166–170). Similar to the severe cases of *P. falciparum* and *P. knowlesi*, severe impacts that are commonly found in *P. vivax* infections include respiratory distress, hepatorenal failure, and shock. Coma cases are rarely reported in *P. vivax* infections (145,152,153,171).

3.7. Plasmodium ovale

P. ovale is currently divided into two different subspecies, namely *P. ovale curtisi* and *P. ovale wallikeri*. These two subspecies cannot be distinguished based on clinical symptoms and treatment. However, they are distinguished by their latency period and genetic sequences. *P. ovale wallikeri* has a shorter latency period than *P. ovale curtisi*. Although the behavior of *P. ovale* is similar to *Plasmodium vivax*, *P. ovale* does not need Duffy's antigens to invade red blood cells. In peripheral blood smears, *P. ovale* tropozoites are comet-shaped and take the appearance of oval-shaped red blood cells after infection (145,172).

3.8. Plasmodium malariae

P. malariae is the most benign form of malaria infection with typical clinical features. Patients will experience fever every 72 hours during infection due to a longer parasitic life cycle. Long life cycles and low-level infections have led to a stronger immune response. Thus, *P. malariae* is often considered a cause of chronic malaria, which can last for long, even decades. *Plasmodium malariae* infection also causes deposition of immune complexes in the kidneys, which can cause nephritis. In peripheral blood smears, the parasite shows a typical schizont picture with some gold merozoites and central pigments and has the shape of a daisy (145,173,174).

3.9. Plasmodium knowlesi

P. knowlesi infection is found with limited distribution in Kalimantan/Borneo Island, in the territory of Malaysia/Indonesia and several other countries in the

Southeast Asian region, including Singapore, Myanmar, Vietnam, Cambodia, Thailand and the Philippines. Long-tailed and/or pig tailed macaques are known as intermediate reservoirs/hosts (175,176). So far, *P. knowlesi* transmission has been reported to occur between macaques and humans without human-to-human transmission (177). *In vitro* studies that have been conducted showed that parasites prefer to infect young red blood cells. However, *P. knowlesi* is able to infect mature human blood cells as well. Clinical symptoms due to parasitic infections show similarities with other malaria infections, which include fever/chills and headaches with unusual symptoms such as nausea/vomiting, myalgia/arthralgia, upper respiratory symptoms, and jaundice (175,176,178). However, fatal complications of *P. knowlesi* have also been reported and in proportionally higher frequencies than those occurring in *P. vivax* and *P. falciparum* (145,176).

Similar to severe symptoms of *P. falciparum* in adults, they usually start with fever, then progresses into more severe symptoms, including hypotension, respiratory distress, acute renal failure, hyperbilirubinemia and shock (179,180). Coma is not always seen in fatal cases due to *P. knowlesi* infection. Other causes of severity that are also commonly seen in *P. knowlesi, P. falciparum* infections, bacterial sepsis, etc. are excessive immune responses in patients that are not treated or with a late treatment. Pathologically, *P. knowlesi* infection has the same effect as *P. falciparum,* which causes sequestration in the brain along with congestion and possible swelling in the brain. In the severe manifestations of *P. falciparum* infection, cythoadherence of infected red blood cells to brain endothelium is causally implicated in malaria coma. Cytoadherence is mediated by specific binding of variant parasite antigens, expressed on the surface of infected erythrocytes to endothelial receptors, such as intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM) and CD36. In addition, *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1) mediates parasites sequestration to the cerebral microvasculature *via* binding of DBLβ domains to ICAM-1 and its clearly associated with severe cerebral malaria. However, the role of (ICAM-1) in the brain associated with clinical manifestations during *P. knowlesi* infection is not yet totally known, but there are similarities in genes of *P. knowlesi* and *P. falciparum* that may play a role, namely *SICAvars* (schizont-infected cell agglutination) (145,181–184).

4. Malaria vectors

4.1. Malaria vectors in Indonesia

Anopheles is a genus of mosquitoes that has been studied in Indonesia since 1897 (185). Various studies have been conducted since in relation to the role of *Anopheles* as malaria vectors in this country. In 1953, Koesoemawiangoen identified 90 species of *Anopheles* in Indonesia (186). Subsequently, O'Connor and Soepanto updated the list of *Anopheles* species to 66 species with one sub-species and 4 varieties in 1979 (187).

Historically, studies of confirmation of *Anopheles* as malaria vectors in Indonesia have been carried out since the early 1900s. There are several versions related to malaria vector records. In 1949, Stoker and Koesoemawinangoen stated that at least 16 *Anopheles* species had been confirmed as malaria vectors (188). Then, Bonne-Wepster & Swellengrebel reported 24 important *Anopheles* species, which were considered as important malaria vectors in Indonesia in 1953 (189). The same number of confirmed malaria vectors was also reported by Knight & Stone in 1977 (190). In 1985, Kirnowardoyo also updated the status of *Anopheles* malaria vectors in Indonesia. He reported 18 *Anopheles* species confirmed as malaria vectors (191). Complementing previously published data, the Directorate General of Disease prevention and control, MoH Indonesia, released an update of the number of *Anopheles* species, which reported 25 species of malaria vectors in Indonesia in 2008 (192).

In 2013, a review of the distribution and bionomics of *Anopheles* malaria vector mosquitoes in Indonesia was conducted by Elyazar *et al.* (125). A total of 259 sources have reported the presence of 20 species or taxa for species complexes/groups (e.g. *An. farauti*, *An. leucosphyrus, An. maculatus, An. subpictus, An. sundaicus*, etc) of *Anopheles* malaria vectors (**Fig. 5**). The data was collected from 755 locations in the period from 1917 to 2011 (3). According to previous published data, the greatest number of sites where vectors have been found in Indonesia were on Java (311 sites; 41%). While the least number of sites (32 sites; 4%) were found on Papua. *Anopheles vagus* is reportedly the most widely distributed across Indonesia and the greatest

number of independent sites (349 sites; 46%). Whereas *An. bancroftii* had the most restricted distribution of *Anopheles* in Indonesia (8 sites in Maluku and Papua; 1%) (3).
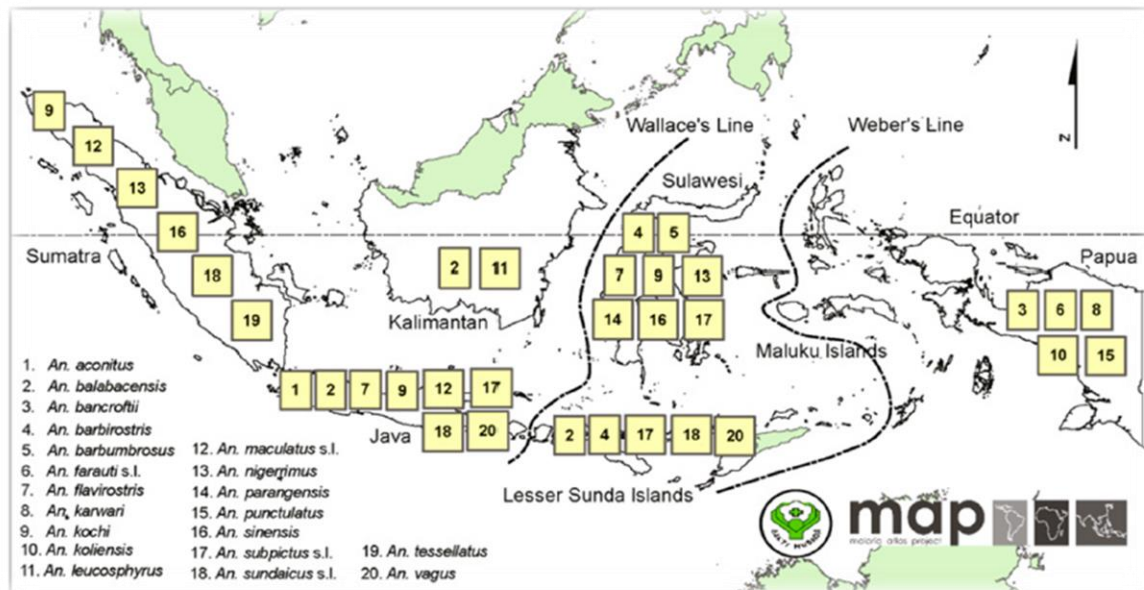
Fig. 5. The distribution map of primary *Anopheles* malaria vectors in Indonesia (3)

4.2. Bionomics of malaria vectors in Indonesia

Understanding the bionomics of malaria vector species is crucial for the study of transmission dynamics and implementation of vector control strategies. An *Anopheles* species is confirmed as a malaria vector if there are sporozoites present in the salivary glands. *Anopheles* species confirmed as important malaria vectors in Indonesia include :

1) *Anopheles aconitus*

Anopheles aconitus is widely distributed in the islands of Java, Sumatra, Kalimantan, Sulawesi and lesser Sunda Archipelago. *Anopheles aconitus* has been confirmed as a malaria vector in Indonesia in the Cianjur region, West Java (1919); Purworejo, Central Java (1954, 1998); Banjarnegara, Central Java (1978, 1982); Jepara, Central Java (1980); and Wonosobo, Central Java (1982) (3,193,194).

Females are zoophilic/zoo-anthropophilic and bite actively before midnight, especially between 8pm -10pm. This mosquito prefers to bite outside the house than indoors (exophagic). In the morning, these mosquitoes are often found resting on the

cliffs of the moat. The larval habitats of this species include rice fields, freshwater pools, river banks, irrigation channels at an altitude of 100-900 m above sea level (asl). In some places, this species is found at altitudes >1,000 m asl. In areas with sufficient water, where farmers plant rice on an irregular planting time, density of *An. aconitus* will be high throughout the year. Before 1990s, malaria transmission was occurring throughout the year in these areas (3,193–195).

2) Anopheles annularis

Similar to *An. aconitus, An. annularis* is distributed in Java, Sumatra, Kalimantan, Sulawesi and lesser Sunda Archipelago. *Anopheles annularis* has been confirmed as a malaria vector in Indonesia, only in Sulawesi (1920). This mosquito prefers to bite outdoor (exophagic), especially around cattle and buffalo. Biting activity occurs before midnight. The larval habitats of this mosquito are freshwater ponds, rice fields and mountain streams (193,194).

3) Anopheles barbirostris s.l.

Anopheles barbirostris s.l. is widely distributed in Java, Sumatra, Kalimantan, Sulawesi, lesser Sunda Archipelago, and also Maluku (1,3). *Anopheles barbirostris* s.l. has been confirmed as one important malaria vector in Indonesia. This taxon was found to positively contain malaria sporozoites in many locations. Kirnowardoyo noted that *An. barbirostris* s.l. was confirmed as malaria vector in 13 locations in South Sulawesi and one location in Southeast Sulawesi during the period of 1929-1942 (191). In 1939, Machsoes found 30 out of 1,041 (2.9%) specimens of *An. barbirostris* s.l. sporozoite positive in South Sulawesi (196). In the early 1990's, several studies in the Lesser Sunda islands (Lombok, Flores and Adonara islands), northern Sulawesi (Meras and Tomohon) have confirmed *An. barbirostris* s.l. as malaria vector of *P. falciparum* and *P. vivax* (197–199).

Females are zoophilic/anthropophilic depending on the species of the complex. This mosquito prefers to bite humans outdoor than indoor (exophagic). Human blood index (HBI) varies, ranging from 12.6% for animal shelter resting collections to 20% from indoor collections. This species is often found resting outdoors (exophilic) and more common around cattle shelters than human settlements. The biting behavior and activity of this species vary depending on its geographical location. This species of

mosquito is reported to more often bite humans on the first half of the night in Java and Sulawesi, but elsewhere, the biting peak is reported to occur during the third quarter of the night (12 pm - 03 am) (200–202).

The larval habitats of *An. barbirostris* s.l. are represented by sunlit water bodies containing fresh and often clean water including lagoons, marshes, pools, slow running streams, along river banks, springs, rice fields, fish ponds, drainage ditches and wells. This species is dispersed from the coastal to hilly areas at altitudes up to 2,000 m asl (3,199,203–206).

4) Anopheles balabacensis s.l.

In Indonesia, *Anopheles balabacensis* s.l. is reportedly distributed in Java, Sumatra, Kalimantan, Sulawesi, and Lesser Sunda islands (1,207). *Anopheles balabacensis* s.l. belongs to the Leucosphyrus subgroup (208). *Anopheles balabancensis* s.l. has been found infected with *P. falciparum* and/or *P. vivax* sporozoites in Purwakarta, West Java (1962), Balikpapan, East Kalimantan (1981), Batulicin, South Kalimantan (1982) (3,192). In addition, this species has also been reported infected with *P. falciparum* sporozoites in Kalimantan (1987). *Plasmodium vivax* infections in *An. balabacensis* were also detected in Central Java (2000). *Anopheles balabacensis* with both *P. falciparum* and *P. vivax* has also been reported in Kenangan, East Kalimantan (2007), Salaman, South Kalimantan (2007), and Menoreh hills, Central Java (2007) (3,191,193,197,209).

High degree of anthropophily of this taxon was reported in the montainous areas in Lombok Island, while low degrees of anthropophilic behaviour have been noted in Menoreh hills, Central Java. Females prefer to bite humans outdoor (exophagic) in Central Java and Lesser Sundas. In contrast, *An. balabacensis* s.l. was mostly feeding indoors (endophagic) in Eastern Kalimantan (3,210). The biting activity varies according to location and most likely species of the complex. On average, the biting peak of this taxon occurs during the second quarter of the night in Lesser Sundas and Java, and in the third quarter of night in Kalimantan (3,210–212). After biting, *An. balabacensis* s.l. rests in shaded locations such as cattle shelters, inside ground pits, and under trees. The larval habitats of *An. balabacensis* s.l. are stream-side rock pools, river banks, puddles, muddy animal wallows, tyre tracks and hoof prints, pools under

shrubs/trees. The habitats of this species is associated to hilly, forested terrain up to 700 m asl (3,188,191,206,213).

5) *Anopheles bancroftii*

Anopheles bancroftii is only distributed in Seram island, Maluku and several sites in Papua (1,3). This species was first reported infected with malaria oocysts in the late 1920s in Tanah Merah, a remote jungle environment in Southern Papua (214). Subsequently, *An. bancroftii* was also confirmed to contain *P. falciparum* parasites in 1935 in Papua (191). It was then confirmed as a vector involved in malaria transmission in Papua after finding 2 specimens out of 982 dissected mosquitoes that contained malaria sporozoites in Merauke in 1957 (215). *An. bancroftii* has not been considered as an important malaria vector due to low vectorial capacity in Papua and no infective *An. bancroftii* has been reported from Maluku (216). Females exhibit endophilic behaviour. Based on Van den Assem's study, mosquitoes rest in the hut after sucking blood in southern Papua (endophagic and endophilic) (217). Immature stages of *An. bancroftii* were typically found in stagnant, clear fresh water pools, ditches, swamps with vegetation, semi-shaded places along the edges of small lakes with lily-like vegetation, and marshes (3,188,191).

6) *Anopheles barbumbrosus*

Anopheles barbumbrosus is reportedly distributed widely in almost all major islands of Indonesia, except Papua (1,3). This species has been reported as closely related to *An. vanus,* mosquito species distributed in Kalimantan, Maluku, and possibly the western tip of Papua (3). *Anopheles barbumbrosus* was found to be *Plasmodium* positive, containing sporozoites in Malili, South Sulawesi in 1941 (194). Subsequently, Bonne Webster and Van Hell reported that this species was also sporozoite positive in the Sulawesi region in 1950 and 1952 (193,218). This mosquito has been identified as anthrophopilic in South Sulawesi, however Sulaeman reported its zoophilic tendency in Central Sulawesi (219). The data describing the bionomics of *An. barbumbrosus* is not well known. Immature stages of *An. barbumbrosus* are typically found in small wells, slow running water in rice-fields and jungle (188).

7) *Anopheles flavirostris*

Anopheles flavirostris is reportedly distributed widely in Sumatra, Java, Kalimantan, Lesser Sundas, and Sulawesi (1). This species is a member of the Minimus subgroup that was previously considered as a subspecies of the Minimus complex. Currently, An. flavirostris has been confirmed as a valid species based on molecular characteristics (3). Sinka et al. assumed that all previous records of An. minimus reported in Indonesia have been misidentifications of An. flavirostris (220). This species was confirmed as malaria vector for the first time in Malili, South Sulawesi in 1949 (194). Since then, An. flavirostris has been confirmed as malaria vector in many locations in Java, Lesser Sundas, Sulawesi. However, this species has been mainly reported as zoophilic with low anthropophilic behaviour and no clear preference for feeding location (221,222). In Flores, the biting peak was recorded during the third quarter of the night with preference for resting indoors after feeding (223).

The larval habitats of An. flavirostris are found in rice fileds, pools, springs, shaded grassy edges of clear, slow-moving small streams, irrigation channels with slow running water (with/without vegetation). This species can be found from coastal plains to hilly areas, up to 600 m asl (3,188,224).

8) Anopheles karwari

Anopheles karwari is reportedly distributed in Sumatra, Java, Kalimantan, Sulawesi and Papua (1). Infective females with malaria sporozoites have only been reported near Jayapura, Papua. Information about the bionomics of this species in Indonesia is also very limited due to infrequent and low population occurrence during field collections, however its alleged to be zoophilic (3). The immature stages of An. karwari are found in marshes, small, slow moving streams, irrigation channels associated with rice fields, small swamps, seepages, ground and rock pools, springs (3,188).

9) Anopheles kochi

Anopheles kochi is reportedly distributed widely in almost all major Indonesian islands, except Papua (1). The role of this species as malaria vector has been confirmed in Nias island, Northern Sumatra, northern Sulawesi and Central Java (197). An. kochi generally reflects a zoophilic feeding behaviour in which females appear more common in cattle shelters than human habitations (200,225). Previous studies indicated a general

tendency for exophagy. Based on human landing collections, *An kochi* specimens reach their peak of blood-feeding activity during the second quarter of the night. Their resting habits appear more exophilic in Central Java and endophilic in Southern Java. This indicates that resting habits from *An. kochi* depends on the study location (3,226,227).

An. kochi prefers to breed either in fresh or brackish habitats, running or stagnant, often with muddy water. The larval habitats of this species also include rice fields, ponds, pools, drain along roads, buffalo-hoofprints, wella, ditches. This mosquito species can be found from coastal to hilly areas with altitute up to 1,100 m asl (3,188,189,228–230).

10) Anopheles koliensis

In Indonesia, *An. koliensis* is only distributed in the Papua region (1). This species is a part of the Punctulatus group and plays a major role as malaria vector together with other members of this group and species of the *An. farauti* complex. *An. koliensis* was first confirmed as malaria vector in Jayapura in 1956 (3,231). *Anopheles koliensis* was also found to be positive, containing *P. vivax* sporozoites in the Mimika area, southern Papua by CSP-ELISA (3,232). Subsequently, *An. koliensis* has been found infected in many locations in Papua (197). This species was found abundant in settlement areas near sago palm and swamp forests (232). Human is the main host for *An. koliensis* due to lack of cattle, buffaloes or horses in Papua. The feeding behaviour of this species varies depending on locations. However, it was suggested as exophagic in some areas (233). In Arso, *An. koliensis* was found biting outdoors in the first quarter of the night. In contrast, the species was found biting indoors between the second and the third quarters of the night (3,233). *An. koliensis* was also reported biting more indoors with biting peak activity during the third quarter of the night in Jayapura (3,233,234). In Entrop, Papua, the biting behaviour was reported both indoors and outdoors (3,233,234).

The breeding habitats of *An. koliensis* can be found in ground pools in grassland, along the edge of jungle, ditches, riverside ponds, footprints, wheel ruts and sometime in pig ruts and wallows. *An. koliensis* can be found from lowland to higlands with altitude up to 1,700 m asl (3,205,235).

11) Anopheles leucosphyrus

Anopheles leucosphyrus is a member of the *Leucosphyrus* complex. This species is considered to be a malaria vector, especially in forest areas of Sumatra. Bionomics of this species remain limited. In 1932, almost all *An. leucosphyrus* specimens collected indoors contained human blood in areas where cattle are scarce. *Anopheles leucosphyrus* was found to be positive containing sporozoites in Southern Kalimantan in 1980 (236). However, the species identification is now in question due to very similar morphology and environmental conditions between *An. latens* and *An. leucosphyrus*. It would need to be confirmed (3,220,237). The larval stages of *An. leucosphyrus* can be found in marshes, small streams, seepage springs, jungle pools, fishponds, ground depressions, wheel ruts, hoof prints (3,224).

12) Anopheles maculatus

Anopheles maculatus s.l. belongs to the Maculatus group. The Southeast Asian mainland presents the highest diversity of the Maculatus Group. So far, only *An. maculatus* (*s.l.*) [presumed (*s.s.*)] was reported in Indonesia (**Fig. 6**) (1). *Anopheles maculatus* has been confirmed as one of the major malaria vectors in Indonesia. *An. maculatus* with *Plasmodium* spp. infections have been reported particularly in the Menoreh hills of Central Java and Tenang, Southern Sumatra (197,209,238,239). The blood-feeding behaviour of females is considered as mainly zoophilic (3,211,222). This species is regularly reported as the most dominant mosquito species in cattle shelters than in human habitations. This species is found to bite humans both inside and outside the house (endophagic and exophagic, respectively). In most study areas, this species generally tends to bite during the first half of night. In addition, several studies of *An. maculatus* in Central Java revealed that the density of mosquitoes increased just before dawn. *Anopheles maculatus* typically rests outdoors (exophily), particularly around cattle shelters, under shade of plants, moist banks of small streams, natural ground pits, amongst low vegetation, and cliff sides (3).

The immature stages of *An. maculatus* can be found in fresh and clean water including slow-moving streams, stream-side rock pools, drying river beds, ground seepages, small pools and puddles, natural springs, ponds, ditches, and rice fields. This species can be found from coastal to hilly areas with altitutes up to 1,100m asl (3,189,204,228,238,240,241).
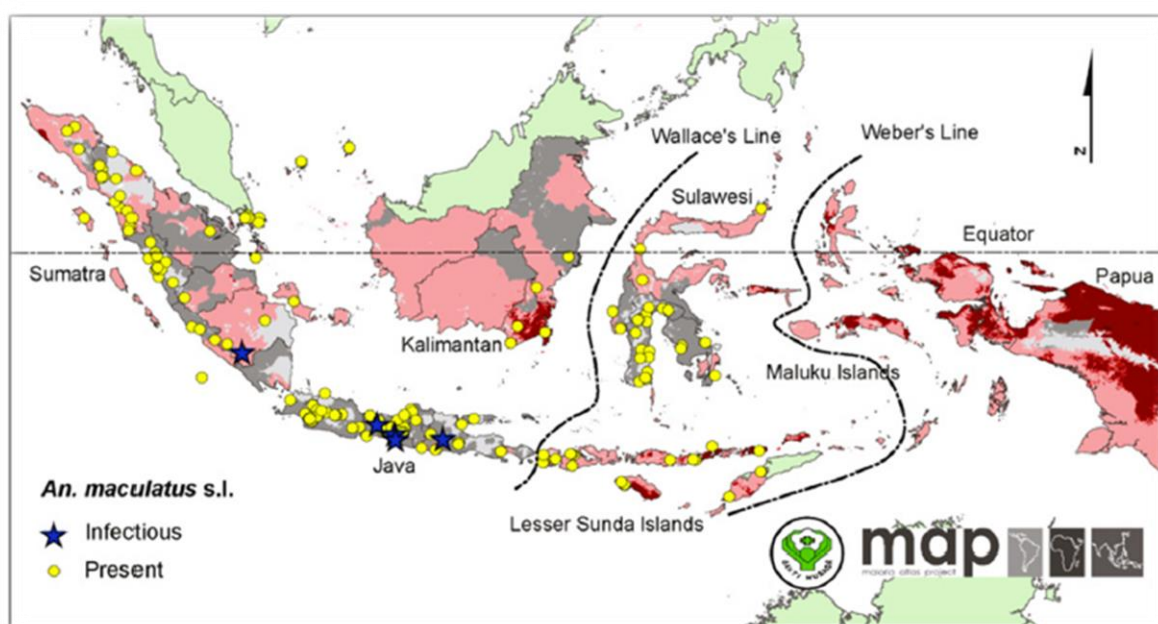
Fig. 6. *Anopheles maculatus* s.l. distribution in Indonesia. The blue stars indicate the confirmed location of *An. leucosphyrus* as malaria vectors. The yellow dots show 188 records of occurrence for this species between 1918 and 2011 (3).

13) Anopheles nigerrimus

    Anopheles nigerrimus is distributed on Sumatra, Java, Kalimantan, Lesser Sundas and Sulawesi (1). *An. nigerrimus* s.l. belongs to the Hyrcanus group (242). *An. nigerrimus* was first reported as malaria vector in Indonesia by Overbeek from Palembang, South Sumatra in 1940 (243). In addition, this species has also been found infected in Sihepeng, Northern Sumatra (197). The host preference for *An. nigerrimus* is still not clear. In Central Sulawesi, this species tends to bite during the first quarter of the night (200). This species was identified to rest around cattle shelters (200,244). After biting indoors, *Anopheles nigerrimus* usually exits immediately to rest outdoors (230). Immature stages of *An. nigerrimus* prefer sunlit, fresh and clean water habitats. The larval sites include marshes, pools, rice fields, irrigation channels, fishponds. This species has been found from coastal to hilly areas at altitudes up to 700 m asl (3,191,245).

14) Anopheles parangensis

    Anopheles parangensis is commonly found in the Sulawesi region. The density of this species was lower than other species in southeast and central Sulawesi, but

higher in northern Sulawesi (1,200,246). The existence of this species in Maluku was also reported in Ternate in 1930 (216). This mosquito has also been found in Sumatra and Kalimantan, however, no detail on location was reported in Kalimantan (1). In Sumatra, this member of the Pyretophorus series, which is an assemblage of mosquitoes including important malaria vectors in Africa and Asia, was recorded in Simeleue island, Aceh in 2005 (3,247).

Anopheles parangensis was first reported with *P. falciparum* sporozoites and was declared as a malaria vector near Manado, North Sulawesi in 1996 (198). The study of *An. parangensis* host preferences is still very limited and the main host of this species is poorly known in Indonesia. According to previous human-landing captures conducted by Widjaya and Marwoto, this species showed a tendency for exophagy (198,201).

The immature stage of *An. parangensis* are found in fresh or coastal brackish water with sunlit or under shade including stagnant pools, fish ponds, ground puddles, and marshes (3,188,189).

15) Anopheles punctulatus

In Indonesia, *An. punctulatus* is only distributed in Maluku and Papua. This species is considered as one of the most important malaria vector in Papua. *Anopheles punctulatus* is proven to transmit *P. falciparum, P. vivax* and *P. malariae* (248,249). This species has been confirmed as a malaria vector in many locations in Papua, from coastal and lowland (Timika, Arso, Armopa, Mapurujaya, Tipuka) to highland areas (Oksibil, near Wamena, and Obio) (197). It is also responsible for several malaria outbreaks in Papua (249,250). *An. punctulatus* is anthropophilic, usually biting human outdoors. When biting humans indoor, peak-biting ativity was reported in the second quarter before midnight (249). *An. punctulatus* typically rests outdoors, especially on the outside of the house walls and amongst vegetation (233).

Larval stages of *An. punctulatus* are found almost everywhere and mostly in sunlit habitats. Larvae have been collected from riverside pools, pig ruts, wheel prints, grasslands, along jungle edges, pools, freshwater coastal marshes, low-lying riverine areas, ground depressions and shallow drainage around houses, footprint, ditches,

earthen drains and rockpools in drying stream beds (3,188). This species has been found from coastal to hilly areas at altitudes up to 1,260 m asl (3,249).

16) Anopheles sinensis

Anopheles sinensis is distributed in Sumatra and Kalimantan. *An. sinensis* belongs to the Hyrcanus group (187). This species was first reported as a malaria vector in Indonesia by Boewono in Nias, Northern Sumatra in 1997 (251). This species is always found in low density compared to other *Anopheles* species, both in Sumatra and Kalimantan (230,251–253). The host preference for *An. sinensis* is still not clear, but assumed to be mostly zoophilic and exophagic (254). This species tends to bite during the first quarter of the night. It was identified to rest around cattle shelters (3).

After biting indoors, *Anopheles nigerrimus* usually exits immediately to rest outdoors (230). Immature stages of *An. nigerrimus* prefer sunlit, fresh and clean water habitats. Larval sites include marshes, pools, rice fields, irrigation channels, fishponds. This species has been found from coastal to hilly areas at altitudes up to 700 m asl (245).

17) Anopheles subpictus

Anopheles subpictus is quite widely distributed in Indonesia, from Sumatra, Kalimantan, Java, Lesser Sundas, Sulawesi, Maluku, to Papua. The *Anopheles subpictus* complex belongs to the Pyretophorus series (187). This taxon was first confirmed as malaria vector in Indonesia in the late 1920s (255). Subsequently, it has also been found infected with sporozoites in Lesser Sunda (Flores, Lombok, Adonara), Sulawesi (north, central and South Sulawesi) (197,256,257). *An. subpictus* females tend to be zoophilic and exophagic, but this depends on the geographic location (226,240,254). This species is reported to be more active during the second half of the night. It was identified to rest more around cattle shelters, bushes and under shaded trees (3). However, several studies have reported this species as endophilic with resting sites varying from hanging clothes, interior wall surfaces, to ceiling (3).

Larval stages of *An. subpictus* prefer sunlit, fresh or brackish water habitats.The larval sites include coastal blocked freshwater rivers and streams, pools, mangrove forests, springs, fish ponds, borrow pits, drains, furrows in gardens, buffalo wallows,

brackish ponds, seaweed ponds, and irrigation ditches. This species has been found mostly along the coast and very few specimens in hilly areas up to 700 m asl (3,245).

18) Anopheles sundaicus

      Anopheles sundaicus complex is reported as a widely distributed *Anopheles* species in Indonesia, from Sumatra, Kalimantan, Java, Lesser Sundas, Sulawesi, to Maluku, except Papua (1). *Anopheles sundaicus* complex belongs to the Pyretophorus series (220). It is considered as one of the most important malaria vector in this country. *Anopheles sundaicus* is the main vector of malaria along coastal areas (3,188,191,258). This taxon is proven to transmit *P. falciparum* and *P. vivax.* (187). *Anopheles sundaicus* s.l. was first reported as malaria vector in west Java in 1918 (229). Then, many studies have also confirmed *An. sundaicus* as an important malaria vector in Java (240,255,259), the Lesser Sundas (Sumbawa, Flores, western Sumba, and Adonara islands) (199), Sulawesi (260), and Sumatra (Sihepeng, Nias, Riau/Bintan island, Lampung) (197).

      An. sundaicus s.l. has an anthropophilic preference, however, the biting location for *An. sundaicus* is unclear. More exophagic habit was identified in Sumatra, Lombok (Lesser Sundas) and Java (240,254,259,260). In contrast, more endophagic behavior was found in Sumba (eastern Lesser Sundas) (191,240,261). In west Java, biting peak activity was reported during the first and last quarters of the night. A lower biting peak activity was reported during the second and third quarters of the night in Central Java. *An. sundaicus* typically rests indoors, especially on clothes, walls or curtains; and outdoors under shaded trees, rock crevices and bushes (3,188,191,233).

      The larval stages of *An. sundaicus* s.l. are mostly found in brackish water, although also occurring in freshwater habitats with sunlit. Larvae have been collected from particular sites such as lagoons with filamentous algae, marshes blocked streams with brackish water, rice fields and irrigation ditches. This species has been found mostly along coastal lowlands to slightly higher elevations at altitudes up to 300 m asl (3,191,230,245,247,261).

19) Anopheles vagus

Anopheles vagus is distributed throughout the main islands of Indonesia, except Papua (1). *Anopheles vagus* belongs to the Pyretophorus series (220). This species has been confirmed as a malaria vector in several malaria endemic areas of this country, i.e in Menoreh hills, Central Java (262) and Kupang, East Nusa Tenggara (197). So far, the taxonomic status of this species in Indonesia is still unclear (197,199,252). However, the national research, which updated disease vectors and disease reservoirs data (Rikhus Vektora) under the Ministry of Health (MoH) Indonesia during the period 2016-2018, revealed two closely related species circulating in Indonesia, namely *An. vagus* and *An. limosus* (263).

An. vagus complex tends be zoophilic, exophagic and exophilic. In addition, this species is often found as the predominant species compared with other *Anopheles* during field collections. *An. vagus* was also more dominant outdoor than indoor in several study sites in Java, Sulawesi and Lesser Sundas. *An. vagus* typically rests around cattle shelters (outdoors), especially from studies done in Central Java, Central Sulawesi and Lesser Sundas (200,227,264). In West Java, blood-feeding activity occurred throughout the night (226) with a biting peak activity reported during the second quarter of the night in East Java. Larvae of *An. vagus* are typically found in grassy ditches, low bushes, ground pits and surrounding salak plantations (3,227,265).

Habitats of *An. vagus* immature stages are typically under sunlit, with fresh or low salinity, stagnant water with warm temperatures, including river edges, small pools, spring, irrigation ditches, wheel ruts, rice fields. This species has been found from coastal lowlands to hilly areas at altitudes up to 1,100 m asl (3,188,204,266).

20) Anopheles tessellatus

Anopheles tessellatus is widely distributed throughout the main islands of Indonesia (1). *Anopheles tessellatus* belongs to the Neomyzomyia series (267). This species has been confirmed as a malaria vector in Nias island, North Sumatra (251). *An. tessellatus* tends to be zoophilic (226,254,268). Feeding behaviour varies with location. In West Java, its behavior is endophagic (biting indoor), whereas exophagic behavior is more common in Sulawesi and Lombok (200,201,245,264). *An. tessellatus* typically prefers to rest outdoors (227). Blood-feeding activity of this species was reported during the second quarter of the night in Sukabumi, West Java (202). The habitats of the larval stages of *An. tessellatus* are typically sunlit, fresh to relative high

salinity, slow-moving water, including fish ponds, small pools, spring, irrigation ditches, wheel ruts, rice fields, and puddles (3,188,191,247).

21) Anopheles farauti

Anopheles farauti is a complex comprising 8 sibling species belonging to the *Punctulatus* group (220). Species of this complex are distributed in Maluku and Papua only (1). At least 5 of 8 *An. farauti* complex members have been identified based on molecular analysis in Papua (3). Most of the data of *An. farauti* study were conducted before it was known to be a complex of sibling species, so it was difficult to distinguish the bionomics of each species of the complex. This species complex is well known as composed of the most important malaria vectors in this country. *Anopheles farauti* s.l. was first reported as a malaria vector near Jayapura in 1950s (235). Since then, several studies and surveys have also confirmed *An. farauti* s.l. specimens with sporozoites (*P. falciparum* and *P. vivax*) in Arso, Armopa, Atuka, Timika, Tipuka, Mapurujaya and Gag islands (197,217). The behaviour of this species complex varies according to the location and sibling species. In Sorong, Jayapura and near Jayapura, *An. farauti* s.l. was reported as being strongly exophagic (234). Biting behaviour may also vary from location. In Entrop, near Jayapura, biting peak ativity was reported during the first quarter of the night, whereas in Arso, biting peak activity was reported during the second and third quarters of the night (233,269). In Jayapura, *An. farauti* 4 was predominant species sampled both indoors and outdoors during early evening hours (234). In the coastal northwest of Papua, *An. farauti* typically rested indoors after feeding but left the house before dawn (269). In contrast, the other sibling species have a strong exophilic behaviour (233).

The larval stages of *An. farauti* s.l are found in brackish or freshwater with sunlit. The specific habitat preference depends on the sibling species. The immature stages of *An. farauti* s.s. were mostly found in brackish habitats in coastal areas. While larvae of the other sibling species of the *An. farauti* complex were collected from a variety of sites such as lagoons, marshes, ponds with vegetation, along river banks, borrow pits, garden pools, large and small streams with grassy margins, fishponds and ditches. This species complex is found from the coastal lowlands to hilly areas at altitudes up to 2,250 m asl (3,205,206,215,235,269).

In brief, the binomics of malaria vectors in Indonesia can be seen in Table 3 below:

Table 3. Indonesian malaria vectors and their bionomics

No.	Species	Distribution	Habitat	Biting habits	Sporozoite rate (%)	Malaria vector confirmation
1	*An. aconitus*	Java, Sumatra, Kalimantan, Sulawesi, West Nusa Tenggara, East Nusa Tenggara	Rice fields, fresh water pools, river banks, irrigation channels, ponds. The species found with an altitude of 100-900 asl, and sometimes found > 1000 asl	Zoophilic/ zoo-antropophilic; Biting activity before midnight; peak biting between 8-10 pm; exophagic	0.1-17.8	1919 West Java (Cianjur) 1954, 1998: Central Java (Purworejo) 1978, 1982 : Central Java (Banjarnegara) 1980: Central Java (Jepara, Wonosobo) 1998 : Central Java (Purworejo)
2	*An. annularis*	Sumatra, Kalimantan, Sulawesi, West Nusa Tenggara, East Nusa Tenggara	Freshwater fish ponds, clear water, rice fields, slow movement mountain streams	abundant in around cattle, buffalo. Biting activity before midnight; exophagic	0.35	1920 : Sulawesi
3	*An. barbirostris* s.l.	Java, Bali, Sumatra, West Nusa Tenggara, East Nusa Tenggara, and Sulawesi	sunlit water bodies containing fresh and often clean water including lagoons, marshes, pools, slow running streams, along river banks, springs, rice fields, fish ponds, drainage ditches and wells swamps with grass (can live in brackish water with salinity <5%). The species can found from coastal to hilly areas (2000 asl)	Zoophilic/anthropophilic. Biting activity before and around midnight; exophagic	0.3-13.3	1929-1942: South and Southeast Sulawesi 1939 : South Sulawesi 1990 East Nusa Tenggara, Sulawesi 2008 : Sulawesi

4	*An. balabacensis*	Java, Sumatra, Kalimantan, Sulawesi, Lesser Sunda islands	stream-side rock pools, river banks, puddles, muddy animal wallows, tyre tracks and hoof prints, pools under shrubs/trees. The habitats of this species is associated to hilly, forested terrain up to 700 m asl	anthropophilic, biting activity from midnight to early morning; exophagic/endophagic		1962 : West Java (Purwakarta) 1981 : East Kalimantan (Balikpapan) 1982 : Central Java (Banjarnegara), South Kalimantan (Batulicin) 1987 : Kalimantan 2000 : Central Java (Purworejo, Magelang, Kokap), East Kalimantan 2007: Central Java (Menoreh hills), South Kalimantan
5	*An. barbumbrosus*	Almost all major islands of Indonesia, except Papua	small wells, slow running water in rice-fields and jungle (260-1370 asl)	anthropophilic		1950 : Sulawesi 1952 : Sulawesi
6	*An. bancroftii*	Seram island, Maluku, Papua	a shady swamp in the forest, stagnant, clear fresh water pools, ditches, swamps with vegetation, semi-shaded places along the edges of small lakes with lily-like vegetation, and marshes	endophilic	0.04-4.3	1920 : Papua (Tanah Merah) 1935 : Papua 1957 : Papua (Merauke)
7	*An. farauti* s.l.	Papua, Maluku, and North Maluku	lagoons, marshes, ponds with vegetation, along river banks, borrow pits, garden pools, large and small streams with grassy	Biting behaviour may also vary from location. In Entrop, near Jayapura, biting peak ativity was reported during the first	0.8	1929 Papua 1950 Papua (Jayapura) 1979 Papua

			margins, fishponds and ditches. This species complex is found from the coastal lowlands to hilly areas at altitudes up to 2,250 m asl	quarter of the night, whereas in Arso, biting peak activity was reported during the second and third quarters of the night		
8	*An. flavirostris*	Sumatra, Java, Kalimantan, Lesser Sundas, and Sulawesi	rice fileds, pools, springs, shaded grassy edges of clear, slow-moving small streams, irrigation channels with slow running water (with/without vegetation). This species can be found from coastal plains to hilly areas, up to 600 m asl	biting peak during the third quarter of the night (in Flores); zoophilic, sometimes attracted to humans	0.07-1.3	1949 : South Sulawesi (Malili) 1979 : East Nusa Tenggara (Flores) 1985
9	*An. koliensis*	Maluku-Papua	grassland, along the edge of jungle, ditches, riverside ponds, footprints, wheel ruts and sometime in pig ruts and wallows; This species can be found from lowland to higlands with altitude up to 1,700 m asl; side by side with *An. farauti* s.l.	Anthropophilic; Biting activity around midnight & third quarter of night; exophagic	0.3-0.63	1956 : Papua (Jayapura) 2007 : Papua (Mimika)
10	*An. kochi*	Sumatra, Java, Sulawesi, Kalimantan, Maluku	fresh or brackish habitats, running or stagnant, often with muddy water, rice fields, ponds, pools, drain along roads, buffalo-	zoophilic, there are also interested in humans. Biting activity before 9 pm	1-12.5	1958

			hoofprints, wells, ditches. This species found from coastal to hilly areas with altitute up to 1,100 m asl			
11	*An. karwari*	Sumatra, Java, Kalimantan, Sulawesi and Papua	marshes, small, slow moving streams, irrigation channels associated with rice fields, small swamps, seepages, ground and rock pools, springs	zoophilic		1958
12	*An. leucosphyrus*	Sumatra, Sulawesi, Kalimantan	marshes, small streams, seepage springs, jungle pools, fishponds, ground depressions, wheel ruts, hoof prints	anthropophilic, biting activity around midnight	1.7-6.4	1951 : Kalimantan 1980 : Southern Kalimantan 1982 : Sulawesi
13	*An. maculatus* s.l.	Almost all major islands of Indonesia, except Papua	fresh and clean water including slow-moving streams, stream-side rock pools, drying river beds, ground seepages, small pools and puddles, natural springs, ponds, ditches, and rice fields. This species can be found from coastal to hilly areas with altitutes up to 1,100m asl	zoophilic / anthropophilic, biting activity 9pm-3am. Endophagic / exophagic	1.4-11	1982 : Central Java (Wonosobo, Kokap, Jepara) 1998 Central Java (Purworejo)
14	*An. nigerrimus*	Sumatra, Java, Kalimantan, Lesser Sundas and Sulawesi	prefer sunlit, fresh and clean water habitats. The larval sites include marshes, pools,	zoophilic / anthropophilic, endophagic / exophagic,	2.1-3.5	1996 : North Sumatra (Sihepeng)

			rice fields, irrigation channels, fishponds. This species has been found from coastal to hilly areas at altitudes up to 700 m asl	peak biting density after sunset		
15	*An. punctulatus*	Papua, Halmahera, North Maluku	riverside pools, pig ruts, wheel prints, grasslands, along jungle edges, pools, freshwater coastal marshes, low-lying riverine areas, ground depressions and shallow drainage around houses, footprint, ditches, earthen drains and rockpools in drying stream beds. This species found from coastal to hilly areas at altitudes up to 1,260 m asl	usually bites humans outdoors; When biting humans indoor, peak-biting ativity was reported in the second quarter before midnight	1.5-5	1929 : Papua 1979 : Papua (Timika, Arso, Armopa, Mapurujaya, Tipuka) Papua (Oksibil, near Wamena, and Obio)
16	*An. parangensis*	Sulawesi, North Maluku	fresh or coastal brackish water with sunlit or under shade including stagnant pools, fish ponds, ground puddles, and marshes	exophagic		1996 North Sulawesi (Tomohon, Manado)
17	*An. sundaicus* s.l.	Sumatra, Kalimantan, Java, Lesser Sundas, Sulawesi, to Maluku, except Papua	brackish water, although also occurring in freshwater habitats with sunlit. Larvae have been collected from particular sites such as lagoons with filamentous	Anthropophilic; biting peak activity during the first and last quarters of the night.	0.5-35	1919 Java, Riau islands, Lampung, East Nusa Tenggara (Flores, Adonara, Alor island)

			algae, marshes blocked streams with brackish water, rice fields and irrigation ditches. This species has been found mostly along coastal lowlands to slightly higher elevations at altitudes up to 300 m asl			
18	*An. subpictus*	Sumatra, Kalimantan, Java, Lesser Sundas, Sulawesi, Maluku, to Papua	sunlit, fresh or brackish water habitats.The larval sites include coastal blocked freshwater rivers and streams, pools, mangrove forests, springs, fish ponds, borrow pits, drains, furrows in gardens, buffalo wallows, brackish ponds, seaweed ponds, and irrigation ditches. This species has been found mostly along the coast and very few specimens in hilly areas up to 700 m asl. Salinity (> 18% o to 40% o)	zoophilic, endophagic / exophagic, biting activity 11 pm-02 am	0.7 – 3.3	1919 Malaria vectors 1979 Sulawesi, Java, West Nusa Tenggara , East Nusa Tenggara (Sikka, Lembata)
19	*An. sinensis*	Sumatra and Kalimantan	sunlit, fresh and clean water habitats. Larval sites include marshes, pools, rice fields, irrigation channels, fishponds. This species has	zoophilic and exophagic. This species tends to bite during the first quarter of the night	0.3-16.6	1997: Northern Sumatra (Nias)

			been found from coastal to hilly areas at altitudes up to 700 m asl		
20	*An. vagus*	Almost all major islands of Indonesia, except Papua	sunlit, with fresh or low salinity, stagnant water with warm temperatures, including river edges, small pools, spring, irrigation ditches, wheel ruts, rice fields. This species has been found from coastal lowlands to hilly areas at altitudes up to 1,100 m asl	Zoophilic, exophagic, and exophilic; blood-feeding activity occurred throughout the night with a biting peak activity reported during the second quarter of the night in East Java	0.05-0.1	1995 : East Nusa Tenggara 2001 : Central Java (Purworejo) 2003 : West Java (Sukabumi)
21	*An. tesselatus*	throughout the main islands of Indonesia	sunlit, fresh to relative high salinity, slow-moving water, including fish ponds, small pools, spring, irrigation ditches, wheel ruts, rice fields, and puddles	zoophilic, biting activity between 5-6 pm. Endophagic, exophagic,	0.7	1979

1.1. *Anopheles* species complexes in Indonesia

Among *Anopheles* species, specimens within a taxon that share very similar morphological characters and are reproductively-isolated, are known as cryptic, sibling or isomorphic species, and the taxon itself as a species complex. Most of the *Anopheles* species that are implicated in malaria transmission in South and Southeast Asia have been identified as species complexes, including : *An. annularis, An. barbirostris, An. culicifacies, An. dirus, An. farauti, An. fluviatilis, An. leucosphyrus, An. minimus, An. philippinensis-nivipes, An. sinensis, An. subpictus* and *An. sundaicus* (207,270). A comprehensive understanding of *Anopheles* species in Southeast Asia, their role as malaria vectors, their bionomics, gene flow and the nature of isolation in generating divergence or homogenising variation within and among them, is key for better apprehending malaria transmission dynamics and a way to interrupt the disease through appropriate vector control approaches (271).

At least four *Anopheles* species complexes play an important role as malaria vectors in Indonesia (3,270,272–274). *Anopheles barbirostris,* which is considered to be an important vector of malaria and filaria parasites in several parts of Indonesia, has been recognized as a species complex. So far, three out of six sibling species within the *An. barbirostris* complex, with unclear distribution and vector status, have been recorded in this country (3,270,275).

The *Anopheles farauti* complex has also been reported in Indonesia. Recently, at least five out of eight members within the Punctulatus group have been identified in eastern Indonesia, including *An. farauti* s.s., *An. hinesorum, An. farauti 4, An. farauti 8,* and *An. oreios* (former *An. farauti 6)* (276,277). All of them are considered as important malaria vectors. *Anopheles farauti* s.s. reported as the most widely distributed of any members of the group, but its habitat is restricted to coastal areas, whereas *An. oreios* is the most probable major vector in the central highland of Papua and Papua New Guinea (276,277). Unfortunately, the major studies on *An. farauti* s.l. were conducted before the era of molecular (DNA) analysis techniques that could differentiate isomorphic species in the complex (3,278).

Anopheles sundaicus is known as another complex of sibling species in Indonesia. This taxon represents one of the most important malaria vectors in Indonesia. Sukowati and Baimai reported for the first time three cytological forms designated as A, B, and C using cytogenetics and enzymatic analyses that were identified from field collected mosquitoes from different areas in Thailand and Java and Sumatra, Indonesia (279). In Indonesia, *An. sundaicus* form A was collected from costal areas in Java and Sumatra. *Anopheles sundaicus* B form was mostly collected in both freswater habitats (in South Tapanuli, Northern Sumatra) and brackish water habitats (in Purworejo, Central Java). While form C was found on coastal area in Asahan, northern Sumatra (3,277). Recently, using mitochondrial DNA markers, Dusfour et al. analysed specimens collected on Java and Sumatra, different from previous sympatric forms A, B and C, which were found genetically identical and this species was named *An. sundaicus* E (281).

Furthermore, *Anopheles maculatus*, which belongs to the Maculatus group that includes nine species, is also known as an important malaria vector in Indonesia. Only *An. maculatus* s.s. has been reported in Indonesia, but there is evidence suggesting that there is also a species complex based on variations in mitotic chromosomes (282). Three variations (i.e., X1, X2 and X3) in female samples and two variations in males (Y1 and Y2) were reported in Purworejo District, Cental Java (3,206).

2. Malaria control: toward elimination – progress and challenge in Indonesia

Indonesia has succeeded in achieving most of the halfway of malaria elimination targets. Indonesia malaria elimination achievement was mostly the result of accelerated malaria control programme in the past decade. During the period of 2007 to 2017, annual parasite incidence fell by three times from 2.89 per 1,000 inhabitants to 0.9 per 1,000 with 66% reduction in fatals and 50% reduction in malaria confirmed cases. Currently, more than half of the districts have been officially declared malaria free with 72% of Indonesian population living in malaria free areas (280). While national program for malaria eradication was first established in 1952, intensified control efforts just began in 2004. Artemisinin combination therapy (ACT) was introduced and distributed as the first-line treatment due to the widespread chloroquine resistance. Rapid diagnostic tests (RDT) were done to complement microscopy as

standard laboratory confirmation to improve surveillance and report. Long-lasting insecticide-treated bednets were distributed nearly every 2 years in highly endemic areas. Approximately 20 million long-lasting insecticide-treated betnets (LLINs) have been distributed in this country since 2005. The continuous distribution of LLINs was then integrated with routine basic immunization program (BCG, DTP, Polio, Measles, and Hepatitis B) for children and antenatal care services. Indoor residual spraying (IRS) was conducted in high-risk villages with annual parasite incidence > 20 per 1,000 population and areas with malaria outbreaks. Clinical malaria screening for sick children was introduced into clinical management protocols. Strengthening capacity development was also established, including case investigation, case management, surveillance and vector control (283).

Evidence-based policy and advocacy across all levels of government, from national level (Ministerial action) to provincial governors, municipal majors, and district regents, have been successful to drive the effective malaria elimination programme in this country. In addition, community empowerment programme based on local specific conditions, such as community participation to reduce malaria transmission through environmental management and larval control; the role of local volunteers for active surveillance of migrant workers and migrant fishermen, has successfully participated to malaria elimination. Private sector engagement supported aggressive case finding to implement early diagnostic and prompt treatment (283).

National malaria working group with the Ministry of Health as a coordinator supported the budget (estimated US$110 million during 2003 and 2017), capacity development, human resource costs and salaries, procurement and malaria drug distribution. Additional financial and technical supports were also crucial, especially those coming from UNICEF, WHO, community organizations, private sectors, involved in the support for malaria elimination. The largest financial support comes from the Global Fund to Fight AIDS, Tuberculosis and Malaria that invested US $238 million between 2003 and 2017. At present, Indonesia is heading towards the goal of malaria elimination. Comprehensive malaria control efforts continue to be made through strengthening the surveillance system, upscaling diagnostic and treatment interventions, as well as vector control, not only in high-transmission districts, but also in low-transmission areas (**Fig. 7**) (283).

Change in incidence by district, 2009–17*
- New malaria elimination/certification district
- Major reduction in malaria incidence
- No change in malaria incidence
- Increase in malaria incidence†

Estimated intervention coverage‡
- <25% of target areas
- 26–50% of target areas
- 51–75% of target areas
- 76–100% of target areas

	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	2014	2015	2016	2017
All endemic areas														
National Ministerial Decree on Malaria Elimination														
Increased global and domestic financing for malaria														
Introduction and scale-up of artemisinin-based combination therapy														
Laboratory confirmation mandated for all malaria cases														
Quality assurance for diagnosis														
Malaria screening and treatment in pregnant women														
Malaria inclusion into sick child management in endemic areas														
Improved malaria surveillance and reporting														
Cross-district learning and mentorship														
High-transmission areas														
Introduction and scale-up of long-lasting insecticide treated bednets														
Indoor residual spraying in high-risk areas and outbreaks														
Larval control and environmental management														
Low-transmission areas														
Enhanced case investigation														
Transmission foci mapping														
Active surveillance and case finding														
Post-elimination areas														
Malaria screening among migrants														

Fig. 7. Changes in the incidence of malaria and various malaria control efforts in Indonesia in the period of 2004 to 2017 (283)

Monitoring and evaluation efforts have been carried out to support the achievement of the target of malaria elimination in 2030 with the support of national and local governments, national technical components (Directorate general of disease prevention and control-MoH and National Institute of Health Research and Development-MoH), donor agencies (Global Fund for Malaria, WHO, UNICEF), other government components and private sectors. Several activities carried out include monitoring anti-malaria drug resistance, monitoring the accuracy of diagnosis, both of rapid diagnostic test and microscopy, monitoring the resistance of mosquito vector species to LLINs, mapping malaria receptivity, especially those areas that have been and will be eliminated, and monitoring behavior changes of malaria vector populations (284).

Arboviruses

The term of arthropod-borne virus, known as arbovirus, is not a taxonomic term, but rather an ecological term to define viruses that are maintained in nature, multiply and produce viremia of sufficient titer in vertebrate hosts and are transmitted to new vertebrates through the bites of hematophagous (blood-sucking) arthropods, such as mosquitoes, ticks, and sandflies, *via* saliva (285–288).

Arboviruses have became the most important emerging infectious diseases in the world based on their geographic widespread and their public health impact on susceptible human populations (289–291). These viruses generate a significant burden of humans with various clinical manifestations ranging from asymptomatic infection, acute fever, muscle and joint pain, hemorrhagic and/or neurological symptoms to severe undifferentiate fever. They can also progress to worse conditions with long-term physical and cognitive impairment or in early death (292).

Arboviruses replicate in the arthropod vectors, such as mosquitoes, midges, ticks, or sandflies, prior to transmission. In a mosquito-borne viruse transmission, female mosquitoes acquire viruses during blood feeding from an infected animal or human. The virus then replicates in the mesenteronal ephithelial cells. Subsequently, the virus is released from the mesenteronal ephithelial cells and infects the salivary glands, from which it is transmitted to the vertebrate hosts during following blood feeding. Arboviruses consist of several families (**Fig. 8**), i.e. *Flaviviridae* (genus *Flavivirus*), *Togaviridae* (genus *Alphavirus*), *Rabdoviridae* (genus *Vesiculovirus*), *Ortomyxoviridae* (genus *Thogotovirus*), *Bunyaviridae* (genus *Orthobunyavirus, Phlebovirus, Nairovirus* and *Tospovirus*), and *Reoviridae* (genus *Orbivirus* and *Coltivirus*) (293).

Arboviruses have many complex life cycles involving vertebrate hosts/non-human primates and arthropod vectors. Mosquitoes are the main vectors of arboviruses. Although sandflies (*Phlebotomus* spp), ticks and gnats (*Culicoides* spp) are also important vectors of zoonotic viruses. Most human arboviruses were initially isolated

in tropical countries in Asia, Africa, and South America. However, in the past several decades, arboviral diseases have expanded across the world at a fast pace frequently causing outbreaks. Climate, changes in viral genetics, and more specifically increasing exchanges of goods and movements of people across the world, have contributed to the transmission and expansion of viruses, resulting in re-emergence or emergence of arboviral diseases and associated outbreaks in new locations throughout the world (293–295).
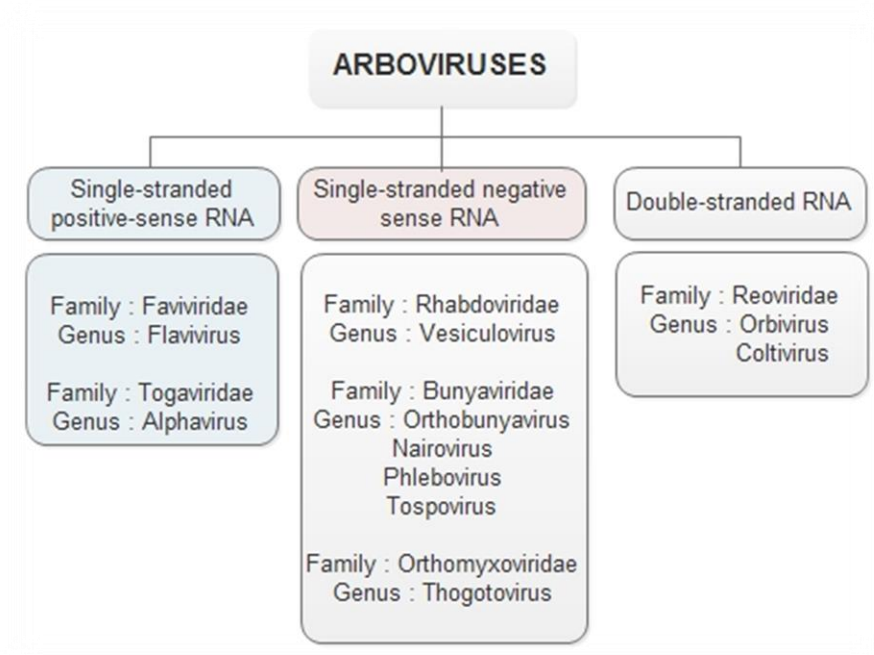
Fig. 8. Classification of arboviruses (293)

Currently, a total of 537 arboviruses have been identified and registered in the International Catalogue of arboviruses with at least 130 among them being the most important human pathogens. Based on the Baltimore classification, arboviruses resulting in human disease belong to three taxonomic groups : double-stranded RNA viruses, positive-sense single-stranded RNA viruses and negative-sense single–tranded RNA viruses. Most of these are included in several taxonomic families, including *Flaviviridae, Bunyaviridae, Togaviridae, Rabdoviridae* and *Reoviridae* families (**Fig. 8, 9**). (285,293,296,297). Many of the important human and animal arboviruses belong to the families *Flaviviridae* and *Togaviridae,* which are transmitted by mosquitoes (293).
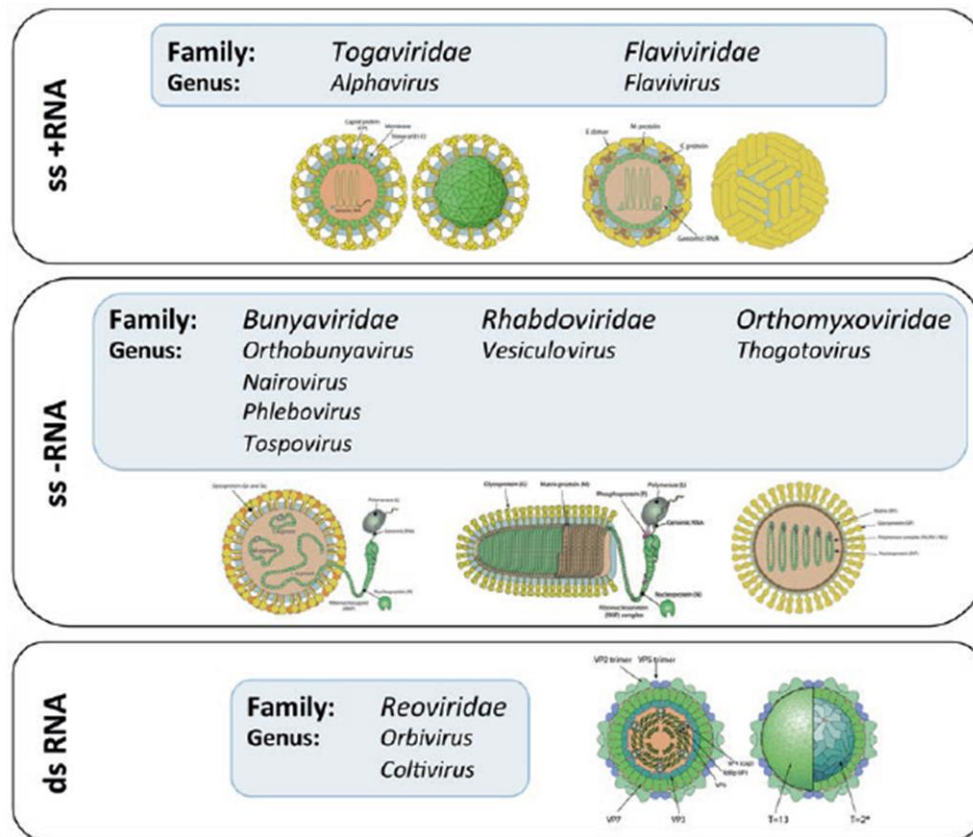
Fig. 9. Arboviruses and virion schematic. Arboviruses related to human disease are found within the Flaviviridae, Togaviridae and Bunyaviridae. The viruses are grouped according to composition of the genome: ss+RNA (single-stranded positive-sense RNA ss); ss-RNA (single-stranded negative-sense RNA); dsRNA (double-stranded RNA). Virus schematics provided by ViralZone, Swiss Institute of Bioinformatics (298).

5. *Flaviviridae*

5.1. Classification

In accordance with the International Committee on Taxonomy of Viruses, a subgroup of the Division of Virology of the International Union of Microbiology Societies, the *Flaviviridae* is a family with several genera including *Flavivirus* (74 species), *Hepacivirus* (1 species), and *Pestivirus* (4 species). Of which, approximately 40 species of *Flavivirus* are mosquito-borne viruses and 16 species are tick-borne viruses. All species within *Herpacivirus* and *Pestivirus* and at least of 18 species of *Flavivirus* are not identified to be transmitted by arthropod vectors (299).

5.2. Structure of *Flaviviridae*

Flaviviridae is a family of small enveloped viruses containing a single-stranded positive-sense, non-segmented RNA genome of approximately 9-13 kb in length (9.2-11, 12.3-13, 8.9-10.5 and 8.9-11.3kb for members of genera *Flavivirus, Pestivirus, Hepacivirus* and *Pegivirus*, respectively) (297,298). These virus members contain a single, long open reading frame (ORF) flanked by 5' and 3' terminal non-coding regions, which specific secondary structures required for genome replication and translation (302).
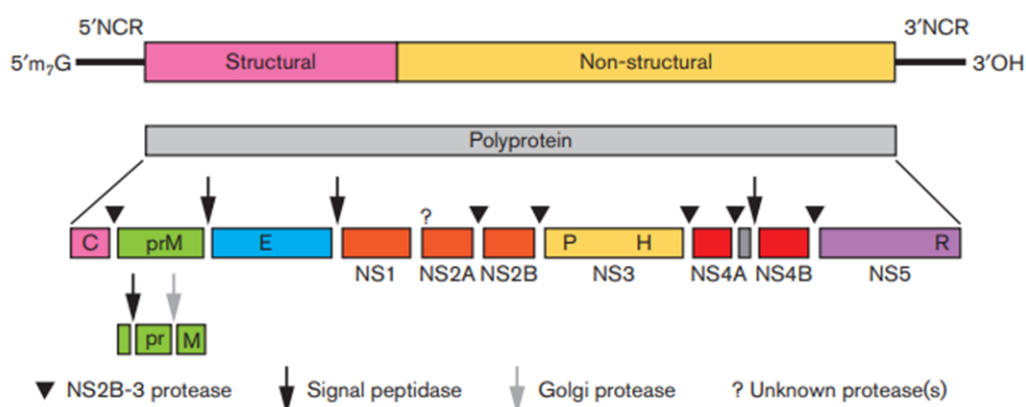
Fig. 10. Organization of genome of the genus *Flavivirus* (302)

Virions of *Flaviviridae* are typically spherical in shape, enveloped, 40-60 nm virions with a single, small core protein (except for genus *Pegivirus*) and 2 (genera *Flavivirus, Hepacivirus* and *Pegivirus*) or 3 (genus *Pestivirus*) envelope glycoprotein (302).

The structure of genus *Flavivirus* consists of a nucleocapsid protein and RNA that is surrounded by a host cell-derived lipid bilayer containing the E and prM/M proteins (300,303). This virus genome encodes three structural proteins (capsid [C], precursor membrane [PrM], and envelope [E] protein), and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) (**Fig. 10**) (293,304,305). Three structural proteins are involved in the mature and infectious virion, while the non-structural proteins have roles in polyprotein processing, viral RNA synthesis, and virus morphogenesis (306). Two of the non-structural proteins, NS3 and NS5 are

multifunctional and essential for the viral replication. The N-terminal one-third of NS3 (NS3pro) and its co-factor, NS4A or NS4B is the main viral protease. A part of NS2B is also involved in its activity (307,308). NS3 has also nucleoside triphosphatase and helicase activities that are important for replication (309). In addition, NS3 plays a role in the post- and co-translational cleavage of the polyprotein in the cytoplasm together with host proteases in the endoplasmic reticulum lumen to yield the mature proteins (306). Due to the role of NS3 having an important part in enzymatic activities, viral replication and polyprotein processing, this gene segment is promising as a drug target for antiviral therapy (310).
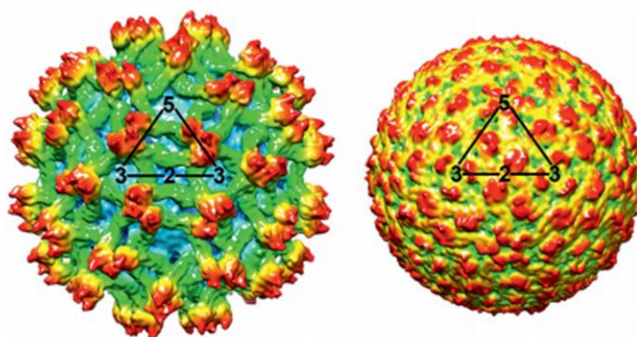
Fig. 11. Reconstruction of three-dimensional cryo-electron of immature (left) and mature (right) particles of a dengue virus isolate. Triangle outline one icoxahedral unit with the 2- ,3- and 5-fold axes of symmetry (courtesy of Richard Kuhn and Michael Rossmann). The picture illustrates a surface rendering of immature dengue virus at 12.5 A° resolution (left) and mature dengue virus at 10 Å resolution (right) (311)

NS5 is a multi-domain protein that contains N-terminal methyltransferase and C-terminal RNA-dependent RNA polymerase (RdPp) (306,312,313). RdPp replicates (+)RNA into (-)RNA that will be used as template to produce large excess of the viral genome (313). In addition, NS5 methyltransferase activity and NS3 5'triphosphatase are thought to be involved in the capping of the viral RNA by removing terminal γ-phosphate and performing sequential N7 and then 2' O methylations (312). The other non-structural protein of the *flavivirus*, NS1, plays a role in the early part of the replication. However, the functions of NS2A, NS4A and NS4B are not well known, but they allegedly play important roles in replication, virus maturation, and modulating NS3 and NS5 activities (306,314).

The *flavivirus* is a highly diverse genus of both vector-borne and non-vector-borne viruses, which comprises more than 70 viruses distributed nearly worldwide, except Antarctica (293,303,315,316). Approximately one-third of them are important human pathogens having a very significant impact on the burden of diseases (317). Some of the major medically important *flavivirus* are mosquito-borne ones, such as viruses of Dengue (DEN) (**Fig. 11**), West Nile (WN), Yellow Fever (YF), Zika (ZIK), Japanese encepalitis (JE), Murray Valley Encephalitis (MVE), St Louis Encephalitis (SLE), Kunjin (KUN) and Edge Hill (EH). According to WHO, flaviviral infections are classified as neglected tropical diseases (NTDs) (310). However, the current epidemiological situation worldwide is of great concern as DEN, ZIK, YF, JE have became a serious public health threat.

5.3. *Flavivirus* cell entry and replication cycle

a. ***Flavivirus*** **cell entry**

The initial step of the *flavivirus* to enter the cell is through the binding of the E glycoprotein to a cellular receptor. *Flavivirus* must recognize particular cell surface molecule or utilize multiple receptors for cell entry as *flavivirus* infection has been inspected from various cell lines derived from different host species. Recently, several studies have indicated that *flaviviruses* may use multiple receptors for cell entry (303).

In mosquito cells, Dengue virus (DENV) has been identified to interact with 45-kDa protein, heat-shock protein 70, R80, and R67. Crystallographic studies on DENV-DC-SIGN complexes revealed that interaction between DENV with DC-SIGN could be mediated through the carbohydrate moiety at Asn67 in EDII. Interaction between West Nile virus with DC-SIGN and DC-SIGNR have also been shown in dendritic cells (300,315,316). In addition, several important arboviruses, particularly WNV, JEV and DEV have been observed to bind to $\alpha v\beta 3$ integrins expressed on mammalian cells, mediated through interaction with EDIII (320,321).

Flaviviruses entry into cells through clathrin-mediated endocytosis. In the cell entry process of DENV, virus particles diffuse along the cell surface towards a pre-existing clathrin-coated pit. Furthermore, the clathrin-coated pit evolves and the

invagination in the plasma membrane is closed by membrane scission mediated by dynamin to form a clathrin-coated vesicle. The clathrin-coated vesicle is transported away from the plasma membrane after which the clathrin coat is released from the vesicle. A study of WNV infection inhibition has documented that inhibition of *flavivirus* infection was observed in cells treated with chlorpromazine (322). This chemical inhibitors prevent clathrin-coated pit formation and expressing dominant-negative mutants of Eps15 in cells (323,324). However, recent study of DENV entry in mammalian cells has revealed that this entry was independent of clathrin, caveolae and lipid rafts. It is important to note that the route of *flavivirus* cell entry depends on the cell type and the virus strain (325).

In the subsequent process, the endocytic vesicle carrying the virus is delivered to early endosomes. Process of internalization of a large fraction of *flavivirus* particles occurs rapidly. In DENV, membrane fusion has been observed within late endosomal compartments. The fusion of the membrane was detected about 10-13 minutes after initiation of infection. In this process, the low-pH environment within endosomes triggers a series of molecular events within the E glycoprotein leading to viral membrane fusion with the endosomal membrane and release of the nucleocapsid into the cytosol cell. Protonation of one or more histidine residues has been postulated to trigger the changes of E glycoprotein conformational (303,326).

The early step in membrane fusion involves protonation-dependent disruption of the E protein rafts at the viral surface. This leads to exposure of the fusion loop at the distal tip of DII to the target membrane. Afterwards, E proteins insert their fusion loops onto the outer leaflet of the membrane and three copies of E interact each other *via* their fusion loops or DII domains to form an unstable trimer. The E trimers stabilize through additional interactions between DI domains of the three E proteins. Subsequently, DIII folds back against the trimer to form a hairpin-like configuration. The energy released by these conformational changes induces the formation of a hemifusion intermediate. The monolayers of the interacting membranes are merged while the inner membranes are still intact. For the final step, a fusion pore is formed, after that, the nucleocapsid is released into the cytosol (303). Besides the low-pH, cholesterol also plays an important role in facilitating efficient cell entry of *flaviviruses*

as viral infectivity was found to be significantly impaired in cholesteral-depleted cells (327).

b. Replication of *Flaviviruses*

Generally, the mechanisms of *flavivirus* infection of the host cells and its life cycle are not fully clear. The current consensus has revealed that endocytosis of the viral particle is an important step in the successful infection of the cell and production of progeny viruses. Attachment of the *flaviviruses* to cells utilizes several potential receptors that are important to facilitate the binding and internalization of the virus (i.e. R80, CD14, heparin sulfate, C-type lectin receptors, DC-SIGN and mannose receptors). The attached virus is internalized into an endosomal compartment, which acidifies to facilitate the fusion of the viral envelope with the endosomal compartment. Subsequently, fusion occurs in the viral envelope due to the rearrangement of the capsid proteins, resulting to virus release into the host cell. Furthermore, the viral RNA is released into the host cytoplasm and transferred into the endoplasmic reticulum (ER) (328). Replication and viral RNA synthesis process of the *flavivirus* occur on an extended network of modified ER membranes (**Fig. 12**). Three membranous structures are known in *flavivirus*-infected cells, they are membranous sacs or vesicle packets (Vp), membrane vesicles (Ve) and convoluted membranes (CM). Vps are used as sites of replication by the virus. Vps are a part of Ve cluster formed by ER membranes modification. Ve are opened to the cytoplasm, whereas the CMs are suggested to form the sites of translation processing of polyprotein and /or storage sites for viral proteins (329).
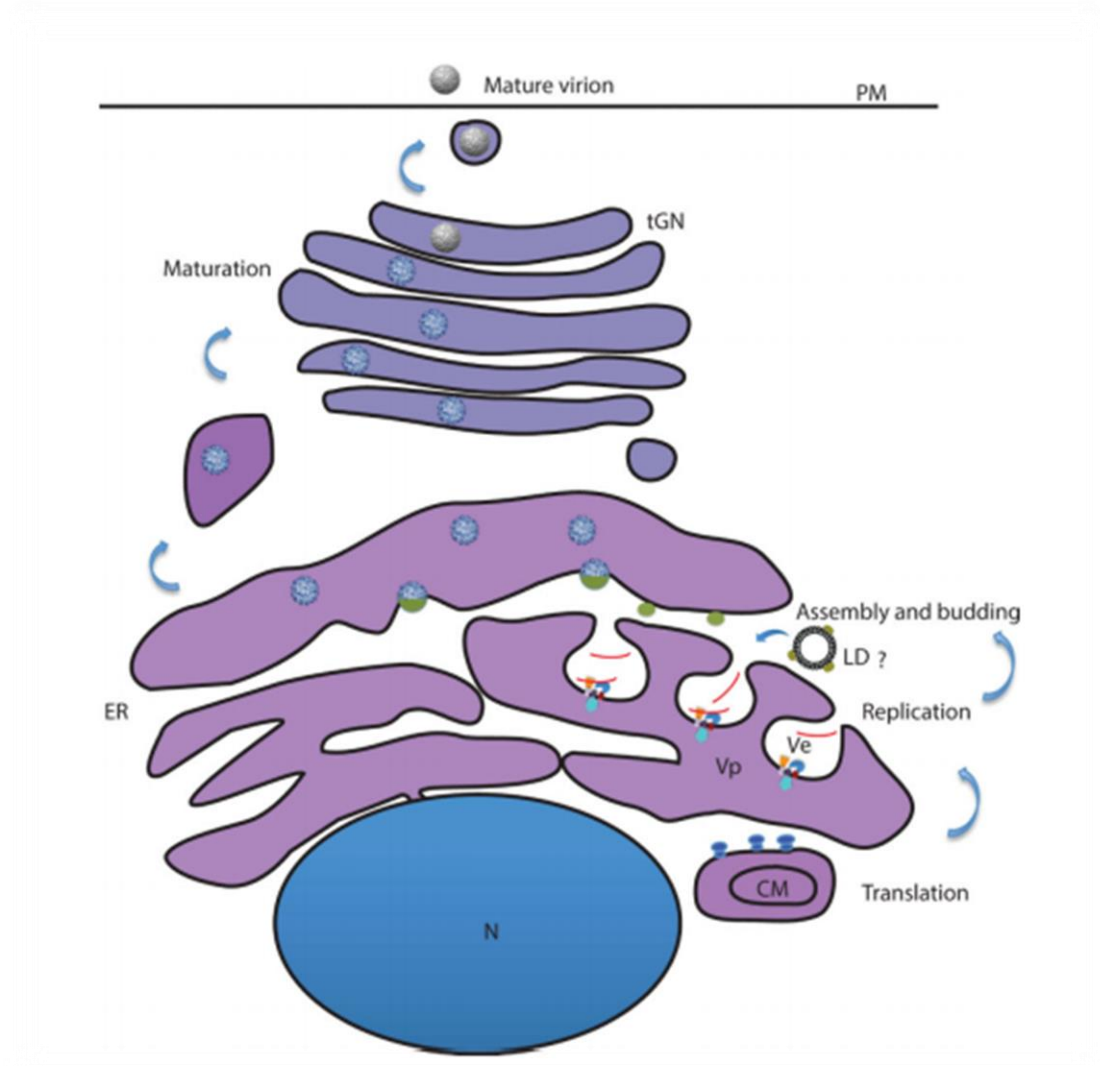
Fig. 12. *Flavivirus* genome replication and assembly (329)

In the subsequent process, the viral RNA undergoes two different mechanisms. First, the positive sense RNA is translated to produce a polyprotein, which is post-translationally cleaved into structural and non-structural proteins. Or second, the genetic material is converted into negative sense RNA by viral NS5 RNA-dependent RNA polymerase (RdRp) and used to produce positive-stranded RNA copies. The viral genome is then packaged within the cytoplasm by support of protein C to form the nucleocapsid. While the PrM and E proteins heterodimerize within the lumen of the ER and initiate viral budding. New virion particles are formed within the ER travel through the secretory pathway and into the Golgi apparatus. Changes in pH within the trans-Golgi network trigger the dissociation of the prM/E heterodimers activating the cellular endo-protease furin. Activation of this protease leads to cleavage of the prM protein to

generate protein M and the peptide pr. The cleavage of this complex protein results in a fully infectious virion (328).

5.4. Phylogenetic analysis of *Flavivirus*

Phylogenetic analysis of the *Flavivirus* reveals a virus family that has evolved rapidly after originating from an ancestor, which possibly appeared in Africa several thousands years ago (330,331). Evolutionarily, *flaviviruses* have shown substantial ecological diversification with different lineages as a result of adapting to various vectors and transmission pattern. They have also developed unique strategies to avoid innate and adaptive immunity from their host (332). According to the phylogenetic tree of the genus *Flavivirus* (**Fig. 13**), all human *flaviviruses* are transmitted by insect vectors (333).

Analysis of the genetic variations of the *flaviviruses* by using coalescent theory and a maximum likelihood (ML) demographic model reveals that *flavivirus*es are growing at an exponential rate, with specific viruses, such as dengue and Japanese encephalitis. The DENV increased rapidly in the recent past, while Japanese encephalitis virus (JEV) changed from constant population size to exponential growth within the last century (334). DENVs are also an example of an emerged *flavivirus* and global health problem that have changed dramatically over the past century. DENV has evolved to a molecular clock that is serotype and genotype specific. Phylogenetic and time analysis suggest that DENV serotypes separated within the last 1,000 years, and the behaviour change of DENV from a sylvatic cycle to sustained human transmission may have occurred between 125 and 320 years ago (333,335,336).
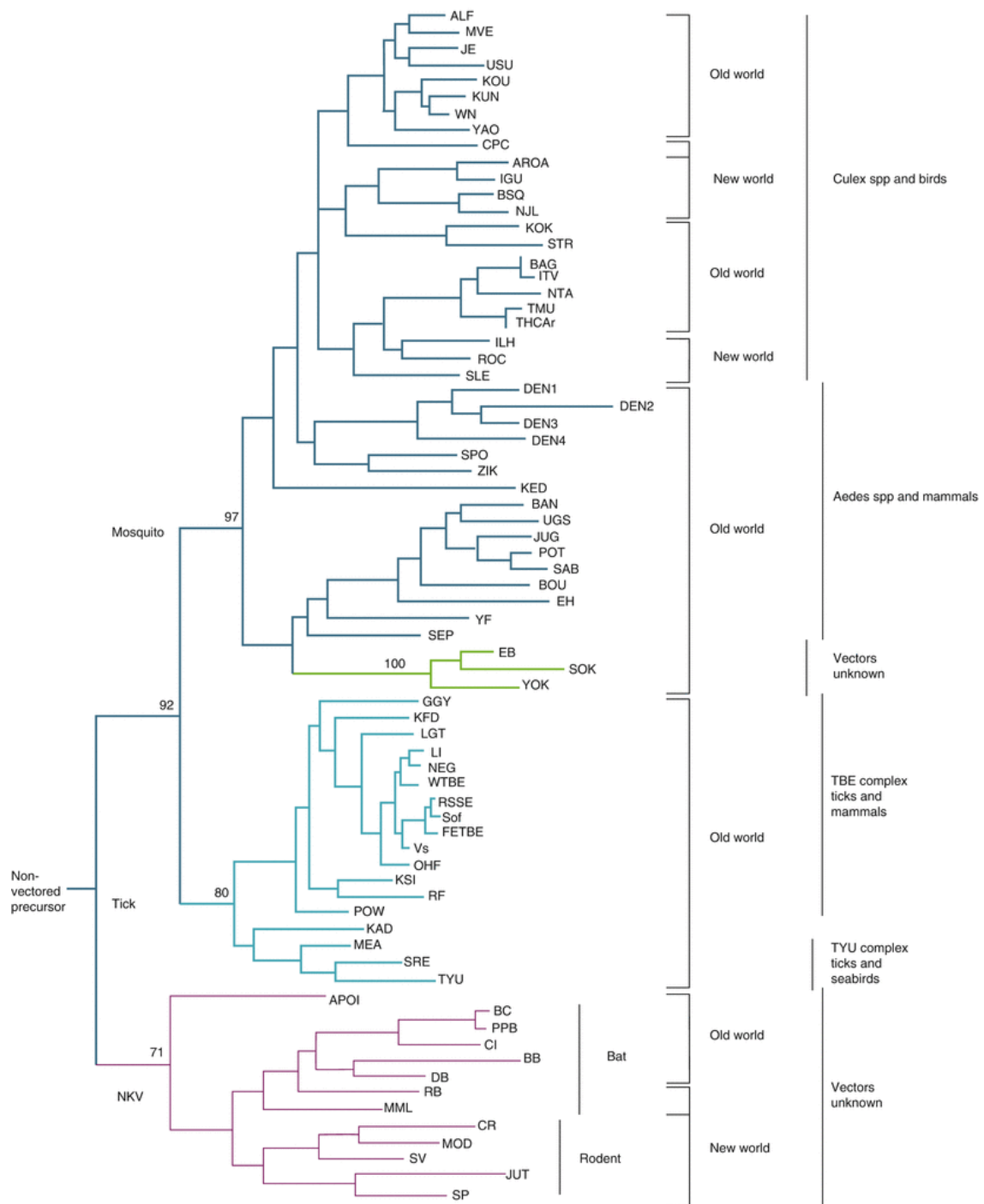
Fig. 13. Phylogenetic tree of the genus *Flavivirus* showing the association of the groups of related viruses with their invertebrate vectors, vertebrate hosts and geographic distribution. Abbreviations of virus names: ALF, Alfuy; MVE, Murray valley encephalitis; JE, Japanese encephalitis; USU, Usutu; KOU, Koutango, KUN, kunjin; WN, West nile virus; YAO, Yaounde; CPC, Cacipacore; AROA, Aroa; IGU, Iguape; BSQ, Bussuquara; NJL, Naranjal; KOK, Kokobera, STR, Stratford; BAG, Bagaza; ITV, Israel Turkey meningoencephalomyelitis virus; NTA, Ntaya; TMU, Tembusu; THCAr, strain dengue; ILH, Ilheus; ROC, Rocio; SLE, St. Louis encephalitis; DEN1-4, dengue ; SPO, Spondweni; ZIK, Zika; KED, Kedougou; BAN, Banzi; UGS, Uganda S; JUG, Jugra; POT, Potiskum; SAB, Saboya; BOU, Bouboui; EH, Edge hill, YF,

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Yellow fever; SEP, Sepik; EB, Entebbe bat; SOK, Sokoluk; YOK, Yokose; GGY, Gadgets gully; KFD, Kyasanur forest disease; LGT, Langat; LI, Louping ill; NEG, Negishi; WTBE, Western european tick borne encephalitis; RSSE, Russian spring and summer encephalitis; SOF, Sofjin; FETBE, Far eastern tick borne encephalitis; VS, Vasilchenko; OHF, Omsk haemorrhage fever; KSI, Karshi; RF, Royal farm; POW, Powassan; KAD, Kadam; MEA, Meaban; SRE, Saumarez reed; TYU, Tyuleniy; APOI, Apoi; BC, Batu cave; PPB, Phnom Penh bat; CI, Carey islan; BB, Bukalasa bat; DB, Dakar bat; RB, Rio Bravo; MML, Montana Myotis Leucoencephalitis; CR, Cowbone ridge; MOD, Modoc; SV, Sal vieja; JUT, Jutiapa; SP, San perlita (333,337).

6. Medically important mosquito-borne *flavivirus*es in Indonesia

In the 1960s, *flaviviruses* were first discovered in Indonesia and since then they have spread in almost all regions of the country. At least seven *flavivirus*es have been serologically reported in Indonesia, including Dengue virus (DENV), Japanese encephalitis virus (JEV), Murray Valley Encephalitis virus (MVEV), West Nile Virus (WNV), Kunjin virus (KV), Edge Hill virus (EHV), and Zika virus (ZIKV). All of these viruses have been responsible for human infections. Among which, DENV is considered to be the most important *Aedes*-borne *flavivirus* causing a major disease burden in Indonesia (338). Since JEV, MVEV, EHV, WNV and ZIKV have been reported to infect humans in Indonesia, these viruses have became additional *Aedes*-borne *flaviviruses* needing much attention and further studies in relation to disease transmission, incidence rates in human and respective vectors (4,339).

6.1. Japanese encephalitis virus (JEV)

JEV is a mosquito-borne *flavivirus* that is endemic in Southeast Asia and South Asia (340,341). JE causes an estimated 68,000 cases every year with case-fatality rate (CFR) of up to 30% and long-term neuropsychological sequelae in 30-50% of survivors. In humans, the viremia is usually very short with low titres, so this virus is considered as a "dead end host". Most JE infections of humans are asymptomatic or result in unspecific flu-like illness. The ratio of symptomatic to asymptomatic cases due to JEV infection varying between 1 in 25 to 1 in 1,000. This may be explained by partial protection due to previous flavivirus exposure in the indigenous population, age related differences, different genetic susceptibility to Japanese encephalitis, or differences in disease surveillance between JE endemic countries(340).

JEV circulation in Indonesia was first documented when serological surveys were conducted in Lombok in 1960 (342). Since then, JEV infections have been reported nearly every year in endemic areas and Indonesia has became a JE endemic country. JEV was first isolated from field collected mosquito species such as *Culex tritaeniorhynchus* in Bekasi, West Java and Kapuk, West Jakarta in 1972 (30,33,342). This species is the major vector of JEV in Indonesia, but also in Southeast Asia (343,344). However, Van Peenen *et al.* have also isolated JEV in *Cx. fuscocephalus* and *Cx. gelidus* (30). These species are extremely common, widespread and breed mainly in paddy fields. These mosquitoes are predominantly cattle blood feeders, and humans are the dead end host. So far, at least of 10 mosquito species, including *Cx. tritaeniorhynchus, Cx. gelidus, Cx. vishnui, Cx. fuscocephala, Cx. bitaeniorhynchus, Cx. quinquefasciatus, Anopheles vagus, An. kochi, An. annularis* and *Armigeres subalbatus* have been confirmed as JEV vectors in Indonesia (345). However, several species of *Aedes* including *Aedes togoi, Aedes japonicus,* and *Aedes vexans* have also been confirmed as JEV vectors in Asia (347). The natural reservoir of JEV is vertebrates. Pigs are the major vertebrate host and considered as amplifying host. However, JE is also reported to be positive in bovine, horse, goat, sheep and avian in several parts of Indonesia (32,342,347,348).

Currently, JE cases were found in at least 29 provinces of the country, including Bali, West Kalimantan, East Nusa Tenggara, West Java and East Java being the areas with high incidence. Although many surveys and studies have been implemented in Indonesia, particularly in high endemic areas, a routine surveillance of JE has not been established at the national level (345). Many evidence of the presence of JEV prompted the need for mass vaccination programme followed by routine tailor-made immunization for specific JE endemic areas in Indonesia. In 2018, a mass vaccination program was initiated in the Bali Province. This program is the initial stage of the national JE vaccination program launched by MoH Indonesia. A total of 890,050 Balinese children aged from 9 months to 15 years were targeted for vaccination with a single dose of the Chengdu SA14-14-2 live-attenuated JE vaccine through a two-phase, school-based and community-based mass campaign. This program was funded and coordinated by the MoH Indonesia and Global Alliance for Vaccines and Immunization (GAVI). JE vaccines are now included in the Balinese immunization routine programs and are administered together with the measles – rubella vaccine in children aged of 9

months (346). In the near future, the mass vaccination program will be carried out in several provinces having high numbers of JE cases (350).

6.2. Edge Hill virus (EHV)

Another *Aedes*-borne *flavivirus* in Indonesia is Edge Hill virus (EHV). This virus is a member of the Yellow fever virus (YFV) group. It is known as an important *flavivirus* in Australia (351). This viral infection have specific clinical symptoms including myalgia, arthralgia, and muscle fatigue. In Southeast Australia, the mosquito species *Ochlerotatus vigilax* has yielded most of the EHV isolates. This virus has also been isolated from other mosquito species. Wallabies and Bandicoots were reported as probable vertebrate hosts. However, studies about EHV reservoirs are still limited (352).

In Indonesia, EHV was detected from human samples in Samarinda, Kupang and Papua. All samples were collected in the period of 1972-1979 (4). However, more recent data on EHV infection in Indonesia are not available.

6.3. West Nile Virus (WNV)

West Nile virus (WNV), also a *flavivirus*, is considered as a public heatlh burden causing epidemic viral encephalitis and known to cause outbreaks of encephalitis in Europe and North America (353,354). WNV was first isolated from the blood of a woman in the West Nile district of Uganda in 1937 (355,356). Since then, the virus has been reported endemic in Africa, Western Asia, and the Middle East. Recently, WNV has expanded its distribution into Europe, Russia, and Americas (357). Clinical symptoms range from mild fever to severe or neuroinvasive disorder, including acute faccid paralysis, meningoencephalitis, encephalitis, meningitis or a combination of them (358).

WNV is maintained in nature among wild birds and mosquitoes. In Africa, *Culex univitattus* has been confirmed as the main WNV vector. In Asia, members of the *Cx. vishnui* complex have been identified as main vectors. In Europe, particularly France, *Cx. modestus* has been reported as the WNV vector. In addition, *Culex pipiens,*

Cx. salinarius, Cx. restuans, Ochlerotatus canadensi, Oc. japonicus, Aedes vexans, and *Culiseta melanura* have also been recorded as WNV vector in Americas (357).

Prior to 2004-2005, Indonesia never reported any WNV endemic area, then WNV was reported twice from a serological study in Lombok, as well as archived samples of 2 acute febrile patients that were hospitalized as suspected hantavirus patients at two hospitals in Bandung, West Java (339,359). Samples were collected from a patient ≥ 10 years old with fever of unknown etiology, hemorrhagic manifestations, renal insufficiency, liver dysfunction and non-cardiogenic pulmonary oedema. The other samples were collected from a 15-years old male admitted for febrile illness with epistaxis, gastrointestinal symptoms, elevated serum transaminases, leucopenia and trombocytopenia. With these findings, more attention should be given to WNV as it may be considered as a serious threat to public health in Southeast Asia, particularly Indonesia (339). Strengthening surveillance studies in humans (dead end host), mosquito vectors and vertebrates, such as birds (amplifier hosts) and horses (dead end host), must be implemented as it is crucial to better investigate this disease in Indonesia.

6.4. Zika virus (ZIKV)

Zika virus (ZIKV) is an emerging vector-borne pathogen characterized by a single-stranded positive RNA of 10,794 bases. This virus is closely related to other members of the *flavivirus*, in particular Dengue, Yellow Fever, West Nile and Japanese encephalitis viruses (360–363). Approximately 20-25% of human cases infected with ZIKV present symptoms, while 75-80% are asymptomatic although ZIKV positive. The reported symptoms of infected persons include fever, cutaneous rash, headache, arthralgia, myalgia, malaise, anorexia, asthenia, lymphadenopathy, non-purulent conjunctivitis, conjunctival hyperemia, arthralgia, myalgia, peripheral edema, gastrointestinal disturbance persisting for a few days and then disappearing. Early clinical manifestations are common and similar to other infections, particularly other arboviral infections including DEN and CHIK (364–366).

ZIKV was first isolated in 1947 from a rhesus macaque monkey in the Zika forest of Uganda (367). The virus was also isolated from pooled specimens of *Aedes*

africanus mosquitoes collected in the same forest in early 1948. The first cases of ZIKV infection in human were reported from Nigeria in 1952 (363,368–370). During the period of 1952 through 2007, serological data and virus isolation from 14 human cases only were reported from Asian countries such as India, Malaysia, The Philippines, Thailand, Vietnam and Indonesia; and African countries including Egypt, Tanzania, Uganda, Central African Republic, Sierra Leone (371–375).

Sixty years after its discovery, an outbreak of ZIKV in humans was reported in the Micronesian island of Yap in 2007, resulting in about 5,000 cases in this area (362,376). Subsequently, ZIKV outbreak was then recognized in French Polynesia in 2013 and 2014 with about 32,000 cases identified as ZIKV infection (374). An increased incidence of the neurological Guillain-Barré syndrome and microcephaly in new-borns from mothers infected with ZIKV were reported concomittantly with the outbreak (378). The virus then spread rapidly from Pacific to Easter island and continued its way to Central and South America and the Carribean in 2015 to 2016 (377).

As of 8 June 2016, a total of 60 countries and territories reported continuing ZIKV transmission, of which 46 countries have experienced a first outbreak of ZIKV since 2015, with no previous evidence of circulation of this virus, and with ongoing transmission by *Aedes* mosquitoes. Between 2007 and 2014, at least 14 countries reported evidence of ZIKV ongoing transmission. The evidence of person to person transmission, probably *via* sexual route was also reported in 10 countries. Eleven countries have reported evidence of microcephaly and other central nervous system malformations associated with ZIKV infection. In addition, a total of 13 countries and territories have reported an increased incidence of Guillain-Barré syndrome. These countries also reported laboratory confirmation of the association ZIKV infection - Guillain-Barré syndrome (379).

Indonesia has been known as a country with a ZIKV transmission history. Seven people with serological evidence of ZIKV illness were reported by hospital-based surveillance in Klaten, Central Java during 1977 through 1978 (6). A subsequent serological study of arboviruses was also conducted in Lombok, West Nusa Tenggara in 1979. Results showed that 31% among 71 human volunteers had neutralizing

antibodies to ZIKV (359). In 2012, a case of ZIKV infection was also reported in an Australian traveler who returned from Jakarta with fever and rash (380). One more human case with ZIKV infection was also detected during a dengue outbreak in Jambi in the period of December 2014 – April 2015. This positive sample was confirmed among 103 case-patients clinically diagnosed with dengue but negative for DENV infection by RT-PCR, NS1 antigen detection, or evidence of seroconversion by ELISA (381). In the same year, a traveler returning to Australia with fever, rash and conjunctivitis was also confirmed as ZIKV case after being infected in Bali (382).

6.5. Dengue (DEN)

6.5.1. Dengue epidemiology

Dengue is an arbovirus caused by four dengue serotypes (DENV-1, DENV-2, DENV-3, and DENV-4) transmitted to humans through the bite of infected *Aedes* mosquitoes (383). Dengue is a complex disease with a wide spectrum of clinical symptoms, ranging from asymptomatic, especially in endemic areas of high dengue hemorragic fever (DHF), to a life-threatening DHF, which is often unrecognized or misdiagnosed with other fever-causing tropical diseases (384,385). There is the most rapidly spreading mosquito-borne viral disease in the world (386).

6.5.2. History and spread of Dengue

Historically, first clinical descriptions of dengue-like syndrome were recorded approximately at A.D. 992 in China, although the first well documented cases of what are believed to be dengue occurred in 1779-1780 (64). Then, the ethiology of DENV was experimentally suggested in 1907 (387).

After World War II, DENV was formally discovered by scientists from Japan and US. The DENV was isolated by inoculation of serum of patients in suckling mice. The blood samples for this experiment were collected during a dengue epidemic in Nagasaki, Japan in 1943 (388–390). Subsequently, DENV was recorded in Calcutta, India from serum samples of US soldiers in 1944 (391). The serotype isolated in Japan and Calcutta, India was then known as the first serotype of DENV (DENV-1) (392). DENV-2 serotype was first identified in Trinidad in 1953 (393). The third (DENV-3)

and fourth (DENV-4) serotypes were first found in 1954 when DHF/DSS (Dengue shock syndrome) emerged in cities of the Philippines and Thailand (394). DENV-3 was also isolated in Puerto Rico, Caribbean in 1963 (395). DENV-3 serotype caused epidemics in the eastern Caribbean and Jamaica during the same year (393). In 1981, DENV-4 was first reported in Americas. This DENV-4 was also of Asian origin (397).

It has been hypothesized that the movement of troops, destruction of both human settlement and the environment during Word War II have contributed to the spread of Dengue viruses and their mosquito vectors throughout Southeast Asia and Western Pacific (395,396). Currently, increases in human population and movement, global urbanisation, climate change, and enlargement of the range of vector habitats have led to the rapid and wide spread of dengue virus to new geographic areas (400).

6.5.3. Global burden

In the past 50 years, dengue has evolved from a sporadic disease to a major public health problem worldwide. Currently, more than 120 countries have been reported as dengue endemic areas, particulalry in South America, Southeast Asia, Western Pacific, Africa and Eastern Mediterranean region. The global burden of dengue was estimated to reach 390 million people being infected with 96 million cases annually worldwide (401). However, the true disease burden is not well known, particularly in Indonesia, Brazil, China, Africa, and India. Several studies calculated that the true number of dengue cases is 2 to 28 fold than what is being reported by national surveillance systems (383,401–403).

Dengue illness has caused a high economic burden on both individuals and governments. In Americas, the average cost needed to overcome this disease reaches US $ 2-1 billion per year, excluding vector control (403). In Southeast Asia, an estimated 2-9 million cases with 5,906 deaths each year are caused by dengue infection. The annual economic burden is estimated at US $ 950 million in the region (404).

6.5.4. Dengue situation in Indonesia

Indonesia is one of the highest dengue endemic countries in the world. Dengue was first described in Indonesia in 1968 in Surabaya and Jakarta (9,10). In the past 45 years, annual Dengue haemorrhagic fever (DHF) incidence increased significantly from 0.05/100,000 in 1968 to 50.75/100,000 in 2015. By contrast, the case fatality ratio of DHF decreased considerably from 41% in 1968 to 0.83% in 2015. The areas affected by the disease in 2015 included 412 districts/municipalities (82.9%) of the total of 497 districts/municipalities in the country) (338). The prevalence of the disease follows a seasonal trend, which corresponds to the seasonal upsurgence of *Ae. aegypti* and *Ae. albopictus*. All four serotypes (DENV-1 to DENV-4) are endemic in most large cities of the country (9). The prominent virus serotype varies every year, however, DENV-1 and DENV-2 have been the most frequently ones during the last decade (405,406).

Dengue is predominantly endemic in urban areas where more than 35% of the country population live. Rapid development of industrial and economic sectors over the past three decades had an impact with massive infrastructural development in both commercial and housing sectors. Large scale migration from rural to urban in many cities of Indonesia has created slum areas and settlements with inadequate water and sanitation facilities. Limited water storage management has resulted in many potential breeding places for both vectors, *Ae. aegypti* and *Ae. albopictus.* Previous studies have reported that *Ae. aegypti* and *Ae. albopictus* larvae are predominantly found in container holding water for bathing, cooking and toilet flushing (345,407). These factors, in addition to others such as rapid transportation, favourable temperature and humidity have led to a rapid spread and rise of dengue transmission in Indonesia (9).

The government of Indonesia, which is coordinated through the Directorate General of Disease Control and Prevention, Ministry of Health, has undertaken various efforts to prevent and control dengue transmission *via* the national DF/DHF prevention and control programme. Control activities have been implemented since 1968 with the objectives to prevent and reduce morbidity and mortality due to DHF at the family and community levels. However, dengue transmission is still ongoing due to the complexity of the dynamic of this disease in Indonesia (9).

6.5.5. Dengue virus evolution

A phylogenetic study of various DENV strains from all serotypes reveals two major related clusters and one out group cluster. Cluster I contains two different subclusters of DENV-1 and DENV-3 strains, while Cluster II is represented by DENV-2. Subsequently, Cluster III includes DENV-4 strains (408).
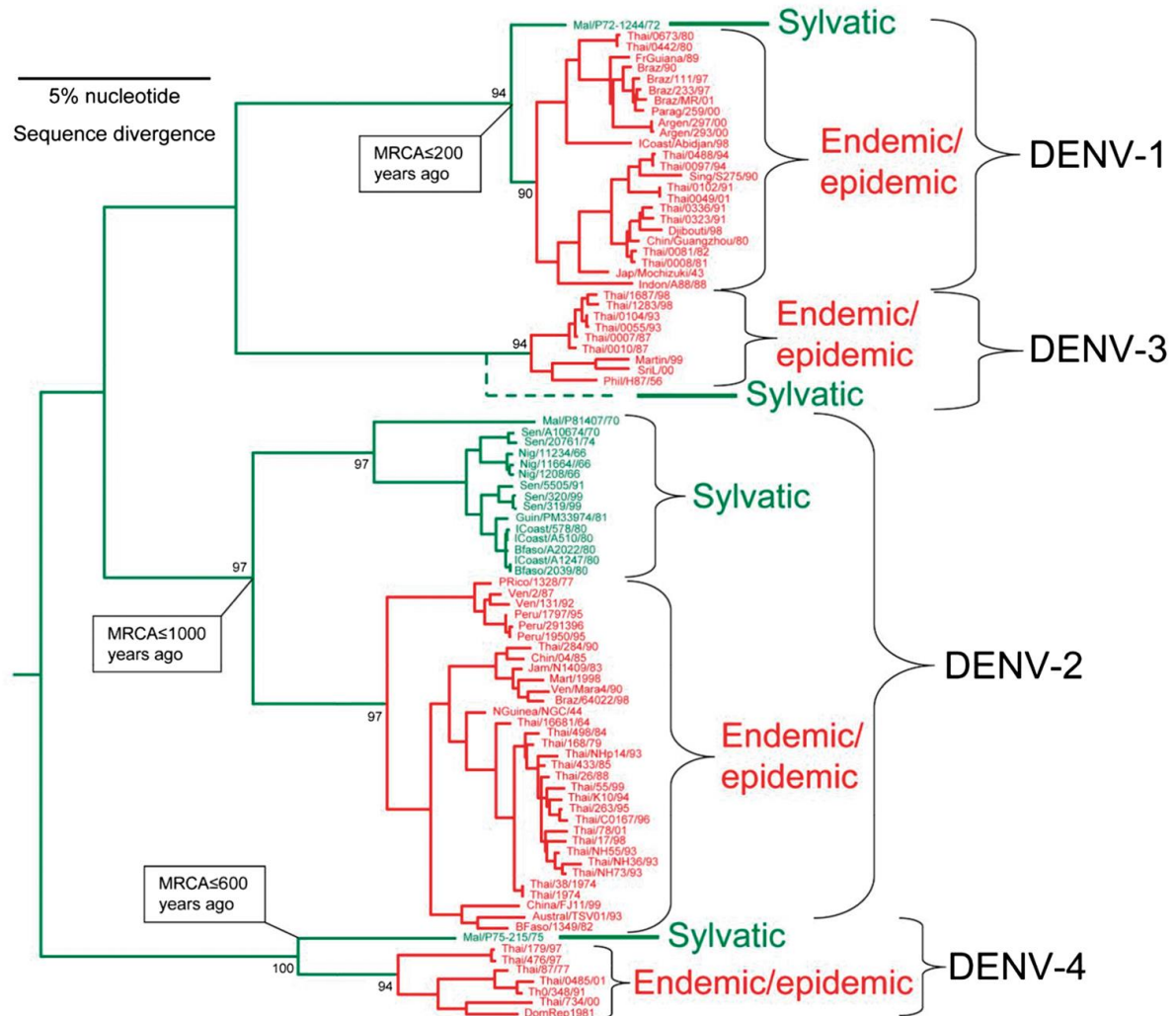
Fig. 14. Phylogenetic tree of DENV strains from four different serotypes derived from complete open reading frames (408)

6.5.6. Transmission cycle of Dengue virus

Dengue transmission occurs through interactions among people, mosquitoes, viruses and environmental factors (383). The DENV is transmitted to humans through the bites of infected female *Aedes* mosquitoes, particularly *Ae. aegypti* as primary vector and *Ae. albopictus* as secondary vector. After blood-feeding an infected person with DENV, viruses mutiply in the midgut of the mosquito. Afterwards, viruses

disseminate to various organs and tissues, including salivary glands. The extrinsic incubation period, which is the time for *Ae. aegypti* to start sucking blood from the host containing the virus until the virus is actually transmitted to the new host, takes about 8-12 days during normal ambient temperature (25-28ºC) (406,407). Besides the temperature, variations in the extrinsic incubation period are also influenced by initial viral concentration, virus genotype, the magnitude of daily temperature fluctuations. These can also alter the time for a mosquito to transmit the virus (411–414). Once infectious, mosquitoes will be able to transmit the virus for the rest of their lives (415).

Mosquitoes become infected from DENV viremic people, such as persons having symptomatic, pre-symptomatic or asymptomatic dengue infection (416). Generally, the transmission from human to mosquito occurs 2 days before someone shows any clinical manifestation of the illness, up to 3 days after the fever has recovered (416,417). The risk of mosquito infection is positively related to high viremia and high fever in patients. In contrast, high levels of DENV-specific antibodies are associated with a reduced risk of mosquito infection. Most people experience the viremic phase for about 4-5 days, but viremia can last for about 12 days (415,418).

6.5.7. Clinical manifestations

After incubation for a period of 3 to 8 days, dengue virus infection may be asymptomatic or may led to undifferentiated fever, dengue fever (DF) or dengue hemorrhagic fever (DHF) with plasma leakage that may led to dengue shock syndrome (DSS). However, most of dengue infections are asymptomatic or subclinical, so that most patients will recover after self-limiting disease (11,419).
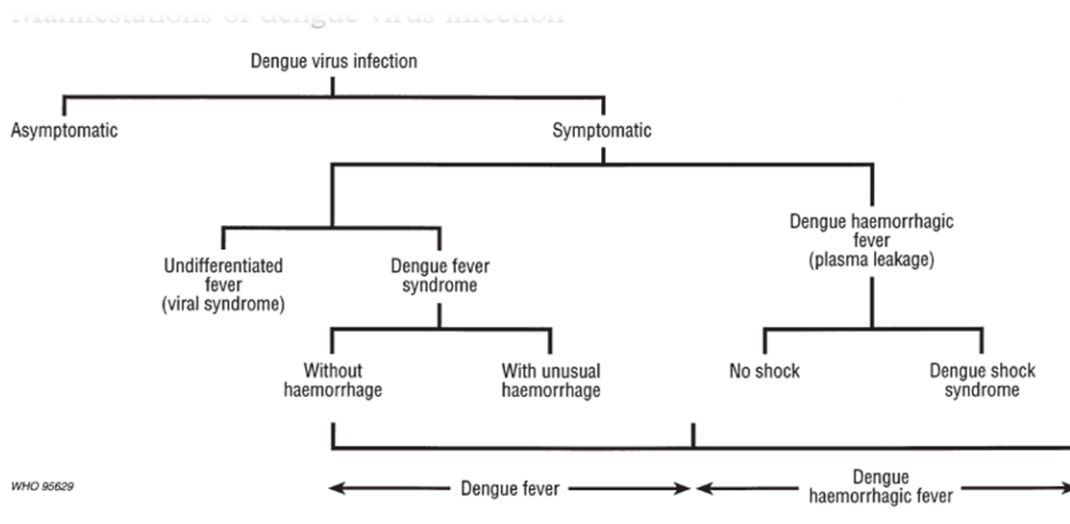
Fig. 15. Schematic manifestations of dengue virus infection (11)

In general, dengue clinical symptoms start unexpectedly and then follow three phases, i.e. febrile, critical, and recovery. The initial phase starts with high fever ($\geq$38.5°C) accompanied by two or more of the following symptoms: severe headache, pain behind the eyes, nausea, vomiting, muscle and joint pains, swollen glands, sometimes with a transient macular rash. During this phase, mild hemorrhagic manifestations such as petechiae, bruising and palpable liver are commonly recorded. This phase lasts for 3 to 7 days. Hereafter, most patients fully recover (11,420).

A small proportion of patients progresses to severe disease, generally characterised by plasma leakage with or without bleeding. The critical phase occurs around the time of defervescence, proven by increasing hemoconcentration, hypoproteinemia, pleural effusions, capillary permeability, leading to hypovolaemic shock that can cause organ impairment, metabolic acidosis, disseminated intravascular coagulation and severe haemorrhage. During the transition from febrile to critical phase, occurring between the 4th and 7th days of the illness, the situation is crucial as the patient may develop vascular leakage. The signs of impending deterioration appears with persistent vomitting, lethargy, restlessness, tender hepatomegaly, severe abdominal pain, increasing hematocrit level accompanying with rapid decrease in the platelet count, and mucosal bleeding. Severe dengue also includes hepatitis, neurological disorders, encephalopathy, myocarditis, or severe bleeding (major skin bleeding, mucosal bleeding, etc) with no obvious precipitating factors and only minor plasma leakage or shock (383,420). If patients stay untreated, mortality can reach 20%,

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however, with appropriate case management and intravenous rehydration, the mortality can be reduced to less than 1% (383).

During the recovery phase, the altered vascular permeability is reverting spontaneously to normal level after 48 to 72 hours. The patient then recovers immediately. A second rash may appear during the recovery phase. Itchy lesion may also appear over a period of one to two weeks. Adults may feel fatigue for several weeks after recovery (420).

6.5.8. Laboratory diagnosis

Laboratory diagnosis of dengue is essential for clinical management and surveillance. Diagnostic is established to detect the virus or its component (genome and antigen) directly or through the host response to the virus (indirectly). The sensitivity of each assay depends on the collection time of samples from the patient and the purpose of testing. During the febrile phase, which correlates closely with viraemia (detectable about 4-5 days after fever onset), detection of viral nucleic acid in patient's serum is done by using reverse-transcriptase polymerase chain reaction (RT-PCR) and real time RT-PCR assay. Otherwise, the detection of the virus expressing soluble non-structural protein 1 (NS1) uses the lateral-flow rapid test or enzyme-linked immunosorbant assay (ELISA), suitable for a confirmatory diagnosis. The sensitivity of NS1 used in febrile phase is high (exceed 90%) for primary infections (persons who have not been infected previously, typically for most travelers from non-endemic countries) and lower in secondary infections (60-80%). The decrease in sensitivity for secondary infections is due to an anamnestic serologic response from a previous dengue virus or other *flavivirus* infection (420,421).

Serological diagnosis of dengue has been used for the detection of high level of serum IgM that binds with DENV antigens in an ELISA test or a lateral-flow rapid test. In a primary infection, anti-dengue virus IgM is typically detected about 4-5 days after fever onset and lasts 2-3 months, whereas anti-dengue virus IgG is detected relatively slowly, with low titres 8-10 days after fever onset. In secondary infection, anti-dengue virus IgM can be undetectable in some cases, in contrast, anti-dengue virus IgG are rapidly detected with high fever soon after fever onset (383,420).

6.5.9. Dengue pathogenesis

Pathogenesis for severe dengue is complex and depends on the balance among viral factors, the host genetic and immunological background (422).

a.   Host risk factors for DHF/DSS

The differences of severity in DENV infection can be observed in both individual and population levels. HLA and non-HLA genetic factors (Fcɣ receptor IIA, vitamin D receptor, G6PD deficiency, tumor necrosis factor α, interleukin 10) have been associated with disease severity (423–427). More specifically, polymorphism in TNFα, Fcɣ receptor IIA, vitamin D receptor, CTLA-4 and transforming growth factor β (TGF- β) genes have been associated with development of DHF (423,428); while G6PD deficiency has contributed to increase replication of DENV in monocytes. In addition, mannose-binding lectin 2 (MBL2) gene may also contribute to increase the risk for developing DHF and thrombocytopenia (429). Diabetes, sickle-cell anemia, bronchial asthma, and other host genetic diseases have also been associated with severe dengue. In addition, major histocompatibility complex class I chain-related protein A (MICA) alleles are associated with symptomatic infection, while major histocompatibility complex class I chain-related gene B (MICB) alleles are associated with asymptomatic infection. Alleles of PLCE1 and MICB have been identified associated with susceptibility to DSS (430,431). Difference in microvascular permeability between children and adults can contribute to case fatality. Recent studies have reported increased rates of hospital admissions and case fatality rate of DHF/DSS in children more than adults during secondary infections (432). Skin color has also shown its effects in relation to the severity of dengue infection. Reduced dengue infection severity in black individuals *versus* white individuals has been observed (383).

b.   Vascular leakage associated with cytokine storm

Cytokines that have been produced in observed patients with DHF / DSS show rapid changes over the course of illness (433). Apart from that, several soluble factors produced by T cells, monocytes, macophages, and mast cells, are thought to contribute

to increased vascular permeability in primary endothelial cells, including TNFa, Interleukin (IL) -6, IL-8, IL- 10 and IL-12, matrix metalloproteinases, macrophage inhibitory factor, MCP-1, and HMGB-1 (422,434–436).

Endothelial permeability can also be affected by NS4B maturation status, which modulates the occurrence of cytokine response in a study of monocytic cell lines (434). Other than that, secreted NS1 protein, along with anti-NS1 antibodies and complement activation, maybe involved in DENV-induced leakage of vascular system (383,438).

c. Autoimmunity

Although considered controversial, autoantibodies that result in platelets and endothelial cell dysfunction have been reported to correlate with DHS / DSS pathogenesis (439,440). Antibodies produced during DENV infection have shown to cross-react with some self antigens. These results have revealed that production of these antibodies is unclearly associated to secondary DENV infection (441). Antibodies against some protein E epitopes can bind to human plasminogen inhibiting activity of plasmin (383). A study in mice revealed that anti-NS1 antibodies specific to cross-reaction with human and mouse platelets lead to transient thrombocytopenia and hemorrhage. In addition, anti-NS1 antibodies with vascular endotelial cells (EC) are causing cell apoptosis (442).

d. Antibody Dependent Enhancement (ADE)

Many previous studies have discussed the role of ADE in the pathogenesis of dengue (443). However, not all studies support this hypothesis (441). ADE is a model of the mechanism of virus-antibody complexes binding to Fcɣ receptor-bearing cells, resulting in increased cell mass and a rise in viremia (383). This model suggests that, at the population level, ADE can provide a competitive advantage to DENV serotypes that have increased antibodies compared to those that do not, providing tangible advantages with natural selection for previous serotypes (445).

e. T-cell response

Both specific serotype and cross-reactive memory T-cell response are generated during primary infection. The expression of viral epitopes in infected cells during secondary infection of the DENV triggers activation of serotype-cross reactive memory T cells, with pro-inflammatory production of cytokines, which can result in plasma leakage in vascular endothelium. Previous studies have reported the activation of memory T cells with low affinity at the time of infection but with a high affinity for previous serotype infections (446). A data study from Vietnamese children suggest that T-cell activation in the blood is incompatible with the commencement of capillary leakage, and the possible activated T-cell sequestration in tissues has been suggested. Other studies have revealed that the ratio of regulatory T cells to effector T-cell responses was increased in patients with mild illness, but not in severe illness patients (422,447,448).

f. Complement activation

Complement activation is an important component in clinical manifestations of DHF, it is reported that the level of protein kapside (C3a and C5a) and complement activation products are correlated with the severity of DHF. When plasma leakage becomes most obvious and decreases in patients with DSS due to accelerated consumption, the levels of C3a and C5a will peak at the time of defervescence. Pre-existing cross-reactive antibodies, high levels of NS secreted, immune complexes are implied in mediating complement activation *via* classical and alternative pathways (449–451).

7. *Togaviridae*

7.1. Classification

The name of Togavirus refers to the envelope and is derived from latin "*toga*", which means a roman mantle or cloak (452). Before April 2019, Togaviridae family

included two genera, *Alphavirus* and *Rubivirus*. Within the family, the genus of Alphavirus includes a large number of diverse species, while the genus *Rubivirus* includes a single species, *Rubella* virus. Most of alphaviruses are mosquito-borne viruses and they are pathogenic in their vertebrate hosts (453). However, after that date, the genus *Rubivirus* has now been moved to the family *Metonaviridae* (453).

Alphaviruses are further divided into two groups, i.e. **1. New world alphaviruses** (e.g. eastern equine encephalitis virus, wester equine encephalitis virus, and venezuelan equine encephalitis virus). These virus group members are distributed across Americas and cause encephalitis in human and equines; **2. Old world alphaviruses** (e.g. chikungunya virus, sindbis virus, ross river virus, o'nyong-nyong virus, mayaro virus, ross river virus, barmah forest virus, and smliki forest virus). These virus group members are present in Asia, Australia, Europe, and portions of Africa. They cause fever, rash and arthritis in humans. Subsequently, the alphaviruses can be further divided into seven groups of alphavirus complexes according to their antigenically differences. (see **Fig. 16** below)
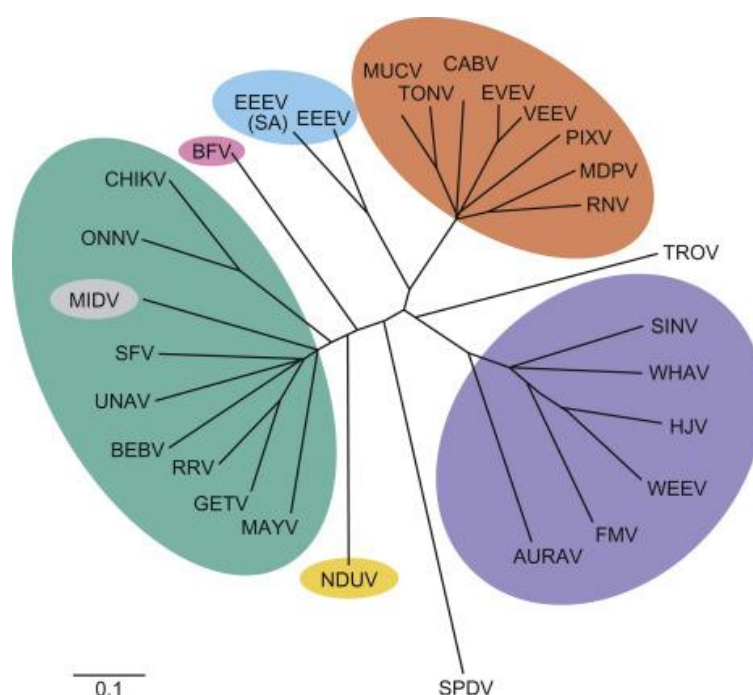
Fig. 16. Phylogenetic tree of representative isolate of all alphavirus species constructed from the E1 nucleotide sequences using the F48 algorithm of the neighbor-joining program. Abbreviations : AURAV, Aura virus; BFV, barmah forest virus; BEBV, Bebaru virus; CABV, Cabassou virus; CHIKV, Chikungunya virus; EEEV, eastern equine encephalitis virus; EVEV, everglades virus; FMV, fort morgan virus; GETV,

getah virus; HJV, highlands J virus; MAYV, mayaro virus; MIDV, middleburg virus; MDPV, mosso das pedras virus; MUCV, Mucambo virus; NDUV, ndumu virus; ONNV, o'nyong-nyong virus; PIXV, pixuna virus; RNV, rio negro virus; RRV, ross river virus; SPDV, salmon pancreas disease virus; SFV, semliki forest virus; SINV, sindbis virus; TONV, tonate virus; TROV, trocara virus; UNAV, una virus; VEEV, venezuelan equine encephalitis virus; WEEV, western equine encephalitis virus; WHAV, whataroa virus (454,455).

7.2. Structure of *Togaviridae*

Togaviridae is a family of small, enveloped, 65-70 nm spherical virions of regular structure with single capsid protein, a nucleocapsid core surrounded by a lipid bilayer that is embedded with glycoprotein spike. The nucleocapsid core comprises 240 copies of capsid proteins surrounding the viral genome. The lipid bilayer is hot-derived from the site budding; this virus buds from the plasma membrane. At least eighty trimeric glycoprotein spikes cover the surface of alphavirions. Each spike consists of three E1-E2 heterodimers. Each E1 and E2 comprises a single trans-membrane domain. E1 has a short cytoplasmic tail, while E2 has a long cytoplasmic domain. This E2 has a function in the interaction with a hydrophobic pocket in the capsid protein. This interaction will mediate between the external glycoprotein spikes and the internal nucleocapsid core that is rare in enveloped virion. This capsid, E1 and E2 proteins are the minimum proteins required for an infectious virion (453).
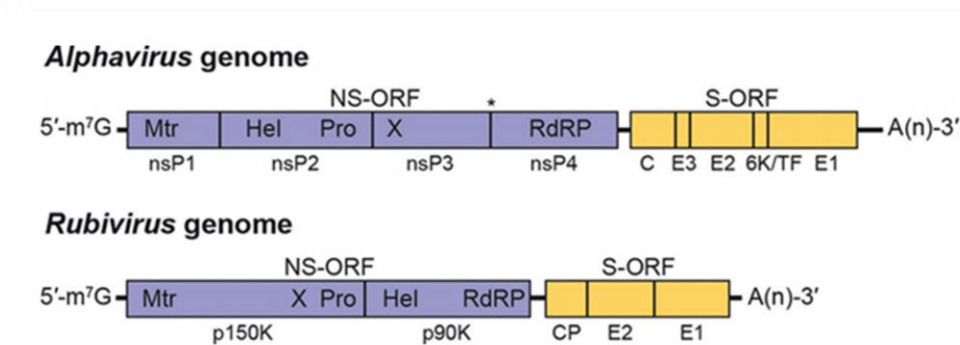
Fig. 17. *Alphavirus* and *Rubivirus* genome organisation (456)

The genome of *Togaviridae* is a single-strand of positive-sense RNA. The virus genome is unsegmented RNA of 9.7-12kb (alphaviruses) or 9.8-10kb (rubella virus). Subgenomic RNA of these viruses encoding the structural proteins contains a 5' cap and a poly-TA tail. The coding sequence consists of two large open reafding frames

(ORFs). Non-structural polyproteins are encoded by the N-terminal ORF, while structural polyproteins are encoded by C-terminal ORF (454). These two polyproteins are cleaved post-translationally by viral (cysteine) and host proteases. The non-structural proteins (nsP1, nsP2, nsP3, and nsP4) and their cleavage intermediates are involved in RNA replication, with the structural proteins (C, E3, E2, E1 and 6K) and their cleavage intermediates required for viral encapsidation and budding (458).
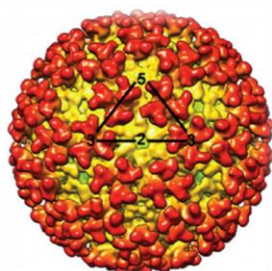
Fig. 18. Three-dimensional cryoelectron reconstruction of Chikungunya virus, a member of *Togaviridae* family, at 10.2 A° resolution (courtesy of JCY Wang) The triangle outlines one icosahedral unit (459)

Alphavirus nsP1 has guanine-7-methyltransferase and guanylyl transferase activities required for capping and methylation of synthesized viral genomic and subgenomic RNAs. The nsP1 protein is also thought to anchor replication complexes to cellular membranes during RNA replication. The protein nsP2 exhibits helicase activity within the N-terminal half and RNA triphosphatase/nucleoside triphosphatase, while the C-terminal half encodes the viral cysteine protease required for processing of the non-structural polyprotein. In CHIKV, crystal structures of nsP3 N-terminus indicate ADP-ribose 1-phosphate phosphatase and RNA-binding activities. Mutagenesis of nsP3 has been documented to play a role in modulation pathogenicity in mice. The nsP4 protein functions as the RNA-dependent RNA polymerase (RdRp), containing the catalytic GDD motif in the C-terminus (458).

Subsequently, the alphavirus capsid protein (C) binds viral genomic RNA *via* N-terminal Arg, Lys, and Pro residues during nucleocapsid formation. During the formation of nucleocapsid-like particles, a Leucine zipper located within this region presumably mediates dimerization during virus assembly. Currently, the role of the structural protein E3 is unclear and varies between different alphaviruses. In CHIKV, SINV, or WEEV, the E2 glycoprotein of these viruses, responsible for receptor binding, is embedded within the membrane of 30 C-terminal residues. Amino acid mutation

identified the structural protein E2 as a neurovirulence determinant. A palmitoylated structural protein, 6K, is essential for alphavirus particle assembly. The 6K protein in the alphavirus has also been identified as a viroporin due to the ability to form cation-selective ion channel and alters membran permeability in mammalian cells. The E1 protein has a role for the alphavirus fusion proteins (458).

7.3. *Alphavirus* cell entry and replication cycle

a. *Alphavirus* cell entry

Initially, viruses enter cells *via* the membrane of plasma, either by fusion with membrane components at the cell surface, or by receptor attachment and internalization. The subsequent process is followed by fusion with intracellular membranes of endocytic vesicles. Receptor-mediated endocytosis is the predominant mode of entry, and mostly mediated by the formation of clathrin-coated pits. Other alternative with several viruses that use clathrin-independent pathways to gain entry cells. The caveolar pathway transports internalised virus to neutral-pH caveosomes, before redistribution to the ER (458,460).

The entry of the viruses into cells is facilitated by interaction of the spikeE2 component with protein receptors on the surface of target cells (461). Subsequent binding of alphaviruses to dendritic cells involves SIGN, which L-SIGN and DC-SIGN acting as receptor molecules. A cell surface glycosaminoglycan, heparan sulfate, may also act as an attachment receptor for alphaviruses. During attachment to cellular receptors, alphaviruses are internalized immediately and then delivered to endosomes (458).

b. Replication cycle

During alphavirus cell entry process, virus particles undergo rupture, releasing genomic RNA into the cytoplasm of infected cells. Furthermore, the viral genome is translated from both open reading frames (ORFs) to generate structural polyproteins and non-

structural (P1234) polyproteins. During early infection, P1234 is cleaved in *cis* between nsP3 and nsP4 to yield P123 and nsP4 (455,459). Then, P123 and nsP4 form an unstable initial replication complex that is able to synthesize negative strand RNA. When polyproteins are at high concentrations, cleavage of P123 to nsP and P23 can occur in *trans.* Furthermore, the polyprotein products nsP1, P23, and nsP4 form a replication complex within virus-induced cytoplasmic vacuoles (CPV I) that are active in negative strand synthesis and synthesis of genomic RNA. After all cleavage to nsP1, nsP2, nsP3, and nsP4 are completed, negative-strand synthesis is inactivated and stable replication complex switches to synthesis of positive-strand genomic and subgenomic RNA (458).

Then, the negative-strand RNA forms a duplex with the positive-strand (genomic) and serves as template in the synthesis of a full-length, positive-strand RNA that will eventually be encapsidated, as well as a subgenomic 26S mRNA that encodes the viral structural proteins (463). Both non-structural proteins and RNAs interact with multiple cellular proteins; some of these interactions are essential for replication (464,465).

Structural polyproteins of alphavirus are translated from a subgenomic mRNA. The predominant translation product is CP/E3/E2/6K/E1, but, at a low frequency, there is a (-1) translational frame shifting event that produces CP/E3/E2/TF. The polyproteins of alphaviruses are then cleaved by both viral and cellular proteases to produce individual structural proteins (466). The glycoproteins that are produced are inserted into the endoplasmic reticulum during translation and are translocated to the plasma membrane. This protein assembles with the viral RNA upon generation of a sufficient amount of CP protein, to form the viral nucleocapsids in the cytosol. Budding through membrane bilayer from the host cell plasma membrane leads to the acquisition of a lipid envelope containing the two main membrane glycoproteins E1 and E2 (467,468).
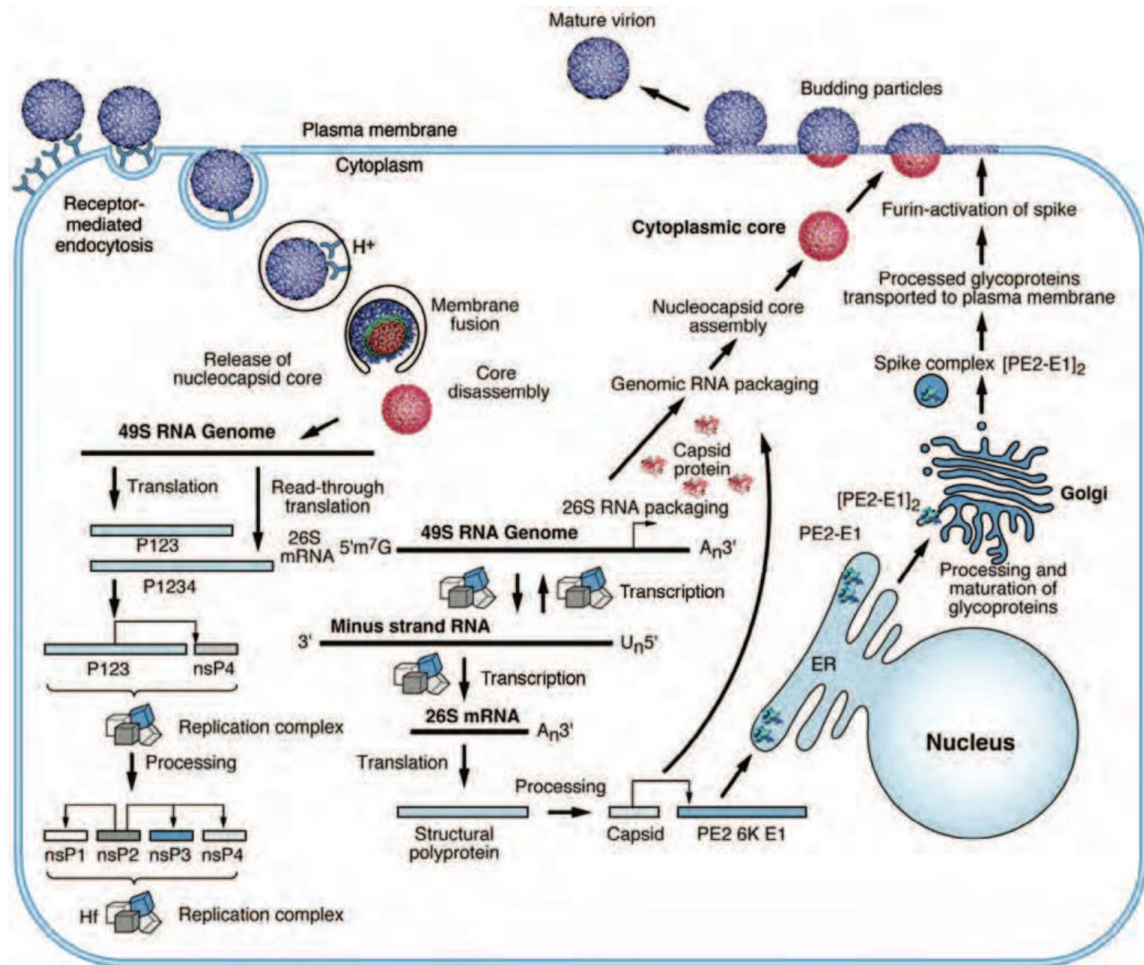
Fig. 19. Alphavirus replication cycle (469)

7.5. Phylogenetic analysis of *Alphaviruses*

Comprehensive phylogenetic analyses of the genus *Alphavirus* have been used to explain evolution and epidemiological pattern. Homology was identified between the E1 glycoprotein of alphaviruses and the envelope glycoprotein of *flavivirus* (470). Sequence analyses also demonstrated homology among the non-structural proteins of alphaviruses and several plant virus groups with different genome organizations (471).
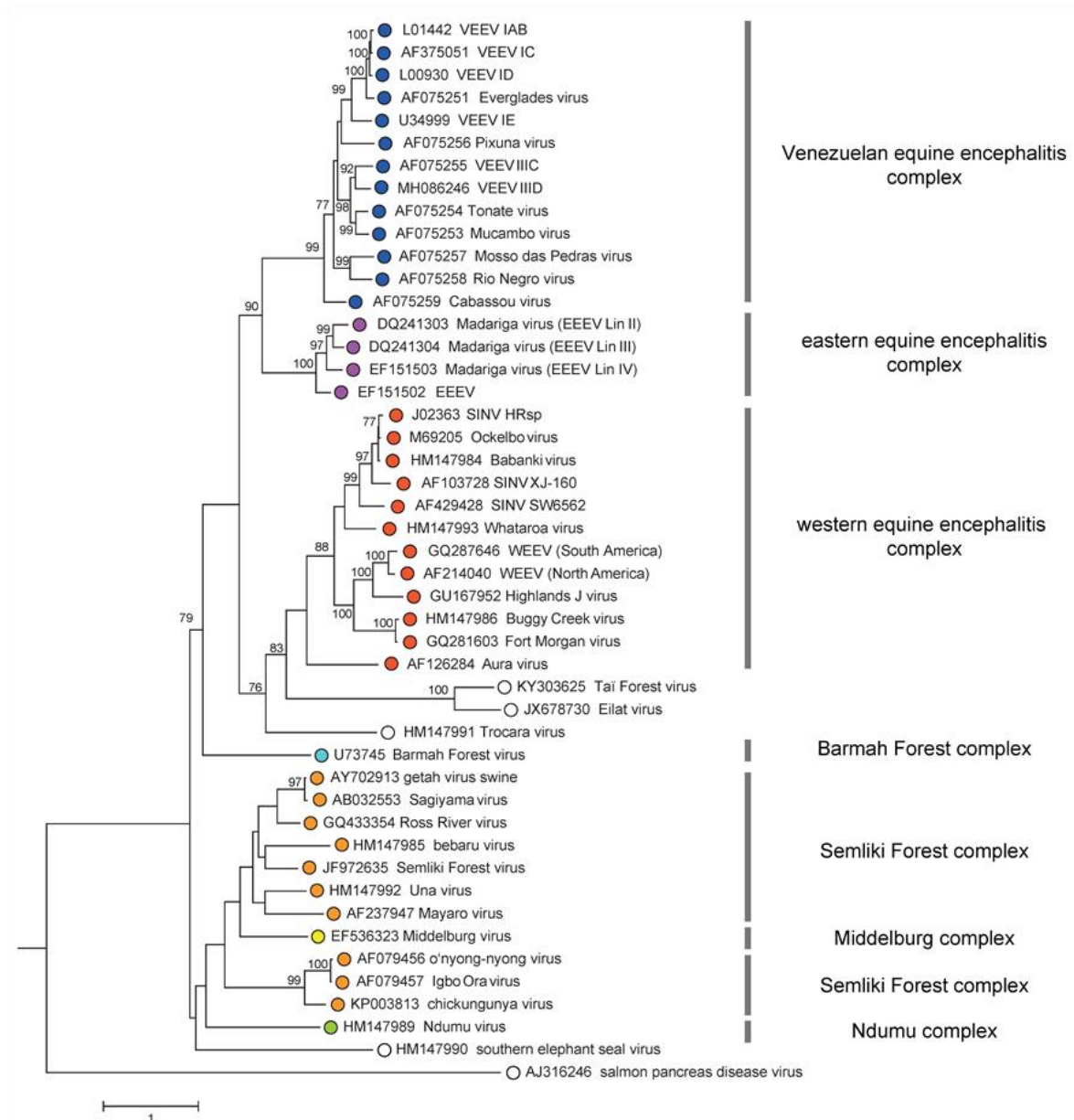
Fig. 20. Mid-point rooted phylogenetic tree of representative isolates of all alphavirus species generated from a conserved region of envelope protein gene nucleotide sequences (2184 nt) using the GTR+1+I substitution model and maximum likelihood methods (453,456).

Sequence alignment among alphaviruses demonstrates a high level of heterogeneity in the hypervariable region (HVR) of the nsP3 gene, the capsid gene, and a few short regions scattered throughout the genome where accurate alignment cannot be conducted. A phylogenetic tree based on the conserved regions of envelope genes reveals 3 major clades in the *Alphavirus* genus tree, all supported by high bootstrap values **(Fig. 20)**. The first major clade further diverges into Venezuelan equine encephalitis (VEE) and Eastern equine encephalitis (EEE) complexes. The second

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major clade bifurcates into Trocara virus (TROV), Eilat virus complex, and Western equine encephalitis (WEE) complex. The third major clade contains Barmah forest virus (BFV), Salmon pancreas disease virus (SPDV), Southern elephant seal virus, ndumu virus (NDUV), middleburg virus (MIDV), and semliki forest virus (SFV) complex (456).

8. Medically important mosquito-borne *Alphaviruses* in Indonesia

At least four alphaviruses have been serologically reported in Indonesia, including Chikungunya (CHIKV), Sinbis (BINV), Getah (GETV), and Ross River (RRV). All of these viruses have been responsible for human infections. Among them, CHIKV is considered to be the most important of *Aedes*-borne alphaviruses causing high disease burden in Indonesia (4,338). Since CHIKV, BINV, GETV and RRV have been reported to infect humans in Indonesia, these viruses become other *Aedes*-borne alphaviruses that need attention and further study in relationship with disease transmission, incidence rates in human and their vectors in Indonesia (4).

8.1. Chikungunya

8.1.1. Epidemiology of Chikungunya

Chikungunya virus (CHIKV) is an arbovirus transmitted primarily by *Ae. aegypti* and *Ae. albopictus* mosquitoes. This virus is a positive–sense single stranded RNA, member of *alphavirus* and belonging to the *Togaviridae* family (469,470). This virus is a causative agent of Chikungunya fever, which is characterized by high fever, rash, nausea and severe arthralgia. The clinical symptoms of Chikungunya fever are difficult to differentiate from dengue fever (474,475). The Chikungunya virus has been reported to cause global public health problems (476,477).

8.1.2. History of Chikungunya

The first well-documented chikungunya outbreak was reported along the coastal plateaus of Mawia, Makonde and Rondo in Southern province, Tanganyika territory of Tanzania in 1952 (478). This fever was later described by Robinson and Lumsden in 1955. The disease was reported very similar to dengue infection (suggested as "Dengue-like fever") (478,479). The name Chikungunya was derived from a Kimakonde word meaning "to become contorted". Kimakonde is the language of the local ethnic group in Southeast Tanzania and northern Mozambique. It refers to the stooped posture developed as a result of joint pains and arthritic symptoms developed with this disease (479). Since then, the virus has been reported to cause outbreaks in eastern and central Africa, Indian Ocean at the Reunion island, Asia, Central and South America, USA and more recently Europe (480).

8.1.3. Global situation of Chikungunya outbreak

a. Africa

b.

In Africa, after the first outbreak in Tanzania in 1952-1953 and prior to 2004, Chikungunya outbreaks were documented in South Africa in 1956 and in 1975-1977; Zimbabwe in 1957, 1961-1962, and 1971; the Democratic Republic of Congo in 1958, 1960 and recent outbreak in 1999-2000; Zambia in 1959; Senegal in 1960, and limited outbreak in 1996-1997; Uganda in 1961-1962 and 1968; Nigeria 1964, 1969, and 1974; Angola in 1970-1971; the Republic of Central Africa in 1978-1979; and Equatorial Guinea in 2002 (481–484).

After 2003, the CHIKV outbreaks were reported in Lamu island, Mobasa and the Comoros, Kenya in 2004-2005; Seychelles in 2005; the Reunion island in 2005-2006; Mauritius, Madagascar, Mayotte, Equatorial Guinea, Senegal and Cameroon in 2006; Gabon in 2007; and Congo in 2011 (482).

c. Asia

The outbreaks of CHIV in Asia were initially associated and confused with dengue epidemics. In Asia, CHIKV was first recognized in Bangkok, Thailand in 1960 with a significant urban outbreak (394,484). Subsequently, confirmation of *Ae. aegypti* as the primary CHIKV vector in urban setting was first documented in Thailand during 1961-1962 (484). The first CHIKV outbreak in Cambodia was identified in 1961 (484). Subsequently, CHIKV seemed to be widespread in Southeast Asia with outbreaks reported in several countries, such as Vietnam, Sri Lanka, Indonesia and Myanmar (485,486). The first CHIKV outbreak in India was first reported in Calcutta in 1963. Afterwards, the disease spread to Chennai and other states of India (483,484).

After 1973, CHIKV outbreaks were only minor with sporadic cases in Asia. A few CHIKV cases were reported in Indonesia in 1982 and Thailand in 1988. The first CHIKV outbreaks in Malaysia was identified in 1998. After 2004, significant outbreaks were noted in several countries in Asia. CHIKV outbreaks were reported in Sri Lanka in 2006, in Malaysia in 2006-2007; Thailand and Singapore in 2008-2009; Thailand in 2010; China in 2010; Cambodia in 2012; Papua New Guinea and Butan in 2012. In 2013, CHIKV outbreaks occurred in a large variety of geographic locations, i.e. the Philippines, Singapore, India (Kerala, Odhisha, Nadu, Tamil, Gujarat states) and Indonesia (East Java and Jakarta) (482).

d. Pacific region

CHIKV was first detected in New Caledonia in early 2011. Subsequent outbreaks were reported in New Caledonia and Yap state in 2013 (485). In 2014 CHIKV outbreaks were reported in Tonga, Tokelau, American Samoa, and The independent states of Samoa. French Polynesia experienced a large outbreak in 2014-2015. The disease also spread further to Cook Island in the early part of 2015 (485).

e. Europe

In Europe, an outbreak was reported for the first time in northeastern Italy (Emilia Romagna region) in 2007 with 217 confirmed cases, *Ae. albopictus* being the

vector (487). Subsequent limited outbreaks were reported in Southern France and Croatia in 2010 (488). Thereafter, other outbreaks occurred in Europe, such as France in 2014, 2017 and 2020 with more than 30 autochthonous confirmed cases reported since 2010 in this country (489).

f. Americas

In Americas, CHIKV outbreak was first detected in the Caribbean island of St Martin on mid-October 2013. Subsequent outbreaks in Americas were reported during the period 2013-2014 (482).

8.2. Chikungunya situation in Indonesia

According to the national disease surveillance database of MoH Indonesia, eleven annual reports of Chikungunya incidence between 2004 and 2014 were documented. The lowest chikungunya incidence was reported in 2005 with IR of 0.16/100,000 population per year (490), while the highest incidence was recorded in 2009 with incidence rate (IR) of 36.2 cases per 100,000 population per year (491). More than 83,000 CHIKV positive cases were reported in 2009 from 17 out of 34 provinces of Indonesia. The disease was not reported in Indonesia Papua and West Papua, while the highest incidence occurred mainly in Java, Sumatra and Kalimantan during the years of 2008 and 2016 (36). In addition, there was no death report related to CHIKV infection in Indonesia during period of 1973 to 2016 (36,490).
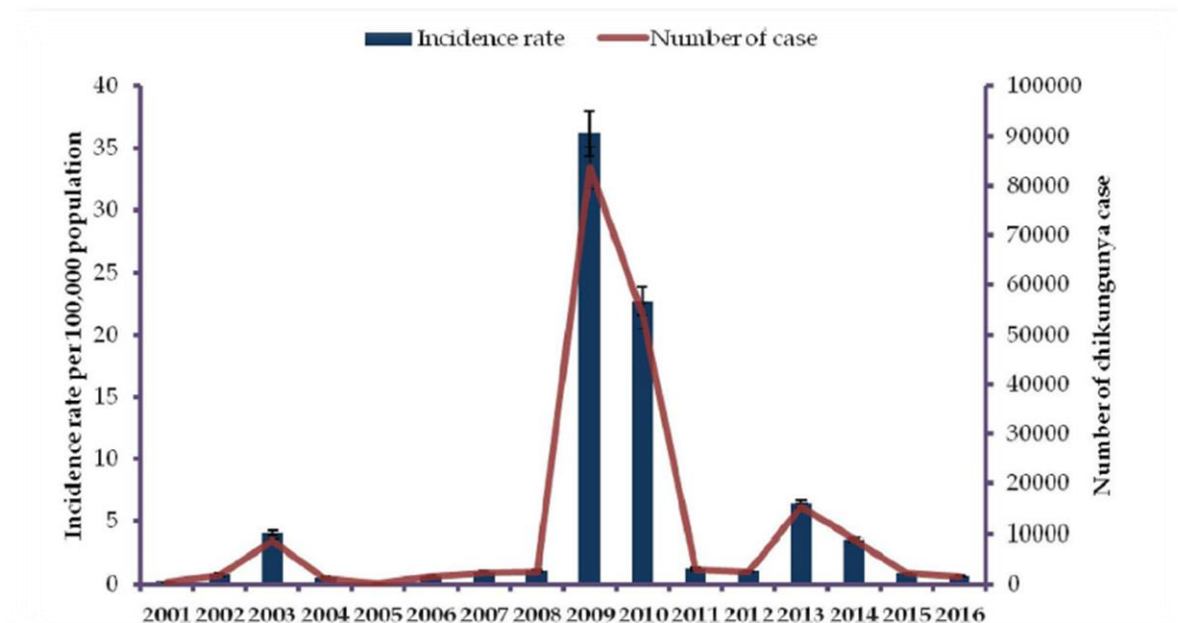
Fig. 21. Fluctuations of the number of Chikungunya cases and CHIK incidence rate (per 100,000 population per year) reported by the Ministry of Health of Indonesia during the period of 2001 to 2016 (36)

During the period from 1989 to 2014, a total of 195 CHIKV cases were reported from travellers from Japan (4 cases) (492,493), Australia (128 cases) (494,495), Taiwan (47 cases) (496) and other countries in Asia, Europe and the Pacific region, all were just returning from Indonesia. These cases were diagnosed with a combination of serology and molecular detection (36).

8.3. Chikungunya virus (CHIKV) evolution and distribution

Globally, the existence of three distinct CHIKV genotypes have been reported, i.e. West Africa, Asia, and Eastern, Central and Southern African (ECSA) genotypes (497,498). CHIKV likely originated from Central/East Africa, where ECSA genotype was identified and found to circulate in a sylvatic cycle between forest-dwelling mosquitoes and non-human primates. The ECSA genotype of CHIKV was first reported during the Chikungunya (CHIK) outbreak in Tanzania in 1952-1953. This virus genotype was also found in South Africa, in 1956, 1975-1977; Zimbabwe in 1957, 1961-1962, 1971; Congo in 1999-2000; Douala and Yaounde, Cameroon in 2006 ; and Libreville, Gabon in 2006-2007 (498).

Furthermore, Asian genotype was first identified from human samples during outbreaks in Bangkok and other locations in Thailand with an estimated number of 40,000 CHIK cases in 1958 and early 1960. This genotype was also recorded in India in 1962-1965; again in Bangkok, Thailand in 1962-1964; Bagan Panchor, Malaysia in 2006; Tangerang, Bali, Mataram, Indonesia in 2011; and Carribean islands in 2013 (34,499).
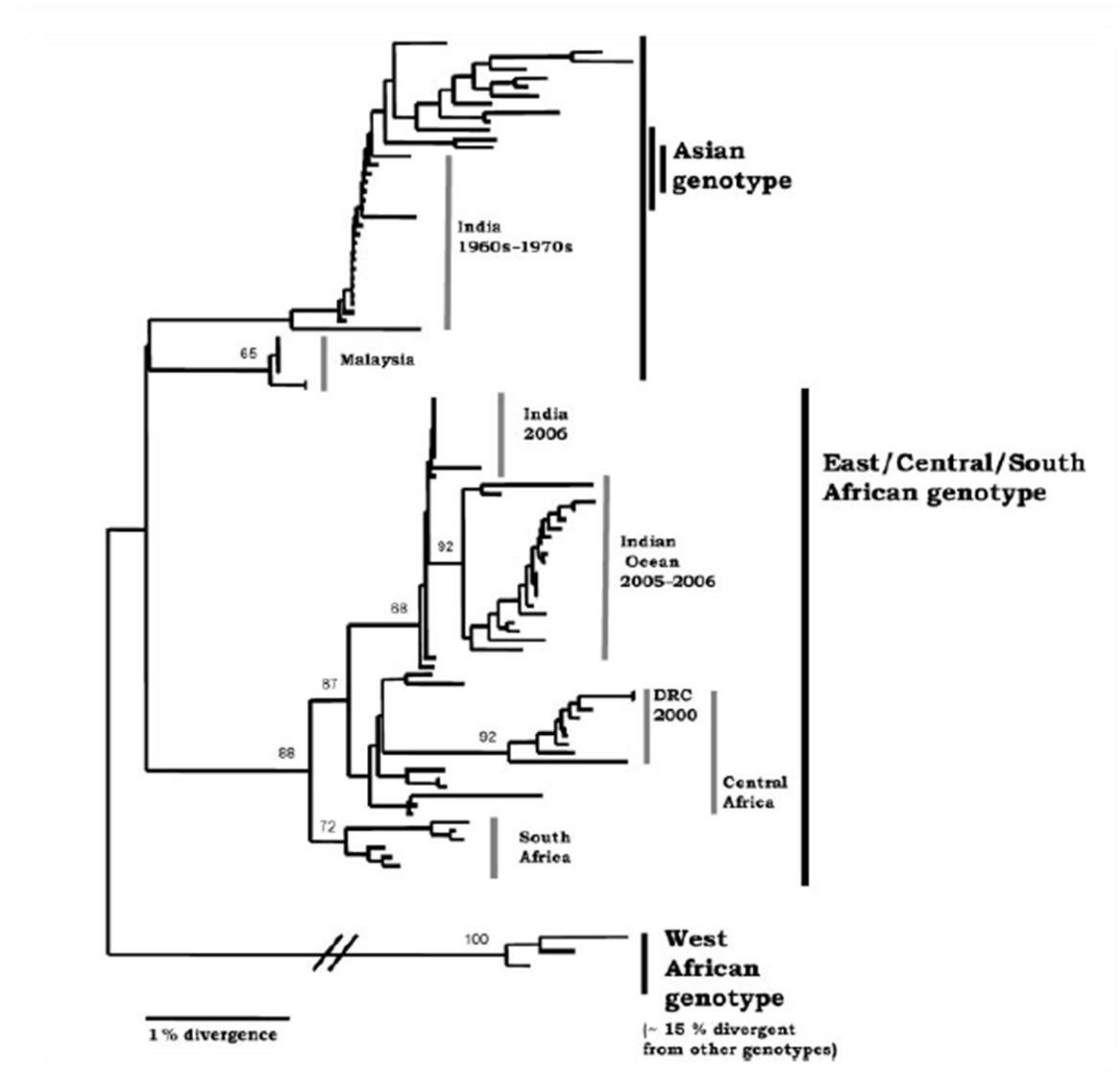
Fig. 22. Phylogenetic analysis of 99 CHIKV E1 sequences that demonstrate the main genotypes and their lineages based on geographic distribution and time of outbreaks. Numbers at nodes indicate bootstrap support of 1,000 replications (484)

The other CHIKV strain is originated from an enzootic ECSA strain and was documented for the first time during the CHIK emergence reported in coastal Kenya in 2004 (500). This strain spread independently into islands of the Indian Ocean and to

India, possibly *via* air travelers (501). Afterward, authochthonous transmission occurred in Italy and France (487,488), initiated by infected Indian travellers. A previous study revealed many imported cases with this strain that were also detected in areas with presence of *Ae. aegypti* and *Ae. albopictus* in USA. However, local transmission was not detected (499). As with the Asian lineage and the ethiology of CHIKV strain, it was called the Indian Ocean Lineage (IOL) (503).

However, some IOL samples contained a genetic change in position 226 of the gene coding for the membrane fusion glycoprotein E1 (E1-A226V). This mutation was shown to increase capability of this CHIKV strain to better infect and replicate in *Ae. albopictus.* In addition, the mutation at position 211 of domain B of the E2 glycoprotein (E2-I211T) was also identified from the IOL CHIKV strain around 2004-2005. This mutation provides a suitable background to allow CHIKV adaptation to *Ae. Albopictus via* the subsequent E1-A226V substitution (504,505). Both mutations allowed the new epidemic IOL to use *Ae. Aegypti* and *Ae. Albopictus* as vectors and impacted millions of human cases. IOL CHIKV strain adapted to *Ae. albopictus,* circulated into temperate climates and rural habitats where this mosquito species is now well installed (498,506). In Africa, IOL strain was identifed on coastal Kenya, Lamu Island in 2004-2005; Comoros, Mauritius, La Reunion in 2005-2011; India and Sri Lanka in 2005-2008. In Asia, this strain was recognized in Thailand in 2008; Malaysia in 2008; Singapore in 2008; Guangdong province, China in 2010; Cambodia and Indonesia in 2011, and Bhutan in 2012. In Europe, the IOL strain of CHIKV was identified in Emilia Romagna, Italy in 2007, and Fréjus, France in 2010 (498,499).
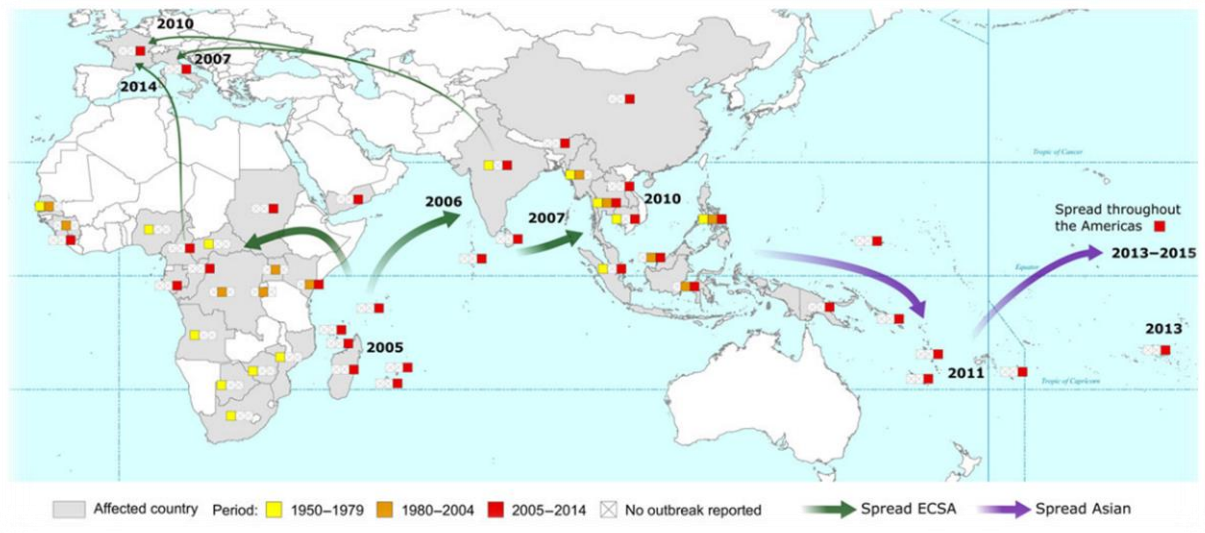
Fig. 23. The geographic distribution of East/Central/South Africa (ECSA) Chikungunya virus and Asia Chikungunya virus genotypes in Africa and Asia in the period of 2005-2015 (482)

8.4. Transmission cycle of Chikungunya virus

In West and Central Africa, CHIKV is believed to be maintained in a sylvatic cycle involving non-human primates and many species of *Aedes* mosquitoes. Longitudinal studies conducted in the Zika forest in Uganda in 1950s detected CHIKV antibodies and viremia in monkeys (482,497). A study conducted in Southeast Senegal revealed a periodicity of CHIKV associated with alignment with renewal of the monkey population in every 4-7 years (507). However, increase of the CHIKV circulation in *Aedes* mosquitoes every 4-7 years was not systematically concomittant with outbreaks in humans. In Asia, there is very limited evidence of an enzootic cycle of CHIKV. So far, only three reports have revealed the existence of CHIKV in nonhuman primates. In 1999, about 59.3% of 54 monkeys (*Macaca fascicularis*) were serologically CHIKV positive in Luzon Island, the Philippines (508). CHIKV was also isolated in several long-tailed macaques in Kuala Lipis Pahang, Malaysia in 2007. In addition, there is evidence that CHIKV collected from human serum during outbreaks in Klang in 1998 and Bagan Panchor in 2006 are closely related to CHIKV isolates from monkeys in Africa, suggesting the existence of a sylvatic cycle for CHIKV in Malaysia (509).

In Africa, the virus was isolated from several sylvatic mosquito species in different countries including Cote d'Ivoire, South Africa, Senegal, and Central African Republic. These major mosquito species involved are *Ae. furcifer, Ae. taylori, Ae. neoafricanus, Ae. dalzieli, Ae. cordellieri, Ae. vittatus, Ae. luteocephalus,* and *Ae. africanus*. Of which, the principal vector of CHIKV was the *Ae. furcifer-taylori* group (483,507).

In Asia, CHIKV transmission was well documented to occur mainly in urban areas. *Ae. aegypti* was identified to be the most significant vector. This species is an anthropophilic mosquito, urban, and peridomestic. Besides being dominant in this region, this species was also responsible for large outbreaks in East Africa and Comoros, Africa, during 2004-2005 (484).

Another CHIKV vector, *Ae. albopictus,* has shown a remarkable capacity to adapt to peri-domestic environments, enabling it to displace *Ae. aegypti* in some areas. *Ae. albopictus* has a wide distribution and could introduce CHIKV into many new ecological niches. Currently, *Ae. albopictus* has become a significant vector of CHIKV and DENV (510,511). This mosquito species was identified as CHIKV vector during outbreaks in Comoros, Mauritius, and La Reunion during the period of 2005-2011; India and Sri Lanka in 2005-2008; Libreville, Gabon in 2006-2007; Emilia, Romagna, Italy in 2007; Thailand in 2008; rural Malaysia in 2008; southern France in 2010, 2014 and 2017 and; Guangdong province, China in 2010 (498).

8.5. Clinical manifestations of Chikungunya infection

After being infected with CHIKV, there is an incubation period lasting about 2-4 days with a range of 1-12 days (512). Clinical onset is sudden including high fever, headache, back pain, myalgia, and arthralgia, particularly affecting ankles, wrists, phalanges and the large joints (513). About 40-50% of CHIKV cases show maculopapular rash. Clinical symptoms may also involve facial oedema and, in children, bullous rash with pronounced decay, localized petechiae and gingivorrhagia. Iridocyclitis and retinitis are the most common ocular manifestations associated with CHIK. Retinitis shows gradual resolution over a period of 6 to 8 weeks. The hallmark

of CHIK infection is arthralgia, which can cause instantaneous paralysis. However, it rarely affects children. This manifestation usually migrates and involves the small joints of the hands, wrists, ankles, and feet with pain when moving. The onset of the disease is associated with an increase in viral titer, which trigger to activate an innate immune response by the production of type I interferons (IFNs). Intermediates during replication, a single-stranded RNA have potential to engage the pathogen recognition receptor Toll-like receptor 3 (TLR3), TLR7 and TLR8 and the retinoic acid-inducible gene I(RIG-I) like receptors (RLRs) melanoma differentiation-associated protein 5 (MDA5) and RIG-I. These receptors activate a signaling cascade that leads to activate interferons (IFNs) and transcription of cytokines and chemokines. Patients successfully clear the virus and recover within 7-10 days (473,513).

Although few, meningo-encephalitis cases were also reported among confirmed cases during CHIK outbreaks in India in 1973 and 2006. Rare cases of Guillain-Barre syndrome associated with CHIK infection have also been described during the Indian Ocean CHIKV outbreak in 2005-2006. Mild hemorrhage, myocarditis and hepatitis are the rare complications described after CHIK infection (513).

Generally, CHIK infection is not considered a life-threatening disease. However, fatal cases associated with CHIKV were observed during the Indian Ocean outbreak (2005-2006). The important report of a mortality associated with CHIKV epidemics was recorded in La Reunion, Mauritius and India. In La Reunion, the monthly crude death rates during the outbreaks were 34.4% and 25.2% in February and March 2006 respectively (513).

8.6. Laboratory diagnosis

In the clinical diagnosis of a patient who has lived in or visited CHIK endemic areas in a timeframe during the incubation period, an acute fever and severe arthralgia or arthritis, that is not explained by the other medical disorders, is considered a possible CHIKV case. Laboratory diagnosis and confirmation is essential to distingush the CHIKV infection from various disorders with similar clinical symptoms, such as dengue, other alphaviruses, arthritic diseases or malaria. Viral nucleid acid detection of

sample serum of suspected CHIKV cases is useful to identify the initial viremic phase, at the onset of symptoms and normally for the following 5-10 days when CHIKV RNA reaches very high levels of viral loads (3.3×10^9 copies/ml). After the viremia phase, further diagnosis is based on serological methods (513). Molecular diagnosis constitutes a rapid and sensitive technique for CHIKV infection diagnosis during the early stages of illness. Conventional RT-PCR, real time loop-mediated RT-PCR and real time TaqMan RT-PCR assay are available to target the envelope E1 gene or the non-structural nsP1 gene (514–518).

Besides the molecular detection, viral isolation is useful for various epidemiology and pathogenesis studies. Virus isolation can be performed from infected patient' serum on insect cell line (c6/36) or mammalian cells (VeroE6, MDCK) or by intracerebral inoculation of 1-day-old mice during viremic phase of the disease when the viral load is very high and before an immune response is evident (519,520).

Furthermore, some serological methods for detecting CHIK-specific immune responses include enzyme-linked immunosurbance assays (ELISA), indirect immunofluorescence assyas (IFA), hemagglutination inhibition (HI) and micro-neutralization (MNt). ELISA and IFA are rapid and sensitive serological methods to detect CHIKV-specific antibody. IgM are detectable during 2-3 days after onset of symptoms and persistent up to 3 months. IgM is rarely detected in a longer period of time (more than 1 year) (521,522). Comparison of the commercial IgM serological assays suggested that the sensitivity for detection of an early antibody response before the 5th day is dependent on the virus strains used for the assay or the source of antigen. Assay based on recombinant antigens might be more specific to mutations (522–524).

Other serological methods to diagnose a couple of sera collected in the acute and convalescent phases that cannot distinguish IgG Ab from IgM Ab (i.e. HI and MNt) is mandatory for the identification of CHIKV recent infection. These methods are also useful to confirm results obtained with other methods, especially in the situation of rare persistence of IgM antibodies (525).

9. Dengue and Chikungunya Vectors

9.1. *Aedes* taxonomy

Aedes is one of the most important mosquito genus including species that are known as main vectors of *flaviviruses* infecting humans. The current study focused on the *Aedes*-borne *flaviviruses* corresponding to 10 species part of the YFV group, ENTV group, SPOV group and DENV group, including *Wesselsborn virus* (WESSV), Banzi virus (BANV), Edge Hill virus (EHV), Jugra virus (JUGV), Saboya virus (SABV), Potiskum virus (POTV), Sepik virus (SEPV), Uganda S virus (UGSV) and Bouboui virus (BOUV), Zika virus (ZIKV), Spondweni virus, Denv-1, Denv-2, Denv-3, Denv-4 (DENV), and kedougou virus (KEDV) (523). *Stegomyia, Ochlerotatus* and *Aedimorphus* are part of *Aedes* mosquitoes that are often studied as important *flavivirus* vectors in the world (357,527–529).

Previously, *Stegomyia* Theobald was part of the *Aedes* genus reported as the most important medical genus since it includes vectors of a large number of arboviruses. It is also one of the most dominant subgenus within the genus *Aedes* Meigen in the Oriental region. So far, at least 37 species and subspecies of this subgenus have been identified in this region (527). However, the Tribe Aedini has been reclassified in 2000-2009. The reclassification of tribe *Aedini* began with removal of *Verallina, Ayurakitia,* and *Ochlerotatus* from the genus *Aedes* and it was followed by a series of phylogenetic analyses of *Aedini* that resulted in 80 genera within the tribe (529).

In Dengue, Chikungunya, Zika and Yellow Fever, two key species of *Stegomyia* are involved in the transmission of the corresponding viruses, *Aedes (Stegomyia) aegypti* and *Aedes (Stegomyia) albopictus.* Morphologically, the scutum of the thorax of *Ae. aegypti* is black or brown with a pair of submedian-longitudinal white stripes, but without median-longitudinal white stripe, or with white lyre-shaped markings. Mesepimeron shows two well separated white scale patches. Anterior portion of the midfemur has longitudinal white stripe, and head clypeus with white scales. In addition, paratergite presents broad white scales and head palpomere 4 with white scales at apex (528). The morphological feature of *Ae. albopictus* is slightly different than *Ae. aegypti.* Scutum thorax of this species has a narrow median-longitudinal white stripe.

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Mesepimeron has white scale patches not separated, forming V-shaped white patch. Anterior portion of midfemur shows longitudinal white stripe. Head clypeus has no white scales. Paratergite and head palpomere are equal as those of *Ae. aegypti* (528).

9.2. Bio-ecology of *Aedes aegypti* and *Aedes albopictus*

Aedes aegypti occurs in urban, rural and forested areas in both artifical and natural containers closely related to human populations. However, its prefers to breed in domestic environment, such as water storage tanks and jars inside and outside houses, leaf axils, bamboo stumps, roof gutters and temporary containers such as jars, drums, used car and motorcycle tyres, tin cans, bottle and plant pots. Adult mosquitoes bite during the day (531,532).

Aedes albopictus is an indigenous forest species from Asia that recently invaded the American, African and European continents (531,532). This species breeds in temporary containers, as well as natural breeding places in forests and bushes such as tree holes, leaf axils, ground pools and coconut shells. It prefers to bite outdoors than indoors (531–534).

9.3. Population genetics of *Aedes aegypti*

Population genetics is the study of genetic variations within and among populations of organisms and the evolutionary factors that explain these variations. This study is concerned with the origin, amount, frequency, distribution in space and time, phenotypic significance of genetic variations, and with the microevolutionary forces that infuence the fate of genetic variation in reproducing populations. Population genetic is a way to understand how and why the frequencies of alleles and genotypes change over time within and between populations (535–537).

Currently, *Aedes aegypti* is one of the focuses for research on the evolution of human association with public health consequences. This species has great epidemiological importance because of its roles as vector of several major pathogens of pandemic viral diseases : yellow fever, dengue, chikungunya and zika fever (538). Knowing the existence of *Ae. aegypti* in recent past and genetically analyzing populations of this species is important to understand how and when it comes to occupy its current distribution, which determines the human population at risk for its transmitted diseases. This will not only help to understand historical disease patterns, it highlights the threat of this dynamic mosquito in the future (538).

Historically, *Ae. aegypti* originated from the African continent, particularly the Egyptian region. This species became established outside Africa after arriving in the Americas aboard slave ships and spread throughout the new world during the 17th and 18th centuries (539,540). Genetic studies of mosquitoes have been carried out since 1950, especially on specific species of *Aedes, Anopheles* and *Culex*, reporting information on chromosome numbers and heterochromatin distribution, as well as on genome size and organization (541). Subsequently, initial population genetic studies of *Ae. aegypti* have been conducted by Powell, Tabachnick, Munstermann and Wallis in 1980s. These studies revealed that *Ae. aegypti* populations are divided into two clades. The first clade consisted of *Ae. aegypti aegypti* from South America, East Africa, and the Caribbean. This clade was suggested as the new world population originated from East Africa. Whereas the second clade consisted of *Ae. aegypti aegypti* population from Asia and Southern USA with a basal *Ae. aegypti formosus* from both East and West Africa (542). This result suggested two introductions into the new world, one from West Africa and another from East Africa (542–546).

Currently, various molecular analyses have been undertaken to identify the genetic structure and gene flow among *Ae. aegypti* populations to provide informative data to help track and prevent movements of associated genetic traits that is useful to interrupt arboviruses transmitted by *Ae. aegypti* (547,548). Some methods have been undertaken for extensive DNA-based genetic studies and population genetic analysis of *Ae. aegypti* including: microsatellites, restriction fragment length polymorphism (RFLP), random amplification of polymorhpic DNA (RAPD) (549–555). In 2007, the

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complete genome of *Ae. aegypti* was sequenced (556). More recently, DNA sequences have been widely used to evaluate the genetic variability of *Ae. aegypti* (540).

Mitochondrial DNA (mtDNA) is particularly used as genetic marker in evolutionary biology and population (540). Mitochondria are parts of cellular organelles that have the function of the oxidative phosphorilation and ATP formation. The mtDNA is inherited as a haploid from the mother and heteroplasmy has rarely been found. According to a populational perspective, mtDNA could be considered as a small system, sexually isolated demes, or clonal lineages, with an evolutionary rate of 5 to 10 times faster than nuclear genome. Based on all these characteristics, the patterns of nucleotide variations is ideal to be used to infer the evoluationary studies of populations and closely related species (557). In addition, many studies concerning mtDNA focused on determining the levels of polymorphism in natural populations (558). Polymorphism of mtDNA is a widely used tool for assessing species gene flow and has been widely applied in population genetic studies of *Ae. aegypti* from different geographic regions where several arboviruses transmitted by this species are endemic (540,551,552).

Chapter 2. Epidemiology of Japanese Encephalitis and its vector distribution in Indonesia

Introduction

Indonesia has been recognized as a country playing a major role in the global transmission of Japanese encephalitis (JE) (559,560). Genetic studies suggest that Japanese encephalitis virus (JEV) originated from the region between the Indian and the Pacific Ocean, including Indonesia. This region is the only area where all five JEV genotypes (GI-GV) occur. JEV was first reported in 1972 from West Java, Indonesia. Since then, this infection, causing neurologic, cognitive and behavior sequelae to human, has been reported in several hospitals and currently JEV has spread in most provinces of Indonesia (31,561). The Ministry of Health of Indonesia has declared Japanese encephalitis as a national priority program. As a follow up, the government has shown its commitment to implement disease prevention and control activities and strengthen the diagnostic capacity in established JE diagnostic laboratories. The government is committed for better clinical management and treatment of disease. In addition, a national JE diagnosis protocol has also been developed (562). To have effective JE prevention and control strategies, JE vaccination program is being implemented in Indonesia. Currently, a total of 890,050 Balinese children aged from 9 months to 15 years were targeted vaccination with single dose of the Chengdu SA14-14-2 live-attenuated JE vaccine through a two-phase, school-based and community-based mass campaign. JE vaccination has now been included in the Balinese immunization routine programs (349,563). In the near future plans, the government is committed to expand the coverage of JE immunization in other different JE endemic areas (561,562).

In order to support an effective national JE prevention and control strategy plan, an updated comprehensive information about epidemiology, virus genotypes, and vector distribution and their ecology is required. This chapter is aiming at updating the epidemiological situation and transmission ecology of JE in Indonesia. In addition, this chapter also provides current information on the first evidence of a new JE genotype

circulating from field-caught mosquitoes and the associated potential risk to public health in Indonesia.

Articles displayed in Chapter 2

Article 1. Garjito TA, Widiarti, Anggraeni YM, Alfiah S, Satoto TBT, Farchanny A, Samaan G, Afelt A, Manguin S, Frutos R, Aditama TY. Japanese encephalitis in Indonesia: An update on epidemiology and transmission ecology. *Acta Trop*. 2018;187:240-247.

Article 2. Garjito TA, Prihatin MT, Susanti L, Prastowo D, Sa'adah SR, Taviv Y, Satoto TBT, Waluyo J, Manguin S, Frutos R. First evidence of the presence of genotype-1 of Japanese encephalitis virus in *Culex gelidus* in Indonesia. *Parasites Vectors*. 2019; 12(1):19.

Article 1.
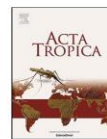
Japanese encephalitis in Indonesia: An update on epidemiology and transmission ecology

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A B S T R A C T

The Japanese Encephalitis (JE) virus circulation in Indonesia was first documented in Lombok in 1960, and the virus was first isolated in 1972 from *Culex tritaeniorhynchus* in Bekasi, West Java and Kapuk, West Jakarta. Since then, Indonesia has been recognized as an endemic country for JE transmission. Up to now, JE cases have been found in at least 29 provinces, with Bali, West Kalimantan, East Nusa Tenggara, West Java and East Java, being the areas of highest incidence. However, routine surveillance on JE has not been established at the national level even though many surveys were conducted. JEV has been isolated from 10 mosquito species: *Culex tritaeniorhynchus, Cx. gelidus, Cx. vishnui, Cx. fuscocephala, Cx. bitaeniorhynchus, Cx. quinquefasciatus, Anopheles vagus, An. kochi, An. annularis*, and *Armigeres subalbatus*. *Culex tritaeniorhynchus* is the main JE vector in Indonesia. JE has been detected throughout the Indonesian archipelago from West to East. However, due to a lack of routine, systematic and standardized diagnostic approaches, the JE burden has still not been clearly established yet. Long term and systematic JE surveillance across Indonesia is a priority, the burden needs to be better assessed and appropriate control measures must be implemented.

1. Introduction

According to the World Health Organization (WHO), more than 3 billion people are exposed to the risk of Japanese encephalitis (JE) infection over 27 Asia-Pacific countries (Wang and Liang, 2015). Approximately 68,000 clinical cases occur each year with a total morbidity rate of 1.8 per 10,000 (Gao et al., 2013; Wang and Liang, 2015; WHO, 2015). The fatality rate ranges from 20% to 30%, and between 30% to 50% of surviving patients experience paralysis, seizures, behavioural changes, and some may remain severely disabled with permanent neurologic or psychiatric sequelae (Maha et al., 2009; Solomon et al., 2000; Wang and Liang, 2015; WHO, 2015). The estimated global impact of JE was 709,000 disability-adjusted life years (DALYs) in 2002 (Campbell et al., 2011; Erlanger et al., 2009; Solomon, 2006; Tarantola et al., 2014). However, this is likely to be underestimated as JE surveillance systems are not effectively implemented in many affected countries.

JE was initially identified in Japan in the 1870s (Chuang and Chen, 2009; Pattan et al., 2009) but the causative virus was formally isolated in 1933 (Rosen, 1986). Currently, JE virus (JEV) transmission has been mainly reported from Asian countries with 2 epidemiological patterns. (i) Endemic sporadic cases of JE are observed in Southern Asian and Australasia, including Southern India, Vietnam, Southern Thailand, Malaysia, Philippines, Indonesia, Cambodia, Lao PDR, Sri Lanka, Myanmar, Brunei Darussalam, Papua New Guinea, Australia, and Timor Leste (Saxena et al., 2011; Solomon et al., 2000; Vaughn and Hoke,

1992; Wang and Liang, 2015). (ii) Epidemic outbreaks are reported mainly in subtropical and temperate regions, including Northern India, Northern Thailand, Nepal, Burma, Butan, Northern Vietnam, Bangladesh, China, Korea, Taiwan, Southern Russia, Pakistan, Afganistan, Madagascar and Oriental Africa (Saxena et al., 2011; Solomon et al., 2000; Vaughn and Hoke, 1992; Wang and Liang, 2015).

JEV belongs to the family *Flaviviridae* and to the genus *Flavivirus*. It is a single-stranded, positive sense RNA virus, transmitted to human through mosquito bites from amplifier animals, such as pigs, birds, bats and other vertebrates. The primary JE vector is *Culex tritaeniorhynchus*. Other species from the genera *Culex, Armigeres, Aedes, Mansonia* and *Anopheles* have also been reported as JE vectors in endemic countries (Banerjee, 1994; Kanojia, 2007; Sucharit et al., 1989). Infected pigs are the main source and the major amplifier of JEV infection (Chew, 2014; Kuno and Chang, 2005; Le Flohic et al., 2013; Mackenzie et al., 2004; Obara et al., 2011; Pan et al., 2011; Wang and Liang, 2015). Humans and cattle are considered to be dead-end hosts and do not transmit viruses to biting mosquitoes because of insufficient infective titres and short duration of viremia. However, these accidental infections can have serious health consequences (Schuh et al., 2011). JE transmission is found in rural areas and at the edge of urban areas, however, human cases occur mostly in rural areas (WHO, 2007).

Indonesia is recognized as a country playing a major role in the global transmission of JE, on one hand owing to her geographic position between the Indian and the Pacific Oceans and on the other hand as the place of origin of JEV, which was shown to have evolved from an ancestral virus in the Indomalayan Region (Solomon et al., 2003; Weaver and Reisen, 2010). This region is the only area where all JEV genotypes: GI, GII, GIII, GIV, and GV occur (Uchil and Satchidanandam, 2001). The GIV and GV genotypes are the most divergent ones and remain limited to the Indomalayan Region, whereas the less divergent GI-GIII genotypes have spread widely across Asia (Diagana et al., 2007). However, the genetic patterns do not necessarily correlate with the epidemiological distribution (Chuang and Chen, 2009; Schuh et al., 2011). Recently, following a pre-campaign survey, Indonesia has initiated the first children vaccination program in Bali with the support of international organizations (Im et al., 2018). Owing to the role of Indonesia in the transmission on JE, the occurrence of sporadic human cases all over the country, a limited routine surveillance and a lack of reporting systems (Chew, 2014) making thus the burden difficult to estimate, we conducted a systematic review of JE epidemiology and vector distribution.

2. Method

We conducted a systematic search of international and national published articles using online search engines. Online published articles were searched using the words "Japanese encephalitis", "Indonesia", "Vector", "Distribution", "Epidemiology", "Surveillance", "Genotype", "Genetic", and "Evolution", on the following databases: Medline, PubMed, Portal garuda, IPI indonesian publication index, and google search. Local publications, unpublished JE documents and presentation by JE researchers were also collected. As the number of research institutions and academics conducting studies on JE in Indonesia is limited, searches were conducted in the library of the National Institute of Health Research and Development, Institute for Vector and Reservoir Control Research and Development, to find unpublished research reports and national reports addressing JE. Researchers related to JE studies were asked not only about their available published and unpublished work, but also to identify or provide information on other researchers and groups undertaking JE activities.

Criteria for inclusion in the review were published articles and unpublished JE documents focusing on epidemiology, virology, entomology, socio-economics, and burden of disease. If articles did not relate to Indonesia and JE, they were excluded from the analysis (Fig. 1). All data were screened and then compiled to summarize the
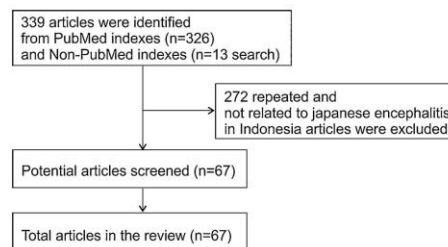
Fig. 1. Flowchart of article selection in the systematic review.

epidemiology of JE and its existing vectors in Indonesia. All data on suspected and confirmed human JE cases, JE seropositive animals and vectors were tabulated and mapped.

Ethical approval was not required for this review. No primary or individual patient data were analyzed.

3. Results

3.1. Human cases and incidence of Japanese Encephalitis in Indonesia

JE transmission was first documented in Indonesia when serological surveys were conducted in Lombok in 1960 and in Surabaya in 1968, and showed the presence of haemagglutination-inhibiting (HI) antibodies to JEV in humans (Soedarmo et al., 1994). In the early 1970s, JE serosurveillance was conducted in various localities in Indonesia. Antibodies against JEV were detected in 52% of human serum samples in Bali, 2–16% in Nusa Tenggara, 22–27% in Kalimantan and about 3% in Sulawesi, Maluku and West Papua (former Irian Jaya) (Widarso et al., 2002). Based on clinical signs and symptoms, suspected cases of JE were also reported in Jakarta (Soedarmo et al., 1994). Kho et al. (1971) found HI antibodies against JE antigen, but not against Dengue (DENV-1). However, other viral antigens were not assessed and it is important to note that cross-reactivity with other *Flaviviruses* may overestimate the burden of JE in this study as well as in others (Peenen et al., 1975b).

High prevalence of JE was reported in Western Indonesia. Kanamitsu et al. (1979) reported that JEV has been active in areas west of the Wallace Line with a rate of neutralizing antibodies in human sera of 27% in Pontianak (Kalimantan), 26% in Samarinda (Kalimantan), 22% in Balikpapan (Kalimantan), and 51% in Bali. Lombok displayed a lower rate, i.e. 16%, of JE antibody and a differing age pattern for the antibody distribution. Reasons are unknown but it seems unlikely to be due to study artifacts as all studies revealed seropositivity in all age groups. A subsequent clinical study, conducted among 118 children admitted to two hospitals in Jakarta in 1981 with JE symptoms, found 25% of seropositivity, while cases displayed a 4-fold antibody increase against JE antigen based on HI and Immune adherence hemagglutination (IAHA) tests (Lubis and Wuryadi, 1981). In 1986–1987, a hundred cerebrospinal fluids (CSF) were collected from suspected JE cases admitted to a hospital in Jakarta and tested them by JE Mac-ELISA. All were seronegative for JE (Soedarmo et al., 1994). In 1985–1987, 130 CSF were also collected from suspected JE cases in Manado and Yogyakarta, and were seronegative (Wuryadi and Suroso, 1989).

Although JE is believed to be endemic in many areas in Indonesia, there has been no outbreak report. However, encephalitis cases have been reported in several hospitals in Indonesia. From 1979 to 1986, yearly encephalitis cases reported from surveillance programs ranged from 932 to 2,189, while case fatality rates (CFR) ranged from 27% to 45%. All cases were based on clinical diagnosis and none were serologically confirmed (Soedarmo et al., 1994).

A study conducted in Bali from October 1990 to July 1995 among

77 JE suspected patients could confirm only 40 cases (52%) based on ELISA examination of CSF samples (Kari et al., 2006). Human serological surveys for JE were conducted from 1993 to 1995 and from 1996 to 1997 in West Java, Central Java, East Java, Riau, Lampung, West Kalimantan, West Nusa Tenggara, and Bali. A total of 1295 serum samples were collected from 8 provinces and 47% to 93% of samples were serologically JE-positive by HI test. Bali was reported as the province with the highest percentage of JE seropositivity in human, i.e. 92.8% (Widarso et al., 2002). Other high rates of seropositivity were recorded in Lampung (88.9%) and Central Java (87.5%) (Widarso et al., 2002).

In 1993–2000, a series of serological survey was conducted among communities living in proximity to rice fields, pig populations and other domestic animals including cows, goats, sheep and horses. The survey was conducted in Bali, Riau, West Java, Central Java, Lampung, West Nusa Tenggara, North Sumatra, West Kalimantan, North and South Sulawesi, East Nusa Tenggara, and Papua. A total of 1830 human sera were examined for JE by IgG and IgM ELISA. Seropositivity ranged between 2%–73%. The highest positive ELISA for JE was reported in West Kalimantan (72.6%). Three other provinces also reported high seropositivity, i.e. North Sumatra (58.8%), South Sulawesi (52.3%), and Papua (54.0%) (Widarso et al., 2002).

In 2001, Konishi (2009) conducted a sero-survey among inhabitants of Jakarta and Surabaya, which detained small swine populations. Overall, 2% of the 1211 sera collected in Jakarta and of the 1751 collected from Surabaya displayed neutralizing antibody titers higher than 1:160 (90% plaque reduction). These results suggest that people in Jakarta and Surabaya were exposed to natural JEV infections with an established transmission cycle through vector mosquitoes despite a relatively small number of pigs.

To estimate the burden of JE in Bali, Kari et al. (Kari et al., 2006) conducted a prospective hospital-based surveillance that included all health care facilities providing care to children under 12 (599,120 children), from July 2001 to December 2003. The annualized JE incidence rate was 7.1 and was adjusted to 8.2 per 100,000 for children less than 10 over the 2.5 consecutive years. Only one JE case was found among 96,920 children 10–11 years old (0.4 per 100,000). In 2005–2006, a hospital-based surveillance system for JE in children under 15 was implemented in 6 provinces: West Sumatra, West Kalimantan, Papua, East Java, West and East Nusa Tenggara with 15 hospitals involved as sentinel sites. The sentinel sites detected clinical cases of acute encephalitis syndrome based on the following definition: a suspected case was defined as a child who experienced acute onset of fever (> 38 °C) with neurological deficits, such as seizure, cranial nerve or sensory deficits, abnormal movements, weakness of one or more limbs; change of mental status and sign of meningeal irritation. All samples (CSF and/or serum) were examined using JE IgM capture ELISA. After 18 months of surveillance, JE cases were confirmed in all 6 provinces and occurred mostly in children under 5. Moreover, the case fatality rate was high (approximately 23%) and almost 20% of survivors displayed disability sequelae (Ompusunggu et al., 2008; Wijaya, 2007).

In a study conducted in 2013, seven human sera were collected from suspected JE cases in Manado (Sulawesi), and all were seronegative based on JE IgM capture ELISA (Garjito et al., 2013). In 2014, subsequent JE surveillance activities were conducted in 11 sentinel sites recognized as JE endemic areas. Routine surveillance was conducted by collecting sera from suspected human JE cases. A total of 8 and 40 JE cases were confirmed in 2014 and 2015, respectively. In 2016, surveillance activities were continued in 11 provinces and a total 43 out of 326 human serum samples from individuals with JE symptoms were confirmed positive by IgM ELISA (Burni, 2017) (Fig. 2).

3.2. Travel-associated Japanese encephalitis cases in Indonesia

Although several occurrences of JE in travelers were reported in Indonesia, cases were very limited, with estimates for U.S. and

European travelers lower than 1 per million (Hatz et al., 2009; US-CDC, 2011). Since 1988, only ten confirmed cases of JE were reported among travelers after a visit to Indonesia, especially in the highly touristic Bali and Java (Anonym, 2015; Berger, 2018; Buhl and Lindquist, 2009; Caramello et al., 2007; Hills et al., 2010; Macdonald et al., 1989; Pavli and Maltezou, 2015; Tappe et al., 2012; Wittesjö et al., 1995) (Table S1). One case out of ten was fatal and one patient survived with sequelae (Berger, 2018; Buhl and Lindquist, 2009; Hills et al., 2010; Pavli and Maltezou, 2015). Four JE cases out of ten were travelers from Australia who spent time in Bali, including a 10-year-old girl in 1988 (Hills et al., 2010; Macdonald et al., 1989), a tourist male in 1991, a 3-year-old girl in 1993 after living in the Balinese jungle for 2 years (Hills et al., 2010), and a 45-year-old male traveler from Southern Australia who developed symptoms 4 days after his return from Bali where he spent 9 days, with 2 days in Seminyak and 7 days in Canggu in 2015 (Anonym, 2015). All patients survived after receiving treatment. Two patients from Sweden were also confirmed with JE infection: in 1994, a 60-year-old woman who was infected after visiting Bali for 10 days (Buhl and Lindquist, 2009; Hills et al., 2010; Wittesjö et al., 1995) and in 2000 an 80-year-old man who visited Java and Bali for 3 weeks (Buhl and Lindquist, 2009; Hills et al., 2010; Tappe et al., 2012). The four additional cases included a 51-year-old male from Denmark who passed away after 12 days on a coastal hotel in Bali with a few trips inland in 1995 (Buhl and Lindquist, 2009; Hills et al., 2010), a 22-year-old man from France who visited Java and Bali in 2000 (Hills et al., 2010), a 29-year-old woman from The Netherlands (Buhl and Lindquist, 2009; Hills et al., 2010) and a 54-year-old woman, german tourist, after a two-week stay in Bali (Caramello et al., 2007).

Risk factors for JE infection among travelers are variable and depend on destination, length of stay, season, itinerary, activities and accomodations (Hills et al., 2010; Pavli and Maltezou, 2015). Outdoor activity, bednets, repellents, protective cloths, well-screened rooms are other important factors that may influence exposure in endemic areas (Buhl and Lindquist, 2009). Long-term visit to JE endemic areas is also considered an important factor for the JE exposure. However, several cases were confirmed with JE infection after traveling to endemic areas for only few days (Barcus et al., 2002; Östlund et al., 2004).

3.3. JE immunization program

WHO recommends the integration of JE vaccination into national immunization programs in all areas where the disease has become a public health problem. This vaccination program was financially supported by the GAVI alliance (WHO, 2016). In 2016, half of the 24 countries with endemic JE had a national immunization program (Heffelfinger et al., 2017). However, although sentinel surveillance and research activities have been conducted in Indonesia, routine JE reports have not been implemented in all provinces. As a consequence, the JE burden as a baseline for implementation of an immunization program could not be determined accurately. As a result, Indonesia did not have any national JE vaccination programs up to 2016. One of the reason for the absence of national routine JE reports is the difficulty of detecting JE infection at the hospital level. Most hospital and provincial laboratories are not equipped with JE diagnostic tools and do not have trained personnel to handle and detect JE specimens (Chew, 2014). In addition, Indonesia has become a country where many flaviviruses are circulating. At least 7 flaviviruses have been detected from human samples from 1970 to 2016 (Kanamitsu et al., 1979; Myint et al., 2014; Olson et al., 1985, 1983; Perkasa et al., 2016). Therefore cross-reactivity with other flavivirus is common. JE diagnosis in humans is usually based on serology using IgM-capture enzyme –linked immunosorbent assay (ELISA). However, one or more other flaviviruses are needed as controls beside the JE positive control to confirm the presence JE antibodies. This is difficult to perform due to the high-routine operational costs of a multiple positive control ELISA test. Reverse transcriptase polymerase chain reaction (RT-PCR) is not sensitive enough to detect JE cases,
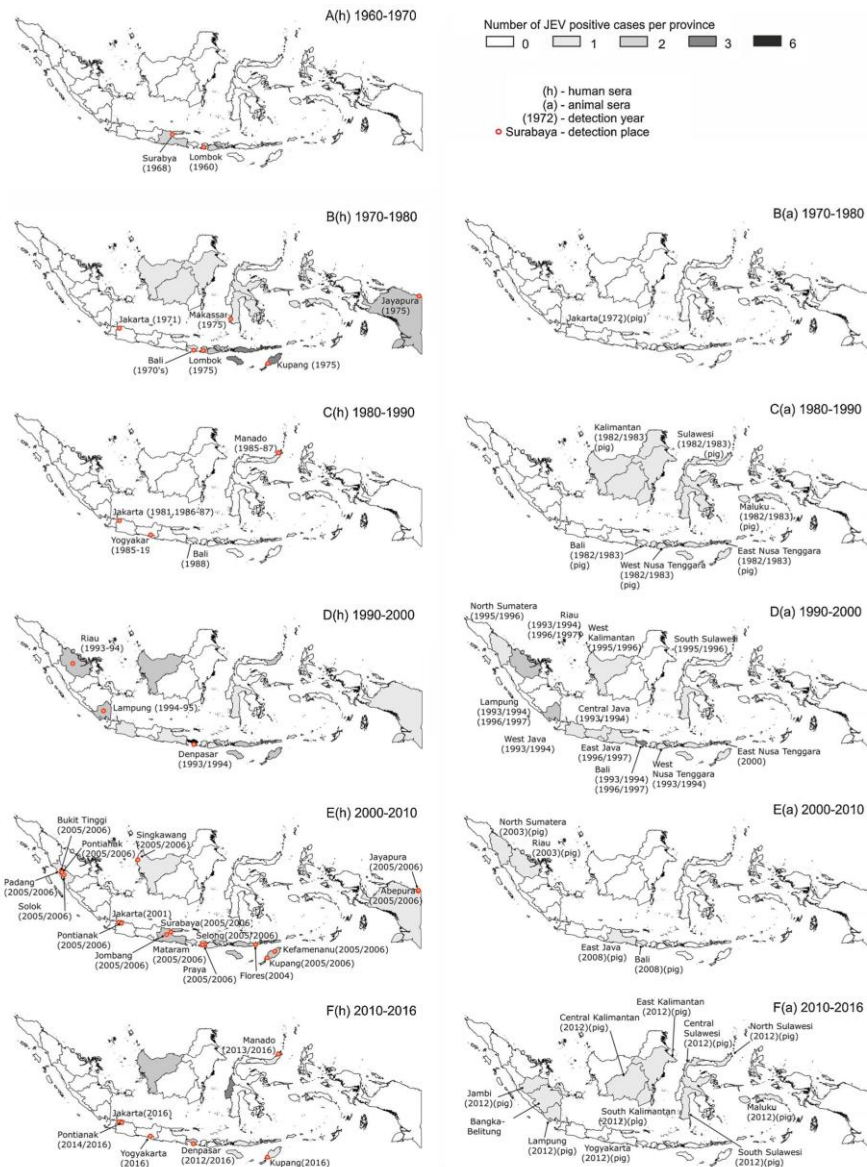
Fig. 2. Maps of Indonesia showing the number of Japanese encephalitis positive cases in human sera (h) and animal sera (a) per province from 1960 to 2016.

particulary from serum samples due to very short viremia period. The plaque reduction neutralizing test (PRNT) is another sensitive detection method for JE. However, this method is time consuming and labour

intensive (Chew, 2014). These reasons have led to a limited-efficiency routine diagnosis of JE in Indonesia.

However, WHO recommends that JE vaccination should be

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integrated into national immunization programs in all JE endemics areas, even though the number of confirmed JE cases is low (WHO, 2016). To initiate the vaccination program, the MoH Indonesia resumed in 2011 a national serum-based surveillance program for JE. The surveillance was conducted in eight sites and Bali became one of the most priority areas (Im et al., 2018). Based on a previous pivotal study of multisite surveillance in 2001, JE transmission in Bali occurs throughout the year, suggesting thus it is an hyperendemic area (Im et al., 2018; Kari et al., 2006). From 2014 to 2016, 408 Balinese children were confirmed with JE infection (Im et al., 2018). Eventually, Bali was chosen as the first location for the implementation of the national JE vaccination program. This program started in March 2018 by using one dose of the Chengdu SA14-14-2 live-attenuated JE vaccine through a two-phase, school-based and community-based mass campaign. It was coordinated and funded by MoH Indonesia and GAVI, and administered together with measles-rubella vaccine. The target population was 890,050 children in Bali ranging from 9 months to 15 years (Im et al., 2018). The JE vaccination program is scheduled to be expanded to North Sulawesi in 2019 (Yosephine, 2017).

3.4. Japanese encephalitis reservoirs

In 1972, pigs were first identified as a reservoir for JE in Indonesia. In that study, pigs did not only have HI antibodies against JE, but also demonstrated viremia (Soedarmo et al., 1994). In 1982–1983, a JE reservoir serosurvey was conducted in several localities of Indonesia. The result revealed considerable seropositivity among pigs. The seropositivity ranged from 34% to 71% in Kalimantan, 27% in Bali, 48% in West Nusa Tenggara and 9–24% in Sulawesi, whereas in Maluku and East Nusa Tenggara all pigs were seronegative. Serological surveys among pigs in slaughter-houses in Jakarta, West Java, Central Java and other provinces suggested a high prevalence of flaviviral antibodies (Table S2) (Koesharyono et al., 1973; Widarso et al., 2002). In several JE endemic areas, such as Bali, West Kalimantan and North Sulawesi, beside the occurrence of JE vectors, the main reason for JE infection incidence is the presence of large scale of intensive pig farming in these areas. However, JE cases also occur in areas where pig farming is low compared to other livestock. A study on JEV mammalian host was conducted in North Sumatera, West Kalimantan, South Sulawesi, West Java, Central Java, East Java and Irian Jaya (Papua) from 1996 to 1997 which showed that 64 cattle samples (51%) out of 126 were positive for JE-antibodies by competitive ELISA. In addition, 23 of 84 goats (27%), 43% (47/110) of chicken, 44% (14/32) of ducks, 14% (2/14) of horses, and 12% (2/16) of dogs samples were found positive, respectively (Sendow et al., 2000). Neutralizing antibodies of JEV were also found in horses (17%) and cows (12%) in Lombok, Timor, West Nusa Tenggara Province (Soedarmo et al., 1994). Although pigs may play a major role as main amplifiers of JEV, there is growing evidence that other vertebrates may have a similar role in JE transmission in certain areas in Indonesia. This is also important to consider for understanding JEV transmission ecology. Ducks and chickens were shown likely to produce a JEV viremia sufficient to infect mosquitoes (Cleton et al., 2014; Lord et al., 2015). However, it has been shown under experimental conditions that cattle are unable to produce a viremia high enough to infect mosquitoes (Lord et al., 2015). Nevertheless, the real role of domesticated birds, cattle, goats, dogs and horses in the transmission JEV remains a challenge that needs to be investigated.

3.5. Japanese encephalitis vectors

3.5.1. Japanese encephalitis virus isolation from mosquitoes

JEV was first isolated in 1972 from *Cx. tritaeniorhynchus* in Bekasi, West Java and Kapuk, West Jakarta. A follow-up study conducted in a pig farm near Bogor (West Java) from 1972 to 1974 successfully isolated JEV from *Cx. tritaeniorhynchus*, *Cx. gelidus*, and *Cx. fuscocephala* (Peenen et al., 1975a; Soedarmo et al., 1994). In 1975, thousands of

mosquito samples were collected from seven Indonesian regions on both sides of the Wallace Line, i.e. Samarinda, Balikpapan, Surabaya, Bali, Lombok, Ujung Pandang, and Pomalaa. The study aimed to identify vector relationships and geographic distribution of arbovirus antibodies in the Indo-Australian archipelago. Potential vectors of JEV were found to be widespread, but virus-specific antibodies were largely confined to regions west of the Wallace Line. This suggests a differential role of Indomalayan and Australasian faunas in the distribution of the JEV (Kanamitsu et al., 1979).

Kanamitsu et al. (1979) detected neutralizing antibodies to JEV in 16% of sera obtained from human residents of Lombok. Olson et al. returned to West Lombok in 1979 to estimate the prevalence of JE viral infection in mosquitoes in an attempt to explain the low prevalence of neutralizing antibodies in human. Three strains of JEV were detected from pools of *An. vagus*, *An. annularis*, and *Cx. tritaeniorhynchus* respectively. The low frequency of JE infection in *Cx. tritaeniorhynchus* and the relatively infrequent raising of pigs may account for the low prevalence of neutralizing antibodies of JE in the human population (Olson et al., 1985, 1983).

In Central Java, Suwasono et al. (1990) conducted a vector risk-assessment in Ungaran in response to historical reports about many patients with fever of unknown origin at the local health clinic. They reported five species of *Culex*, two species of *Aedes* and one species of *Armigeres* in the area that might be potential JEV vectors. A subsequent study was conducted by Tan et al. (Tan et al., 1993) in the same region between September 1986 and August 1988 to assess whether the collected mosquitoes were likely arbovirus vectors. A total of 1385 mosquito pools were collected by the resting method in human dwellings and cattle shelters out of which 25 only (1.8%) were positive for JEV. The majority of JEV were isolated from mosquitoes collected in cattle shelters (*Cx. fuscocephalus*, *Cx. tritaeniorhynchus*, *Cx. bitaeniorhynchus*, *Cx. quinquefasciatus*, *An. vagus*, *An. kochi* and *Armigeres subalbatus*) and only one species was positive from those collected in houses (*Cx. quinquefasciatus*). In this study, 4 new potential JEV vectors were identified in Indonesia: *Cx. bitaeniorhynchus*, *Cx. quinquefasciatus*, *An. kochi* and *Ar. subalbatus*. Interestingly, the percentage of JE positive was higher in cattle sheds (2.17%) than in houses (1.26%), and results were not impacted by seasonality (Tan et al., 1993). Isolates of JEV from *Cx. tritaeniorhynchus* females were obtained during a JE outbreak in Surabaya in 2009 (Widiarti et al., 2014). JEV was also isolated from *Ar. subalbatus* from surrounding cow breeding areas around Surakarta, Central Java (Garjito et al., 2013).

The E gene genotyping of 24 JEV isolates, obtained from mosquitoes collected throughout the Indonesian archipelago from 1974 to 1987, indicated that over that period, three genotypes of JEV circulated. The genotype II (GII) was detected in several mosquito species, i.e. *Cx. tritaeniorhynchus*, *Cx gelidus*, *Cx quinquefasciatus* and *An. vagus* from 1974 to 1980 (Schuh et al., 2013a, b). Genotype III was isolated from *Cx. tritaeniorhynchus* in 1979. From 1980 to 1981, GIV was the only genotype isolated from *Cx. tritaeniorhynchus* in Indonesia (Schuh et al., 2013a, b). Currently, out of 450 mosquito species described in Indonesia, JEV has been isolated from 10 species: *Cx. tritaeniorhynchus*, *Cx. gelidus*, *Cx. vishnui*, *Cx. fuscocephala*, *Cx. bitaeniorhynchus*, *Cx. quinquefasciatus*, *An. vagus*, *An. kochi*, *An. annularis* and *Ar. subalbatus* (Table S3, Fig. 3).

3.5.2. Distribution of Japanese Encephalitis vectors

Cx tritaeniorhynchus is considered the most important JEV vector in many Asian countries, including Indonesia (Banerjee, 1994; Erlanger et al., 2009; Soedarmo et al., 1994; Wang et al., 2007). Isolations of JEV from this mosquito species did not only occur in Java Island, but also in West Nusa Tenggara (Lombok), West Kalimantan, Bali and East Nusa Tenggara. *Cx. tritaeniorhynchus* is found in most main land-mass islands of Indonesia, i.e. Sumatra, Java, Kalimantan, Lesser Sunda archipelago, Sulawesi and Maluku, except Papua. The predominance of *Cx. tritaeniorhunchus* might be due to the wide presence of breeding places,

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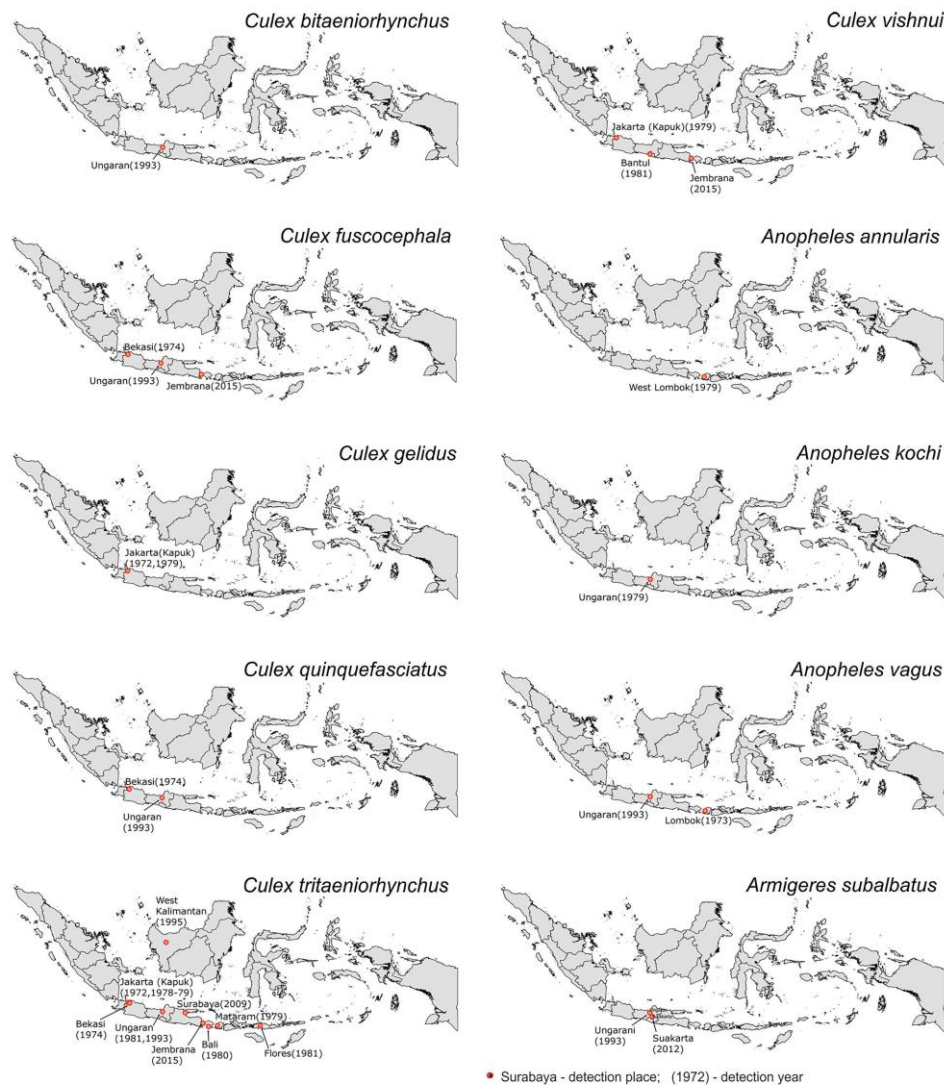
Fig. 3. Occurrence of 10 mosquito vector species reported positive to Japanese encephalitis virus (JEV) in Indonesia and year of detection.

particularly rice fields. A correlation could be established between the increase of JE prevalence and the rice planting season, however this might not be considered important in semi-urban areas, such as Bekasi and Kapuk (O'Connor and Sopa, 1981; Peenen et al., 1975a). Other species including *Cx. gelidus, Cx. vishnui, Cx. fuscocephala, Cx. bitae-niorhynchus, An. vagus, An. kochi, An. annularis, Cx. quinquefasciatus* and *Ar. subalbatus* are also JEV confirmed vectors (Garjito et al., 2013; Soedarmo et al., 1994; Tan et al., 1993).

4. Discussion and conclusions

Sporadic research works and various surveillance studies conducted over the last 40 years have shown that JE continues to circulate in various urban, suburban and rural regions of the Indonesian archipelago. Recently, a sentinel surveillance established in 11 selected provinces (Burni, 2017) confirmed the occurrence of human cases, seroprevalence in humans, presence of various animal reservoir pools and

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multiple vectors (Hills et al., 2010; Kari et al., 2006; Konishi et al., 2009; Kumara et al., 2013; Olson et al., 1983; Ompusunggu et al., 2008; Peenen et al., 1975b, 1974; Sendow et al., 2000; Yamanaka et al., 2010). Pigs are suspected to be the main reservoir (amplifying host) for JEV. However, other animals also play a role in the JE transmission including bovine, horses, goats, sheep and birds. JEV has been documented in 29 provinces located in almost all of regions (Fig. 3). This indicates that JE has been endemic naturally and spread in almost all areas in Indonesia (Solomon et al., 2003; Weaver and Reisen, 2010), which is in agreement with the hypothesis of the Indomalayan region as the place of origin of JE.

The burden of JE in Indonesia is still not clearly established mostly because data on this disease are missing. There is limited information, and particularly no routine JE surveillance data generated at the national, provincial and district levels. The trend of the JE situation in Indonesia from 1960 to present could thus not be established. There is a lack of nation-wide systematic routine surveys, standardized diagnostic approaches and spatio-temporal surveillance of the disease. Therefore, the data included in this study, particularly human cases, JE in animals and JE vector status were derived from various sources. Meta-analysis could not be conducted and comparative analyses between provinces were also limited owing to different survey methods and sample sizes. These are clear limitations of this work. Nevertheless, it appears that Bali, West Kalimantan, East Nusa Tenggara, West Java and East Java display the highest JE incidence. JE burden must be clearly assessed based on longitudinal and standardized analyses, including molecular epidemiology, in order to properly inform decision makers and provide the evidence-based data required to direct public health investment in JE control. In spite of these limitations, this comprehensive review may aid to identify the gaps to fulfill, evaluate the current potential of JE transmission in Indonesia in order to strengthen JE surveillance network and control strategies.

Acknowledgements

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.actatropica.2018.08.017.

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Article 2.

Parasites & Vectors

SHORT REPORT   **Open Access**

First evidence of the presence of genotype-1 of Japanese encephalitis virus in *Culex gelidus* in Indonesia

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Abstract

Background: Japanese encephalitis has become a public health threat in Indonesia. Three genotypes have been recorded in Indonesia, i.e. genotype II (GII), genotype III (GIII) and genotype IV (GIV). Genotype I (GI) and genotype V (GV) have never been reported in Indonesia.

Results: A Japanese encephalitis virus (JEV) belonging to the genotype I-a (GI-a) has been isolated for the first time from a *Culex gelidus* mosquito in the Province of Jambi, Indonesia. This virus is related to a 1983 isolate from Thailand whereas the infected *Cx. gelidus* mosquito belonged to a Chinese haplotype.

Conclusions: Surveillance of JEV and mosquito dissemination is recommended.

Keywords: Japanese encephalitis, Genotype I, Indonesia, *Culex gelidus*

Background

The Japanese encephalitis virus (JEV) is a mosquito-borne flavivirus that has become a public health threat in Asia, including Indonesia. JEV is transmitted to humans through mosquito bites, especially of *Culex* species, from amplifier animals such as pigs. JEV can cause severe central nervous system disorders with high mortality or permanent neurological sequelae [1].

In Indonesia, JEV was first isolated from mosquitoes in West Java in 1972. Since then, encephalitis cases have been reported in several hospitals and currently Japanese encephalitis (JE) has become widespread and endemic across 32 out of 34 Indonesian provinces [2]. JEV originated from the IndoMalayan region and further evolved into five genotypes. Until now, only three genotypes have been recorded in Indonesia, i.e. genotype II (GII), genotype III (GIII) and genotype IV (GIV). Furthermore, GIV has only been described in mosquitoes in Indonesia [3]. Although JEV originated from the Indo-Malaysia

region about 1695 years ago, genotype I (GI) most likely originated in Thailand for clade GI-a and Vietnam for clade GI-b about 193 years ago and has never been reported in Indonesia [3, 4]. Genotype I is associated with human encephalitis in China, Japan, India, Korea, Taiwan, Thailand and Vietnam [4]. GI JEV is an epidemic genotype with equal virulence as GIII JEV, the genotype most frequently associated with outbreaks in Asia [5].

Methods

Jambi, Sumatra, is a province confirmed as a JE endemic area. Entomological investigation was conducted at six sentinel sites in the Bungo, Tanjung Jabung Barat and Sarolangun Districts from May to June 2017 (Fig. 1). Mosquito collections were conducted using human landing, direct collection around cattle and animal baited trap collection. Sampling was conducted from 18:00 h to 6:00 h at every sentinel site. After identification, mosquitoes were sorted according to locality and date, and stored in RNAlater (Ambion-Thermo Fischer Scientific, Waltham, USA) at -80 °C until further analysis. The excised head and thorax of each mosquito were homogenized in a sterile homogenizer, RNA was extracted by

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Fig. 1 Map of Indonesia and of the province of Jambi. The Bungo, Tanjung Jabung Barat and Sarolangun districts where the sampling was conducted are located on the map. Black dots represent the sampling locations in each district (6 per district)

silica-based methods (RNA-easy minikit, Qiagen, Hilden, Germany) and sample aliquots were pooled together by groups of 25. JEV detection was carried out by one step RT-PCR on the NS3 gene [6] using the consensus primers FP (5'-AGA GCG GGG AAA AAG GTC AT-3') and RP (5'-TTT CAC GCT CTT TCT ACA GT-3'). The PCR reaction was performed as previously described [7]. The primers corresponded to a 162-bp product (Fig. 2). JEV genotyping from positive samples was performed using the envelope (E) gene sequence. The E gene was amplified using the primers JEV-Ef (5'-TGY TGG TCG CTC CGG

Fig. 2 Electrophoretic analysis of JEV NS3 PCR products. Lane K+, positive control; Lane K-, negative control; Lane 107, Sample 107 (JE/mosq/jambi107/2017)

CTT A-3') and JEV-Er (5'-AAG ATG CCA CTT CCA CAY CTC-3') [7] using Superscript III one-step RT-PCR with platinum *Taq*DNA polymerase (Invitrogen, Life Technologies, Carlsbad, USA). For amplification of the JEV gene, initial denaturation was carried out at 93 °C for 30 s followed by 40 cycles of 94 °C for 15 s, 53 °C for 30 s and 68 °C for 1 min 30 s, with a final extention step at 68 °C for 5 min. Products were electrophoresed in 2% agarose gel and vizualized by SYBR safe DNA gel staining (Invitrogen, Life Technologies).

The amplification products were then purified using Illustra ExoProSTar (GE Healthcare Life Sciences, Tokyo, Japan). Sequencing of the amplified gene E was performed using the primers JEV-Ef and JEV-Er mentioned above and ABI BigDye terminator Cycle Sequencing Kit v.3.1 (Applied Biosystems, Austin, USA). Prior to sequencing, a PCR product purification step was performed using Big-Dye Xterminator Purification Kit (Applied Biosystems). Sequence data were obtained through the Sanger method using an automatic DNA sequencer (Applied Biosystems 3500 Genetic Analyzer) and analyzed using the Sequencing Analysis 6 program (Applied Biosystems).

The phylogenetic tree was built using the maximum likelihood method with Tamura Nei (TN93) as evolutionary model. Node bootstraps were calculated with 2000

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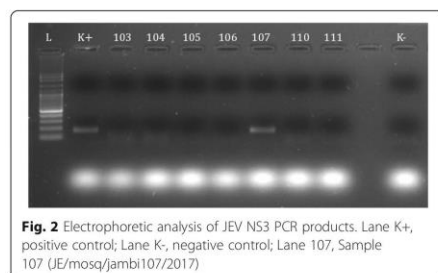
Table 1 *Culex* mosquitoes captured in the Province of Jambi, Indonesia

Species	No. of pools	No. of samples
Cx. fuscocephalus	2	10
Cx. gelidus	34	850
Cx. quinquefasciatus	23	500
Cx. tritaeniorhynchus	2	50
Cx. vishnui	3	75
Total	64	1485

replicates. Sequences were compared to 16 JEV reference sequences from GenBank comprising 6 GI-b, 2 GI-a, 2 GII, 3 GIII, 2 GIV, 1 GV and 2 MVEV (Murray Valley encephalitis virus) sequences used as outgroups. The mosquito in which the JEV was detected was genotyped using the cytochrome *c* oxidase subunit 1 gene (*cox*1) as a target. The *cox*1 gene was amplified using the primers CIN2087 (5'-AAT TTC GGT CAG TTA ATA ATA TAG-3') and TYJ-1460 (5'-TAC AAT TTA TCG CCT AAA CTT CAG CC-3') as previously described [8].

Results and discussion

A total of 1485 *Culex* mosquitoes were collected and analyzed. These mosquitoes belonged to five different species: *Culex gelidus*, *Culex quinquefasciatus*, *Culex tritaeniorhynchus*, *Culex vishnui* and *Culex fuscocephalus* (Table 1). The species displaying the highest prevalence were *Cx. gelidus* and *Cx. quinquefasciatus*. JEV was detected in only one *Cx. gelidus* mosquito. Positive detection of JEV was

confirmed by sequencing and blast analysis. The gene E sequence from JE/mosq/Jambi107/2017 was deposited in GenBank under the accession number MK032889. The gene E phylogenetic analysis (Fig. 3) indicated that JE/mosq/Jambi107/2017 belonged to the clade GI-a of JEV and was closely related to a genotype I-a isolate from Thailand (GenBank: KF192510.1). The GI-a clade of genotype I was described until now only in Thailand and Cambodia. The genotype I of JEV is found only from 10°N up to 35°N with two separate clades [3, 4]. The clade GI-a is found in Thailand and Cambodia from where one case was introduced to Australia [3, 4]. The clade GI-b is found in Vietnam, China, Taiwan, Korea, India and Japan [3, 4].

To our knowledge, this is the first detection of GI JEV in Indonesia. A replacement of GIII by GI was reported throughout Asia and Australia since 1979 [9]. A similar phenomenon could be underway in Indonesia and should be investigated more thoroughly. The mode of introduction of GI in several countries has not been clearly established but a narrower vector host range and a higher replication capacity of GI in mosquitoes has been described [3]. The *cox*1 haplotype of the *Cx. gelidus* mosquito infected with JE/mosq/Jambi107/2017 (GenBank: MK045308) was found to be very close to a haplotype previously described in China (GenBank: MF179173). Blast results for the two best hits were as follows: GenBank: MF179173, maximum score 861, total score 861, total coverage 100%, E value 0.0, identity 97%; GenBank: MF179172, maximum score 856, total score 856, total coverage 100%, E value 0.0, identity 97%. *Culex gelidus* is a good vector of JEV with an invasive capacity and a

Fig. 3 Phylogenetic tree positioning JE/mosq/jambi107/2017 in comparison to 16 JEV reference strains. Reference JEV sequences were obtained from GenBank. Reference sequences are identified by their accession number. JE/mosq/jambi107/2017 (KF192510.1) is shown in a box and marked by a red triangle. The sequence from this study is marked by a red triangle. The phylogenetic analysis was performed with the maximum likelihood method using the Tamura-Nei model (TN93) as evolutionary model. Node bootstraps were calculated with 2000 replicates. Bootstrap values < 70 are not shown. The tree was rooted with 2 Murray Valley encephalitis virus (MVEV) sequences as outgroups. The scale-bar indicates the number of nucleotide substitutions per site

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potential for being transported over long distance by boats, planes or road transportation [10].

Conclusions

The role of specific mosquito populations in the introduction and dissemination of GI JEV through commercial routes should be investigated. GI, which is currently replacing GIII in Asia, could not be detected in cerebrospinal fluid by JEV-specific IgM antibodies raised against GIII JEV [3]. There is thus a risk of misdiagnosis in the presence of GI. Furthermore, all vaccines currently available against JEV are derived from GIII JEV and several studies have reported human confirmed cases with GI JEV infection in areas where effective JEV vaccination programs exist [11, 12]. There is thus, in addition to misdiagnosis, a risk of lack of efficient protection associated with the extension of GI. Further studies and strengthened JE surveillance should be implemented to assess the distribution of GI JEV in Indonesia and health authorities must be alerted in order to address potential risks to public health.

Abbreviations
JEV: Japanese encephalitis virus; G: genotype; *cox*1: cytochrome *c* oxidase subunit 1; MVEV: Murray Valley encephalitis virus

Availability of data and materials
Data supporting the conclusions of this article are included within the article. Raw data are available from corresponding author upon reasonable request. Sequences are deposited in the GenBank database under the accession numbers MK032889 and MK045308.

Authors' contributions
TAG, JW, TTS and LS conceived and designed the experiments. TAG, MTP, LS. DP, SRS, YT, TBTS and DP performed the experiments. TAG, MTP, SM and RF analyzed the data. TAG, TTS, SM and RF wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate
The ethical clearance for primary data was obtained from the authorities of the Ethics Commitee of the National Institute of Health Reserach and Development, Ministry of Health of Indonesia (number LB.02.01/5.2/KE.020/2017).

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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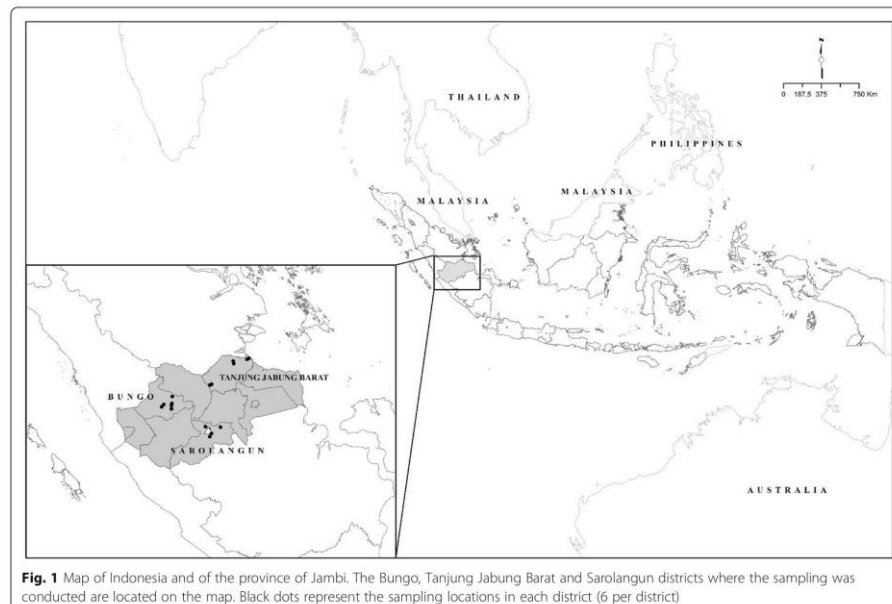
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Conclusions

Japanese encephalitis has been detected throughout the Indonesian archipelago with human cases identified in at least 29 out of 34 provinces, such as Bali, West Kalimantan, East Nusa Tenggara, West Java and East Java, having the highest incidence rates (342). Although limited, records of travel-associated JE cases have also been reported in Indonesia (564,565). Risk factors for JE infection in travelers vary depending on the destination, length of stay, itinerary, activity, and accommodation.

Sentinel surveillance and research activities have been conducted in Indonesia, however, routine JE reports have not been implemented in all provinces. One reason for the absence of JE national routine surveillance reports is the difficulty of JE cases diagnostic at the hospital level due to high-costs for routine operational logistics. As a consequence, data on the number of cases and disease burden, as a basis for implementing the vaccination program, cannot be precisely determined at the national level.

Japanese encephalitis virus (JEV) isolation from mosquitoes were successfully carried out in several places in Indonesia, not only in Java Island, but also in Lombok, Bali, West Kalimantan and East Nusa Tenggara. So far, JEV has been isolated from nine mosquito species: *Culex tritaeniorhynchus, Cx. gelidus, Cx. vishnui, Cx. bitaeniorhynchus, Cx. quinquefasciatus, Anopheles vagus, An. kochi, An. annularis,* and *Armigeres subalbatus. Culex tritaeniorhynchus* is considered the most important JEV vector in Indonesia. Beside confirmed cases with JE infection and the presence of JE vectors, the other important reason for the JE transmission is the occurrence of pig farming and other livestock. Although pigs are reported to have served as the main amplifier of JEV in Indonesia, some evidence also suggests that other vertebrates, such as cattle and goats may also have the same role in JE transmission in certain areas (31). In addition, several studies of JE prevalence in various animal species also revealed that some chickens, ducks, horses and dogs were also positive for JE-antibodies with competitive ELISA. JE reservoirs still need to be further studied and the role of livestock, other than pigs, must be closely investigated in order to better understand JEV transmission and ecology (31,566).

Three genotypes of JEV, i.e. genotype II (GII), genotype III (GIII), and gentoype IV (GIV) have been collected throughout the Indonesian archipelago from 1974 to 1987. However, genotype I (GI) and genotype V (GV) have never been reported from Indonesia. In our study, genotype I-a (GI-a) has been isolated for the first time from a *Culex gelidus* mosquito in Jambi province, Indonesia, in 2017. The phylogenetic analysis of the E gene indicated that this virus is closely related to an isolate of GI from Thailand in 1983. To our knowledge, GI is currently replacing GIII in Asia. This virus genotype could not be detected in the cerebrospinal fluid by JEV-specific IgM antibodies raised against GIII-JEV. This can cause a risk of false-negative and misdiagnosis in the presence of GI. Further study and strengthening of the JE surveillance should be implemented to find out the precise distribution of GI-JEV in Indonesia in order to address potential risks of transmission.

Chapter 3. *Anopheles* species diversity and implications for malaria control interventions in Indonesia

Introduction

Malaria is still one of the most important vector-borne diseases occurring throughout Indonesia (567). National malaria control efforts have been carried out since the 1950s with focus on vector control mainly using indoor residual spraying (IRS) with DDT (dichlorodiphenyltrichloroethane) and chloroquine-based drug treatment. This control program successfully reduced malaria cases in Indonesia during a decade. However, Indonesia was reported to have a resurgence of malaria in the 1960s and 1970s, mainly due to both insecticide resistance and malaria drug resistance. In 2004, the national malaria control program began to intensify malaria control efforts with several approaches. New drug such as artemisinin combination therapy (ACT) was introduced as a first-line treatment after the widespread chloroquine resistance was reported in this country. Improvement and strengthening of diagnosis capacity and vector control were mandated thereafter. These have resulted in better reporting and surveillance. In 2009, Indonesia declared a commitment to achieve national malaria elimination by 2030 with vector control being one of the priority policies.

Currently, Indonesia has made a significant progress and marked a major milestone on malaria elimination, with more than 50% of districts/municipalities officially declared malaria free and with at least 70% of Indonesian population living in areas free from malaria transmission (284). The majority of confirmed malaria cases (76%) occur in eastern Indonesia, particularly in the provinces of Papua, West Papua and East Nusa Tenggara (567).

This is an important achievement for an archipelago country located between the continents of Asia and Australia with a large dispersed population, high diversity of *Plasmodium* spp. and *Anopheles* mosquito species, with various epidemiological contexts and malaria vector habitats. As causative agent of this disease, the duet *P. falciparum* and *P. vivax* is still confirmed as the dominant parasites causing malaria in Indonesia. However, two other species of human malaria parasites (i.e. *P. malariae*,

and *P. ovale*) and the simian *P. knowlesi* species are also identified as associated with the reported malaria cases in Indonesia.

The role of *Anopheles* species as malaria vectors in Indonesia has been identified since 1733. Afterwards, more intensive studies on *Anopheles* species and their role as malaria vectors in Indonesia were initially reported in 1908 (568,569). The checklist of the Anophelines in Indonesia was first published by Swellengrebel in 1921, of which 29 species from 6 groups were listed as being present in Indonesia (567). In 1932, Swellengrebel and Rodenwaldt updated the anopheline checklist, which included 56 species of *Anopheles* from 9 subgenera (570). In 1949, 66 species of *Anopheles* were recorded by Stoker and Koesoemawinangoen (188). Subsequently, in 1981, O'Connor & Sopa described an updated checklist of *Anopheles*, of which 40 species were documented for each subgenus *Anopheles* and *Cellia* (1). In 2005, three new record of species, members of the Leucosphyrus Complex, was made including *An. introlatus, An. latens* and *An. cracens* reported and described by Sallum*, et al.* in several areas of Sumatra and Kalimantan (571). In 2009, Paredes-Esquivel, *et al.* reported the new distribution of *An. saeungae,* members in the Barbirostris Complex, in West Sumatra, Indonesia (274). In 2013, Townson, *et al.* described *An. vanderwulpi* (formerly *An. barbirostris* clade II) as a new species and new member in the Barbirostris Complex from samples collected in Central Java, Indonesia (273,274). In 2014, *An. oreios,* a new species, member of the Farauti Complex, was described by Bangs, *et al.* in Papua following morphological and molecular evidences (276). In 2017, Harbach has upgraded the status of *An. sumatrana*, previously recommended as a subspecies of *An. gigas* to the list of valid mosquito species (572). National research to update mosquito fauna in Indonesia (Vektora) also revealed *An. limosus* as a new record of *Anopheles* species distributed in Indonesia (263). In 2020, Syafruddin, *et al.* also reported the presence of *An. epiroticus* of the *An. sundaicus* complex, in Indonesia (573). Currently, at least of 87 formally named *Anopheles* species, one unnamed putative species and two subspecies of *An. gigas* (*An. gigas* var. danaubento and *An. gigas* var. oedjalikalah) have been identified in Indonesia. Of these 90 *Anopheles* taxa, 25 among them have been documented as being malaria vectors. The primary vector species include *An. aconitus, An. barbirostris, An. balabacensis, An. farauti, An. maculatus, An. sundaicus, An. subpictus, An. sinensis, An. flavirostris, An. nigerrimus, An. punctulatus,* and *An. koliensis* (3).

The distribution and bionomics of the 25 confirmed malaria vector species in Indonesia have also been reviewed (3,192,194). However, before some of them were recognized as sibling species belonging to complexes, most previous studies on bionomics and ecological work on malaria vector species in Indonesia were done based on morphological identifications. Differentiating species using morphological characters raises serious questions and generates misidentifications due to insufficient or overlapping characters, especially in the case of species complexes. Therefore, the ability to differentiate isomorphic species is important as sibling species within a complex may have different roles in malaria transmission from very efficient vectors with high vectorial capacity to not-epidemiologically important species without any role as malaria vector. Therefore, it is essential to use appropriate molecular assays to identify the different sibling species within a species complex or group. The *An. farauti* complex and its bionomics are a case study. Generally, the *An. farauti* complex is known as the most important malaria vector in Papua and West Papua provinces. The *An. farauti* complex comprises 8 sibling species within the Punctulatus Group (574). Several previous studies revealed that Papua has at least 5 of the 8 sibling species based on molecular analysis (3). *An. farauti* s.s. has the most extensive geographic distribution compared to any other members of the group, but it is restricted to coastal areas, while the other sibling species of the complex have a more diverse ecological distribution (220). Biting and resting behaviour among *An. farauti* s.l. may also vary by location and sibling species. This has been noted from some studies conducted on the coastal areas of Sorong and near Jayapura (3,233). In 2011, a study on the behaviour and molecular identification of *Anopheles* malaria vectors was conducted in Jayapura district, Papua province. The result revealed that a single morphological species of *An. farauti* s.l. could be separated into three molecular species within the *An. farauti* complex (i.e. *An. farauti* s.s., *An. farauti* 4, and *An. hinesorum*). All of these species are considered to be important malaria vectors. In this case, without molecular identification, *An. farauti* 4 would have been identified as *An. farauti* s.l. and not considered to be a major malaria vector. No other species of the *An. farauti* complex were found during this study (234).

As noted above, the present study was intended to investigate the genetic diversity of species within the *Anopheles maculatus* group, known to play an important

role in malaria transmission in Indonesia. It is essential to understand the bionomic traits by investigating the species diversity and the geographic distribution of each malaria vector species for implementing effective malaria control methods and elimination efforts since an important biodiversity of *Anopheles* species occur, including major vectors to non-vector species.

Article displayed in Chapter 3

Article 3. Garjito TA, Widiastuti U, Mujiyono M, Prihatin MT, Widiarti W, Setyaningsih R, Alfiah S, Widartono BS, Syafruddin D, Satoto TBT, Gavotte L, Bangs MJ, Manguin S, Frutos R. Genetic homogeneity of *Anopheles maculatus* in Indonesia and origin of a novel species present in Central Java. *Parasites & Vectors.* 2019; 12(1): 351.

Article 3.

RESEARCH **Open Access**

Genetic homogeneity of *Anopheles maculatus* in Indonesia and origin of a novel species present in Central Java

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Abstract

Background: *Anopheles maculatus* (*s.s.*) is an important vector of malaria in Indonesia. Previously it was considered the only member of the Maculatus Group present in Indonesia. A novel species was recently identified in the Kulon Progo District in Central Java. Until recently, few investigations have been conducted looking at *An. maculatus* genetic diversity in Indonesia, including allopatric island populations.

Methods: Indonesian *An. maculatus* (*s.l.*) samples were collected in several locations in Java, Lesser Sunda Island group, Sumatra and in Kulon Progo (Yogyakarta, central Java) where a novel species has been identified. Samples from a 30-year-old colony of the Kulon Progo population were also included in the analysis. Maximum-likelihood analysis established the phylogenies of the ITS2 (nuclear) and *cox*1 (mitochondrial) markers. Putative times of separation were based on *cox*1 genetic distances.

Results: Two species of the Maculatus Group are present in Indonesia. The novel sibling species is more closely related to *Anopheles dispar* than to *An. maculatus* (*s.s.*). *Anopheles maculatus* (*s.s.*) samples are homogeneous based on the ITS2 sequences. Indonesian samples and *An. dispar* belong to the same *cox*1 maternal lineage and differ from all other known members of the Maculatus Group. Divergence time between the different populations found in Java was estimated using an established *cox*1 mutation rate.

Conclusions: A novel species within the Maculatus Group, most closely related to *An. dispar*, is confirmed present in the Kulon Progo area of Central Java. The divergence of this species from *An. maculatus* (*s.s.*) is explained by the stable refugia in the Kulon Progo area during the quaternary period of intense volcanic activity throughout most of Java. This novel species awaits detailed morphological description before applying a formal species name. For the interim, it is proposed that the Kulon Progo population be designated *An. maculatus* var. *menoreh* to distinguish it from *An. maculatus* (*s.s.*).

Keywords: *Anopheles maculatus*, Maculatus Group, Indonesia, Malaria

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Background

Anopheles maculatus (*sensu lato*), in the Neocellia Series [1] of the subgenus *Cellia*, is a widespread species in Asia, ranging from the Indian subcontinent to Southeast Asia and southern China [2–6]. In Indonesia, this species is widely distributed in the western part of the archipelago extending to Weber's Line, a hypothetical biogeographical separation between Sulawesi and the Maluku Islands chain [7]. *Anopheles maculatus* has been recorded in Sumatra, Java, Kalimantan, Bali, Lesser Sunda Islands including East Timor (Democratic Republic of Timor-Leste), and Sulawesi [8].

Prior to the cytogenetic identification of different chromosomal forms, *An. maculatus* was regarded as a single taxon [9, 10]. Currently, based on phenotypic characteristics, crossmating experiments, cytogenetic and molecular analyses, the Maculatus Group [11] is divided into two subgroups and nine species [3, 10, 12]. The subgroups are differentiated by distinct morphological characters. The Maculatus Subgroup [13] includes *An. maculatus* (*sensu stricto*) Theobald, 1901 and *Anopheles dravidicus* Christophers, 1924, while the Sawadwongporni Subgroup [13] comprises *Anopheles sawadwongporni* Rattanarithikul & Green, 1986 [9], *Anopheles notanandai* Rattanarithikul & Green, 1986 [11] and *Anopheles rampae* Harbach & Somboon, 2011 [14]. The four other species in the group include *Anopheles greeni* Rattanarithikul & Harbach, 1991, *Anopheles dispar* Rattanarithikul & Harbach, 1991, *Anopheles willmori* James, 1903 and *Anopheles pseudowillmori* Theobald, 1910 [12, 15, 16].

The Southeast Asian mainland presents the highest diversity of the Maculatus Group, with seven species present in Thailand [3, 17, 18]. *Anopheles greeni* and *An. dispar* appear restricted (endemic) to the Philippines [15]. Five species are found in China excluding *An. notanandai* and *An. rampae* [3, 19]. In Vietnam, four species [*An. maculatus* (*s.s.*), *An. pseudowillmori*, *An. sawadwongporni* and *An. rampae*] are present [20–22]. Until recently, only *An. maculatus* (*s.l.*) [presumed (*s.s.*)] was reported in Indonesia [8]. A second species has been suspected present in the Kulon Progo District area in Central Java since the late 1990s (MJB, personal communication). This putative, as yet undescribed species was recently reported from material derived from a continuously colonized strain reared [23] over three decades at the Indonesian Ministry of Health Institute for Vector and Reservoir Control Research and Development, a component of the National Institute of Health Research and Development (NIHRD-IVRCRD) and described in this work [24]. *Anopheles maculatus* has long been considered a major malaria vector in West (peninsular) Malaysia [25], and areas of Sumatra and Java, Indonesia [26–30], predominately in rural, forested areas [31].

Numerous instances of natural malaria plasmodia infections in *An. maculatus* have been reported in Indonesia [31]. Infection indices have varied from 2.83% in Kisaran (Sumatra) to 3% in Central Java, 17% in Londut (Sumatra) and 11% in Riau Province (Sumatra) [26, 32]. This species is a major public health concern in the Menoreh Hills region, which includes the Kulon Progo District near the border of Central Java Province and the Special Region of Yogyakarta. It is also reported as a major malaria vector in southern Sumatra (Tenang) [33–36]. Interestingly, although present in Kalimantan, Sulawesi, Bali and the larger islands in the Nusa Tenggara (Lesser Sunda Islands) region, *An. maculatus* has either not been reported as a malaria vector or is an epidemiologically insignificant species in these areas [37].

We analyzed the diversity and phylogeny of *An. maculatus* samples collected in different locations and islands in Indonesia. We also analyzed the relationship of the proposed novel species present in Kulon Progo District and reared at NIHRD-IVRCRD with other members of the Maculatus Group to derive its putative origin.

Methods

Mosquito collections and identification

Adult mosquitoes were collected from field settings using standard procedures for human-landing and cattle-landing methods [38] in six provinces of Indonesia between 2012 and 2018. Sampling locations included Cilacap, southern Central Java (samples C1 and C2; October 2011), Belu, West Timor, East Nusa Tenggara (samples NT64 and NT 101; November 2011), Ogan Komering Ulu, South Sumatra (samples S9 and S33; October 2011), Sebatik Island, northern Kalimantan (samples N2 and N44; November 2011), Purbalingga, Central Java (sample P1; September 2011), Kulon Progo, Central Java (samples KP10 and KP72; November 2013) and the NIHRD-IVRCRD laboratory, Salatiga (samples 1x, 2M and 4M; October 2018) (Fig. 1; Table 1). *Anopheles maculatus* samples were initially identified using morphological criteria [39]. Mosquitoes were sorted and labeled according to locality and date, and stored in 1.5 ml Eppendorf tubes under dry conditions over silica gel until further analysis [13, 39]. Additionally, a laboratory strain of *An. maculatus* originating from Kulon Progo and under continuous colonization for greater than 30 years at the NIHRD-IVRCRD laboratory in Salatiga, Central Java [23] was compared with more recent field samples from Kulon Progo collected in 2015. To maintain the colony established at IVRCRD Salatiga, wild type material was re-introduced into the laboratory colony in 2003. This re-introduced wild type material was collected in the exact same location as the initial population, i.e. the village of Hargotirto, Kokap subdistrict, Kulon Progo district, Province of

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Fig. 1 Map of the sampling sites in Indonesia. Each sampling site is indicated as a black spot. The name of sampling sites and samples are indicated. The source of geographical data layers is the Indonesia Geospatial Information Agency who granted the permission and rights to publish this map
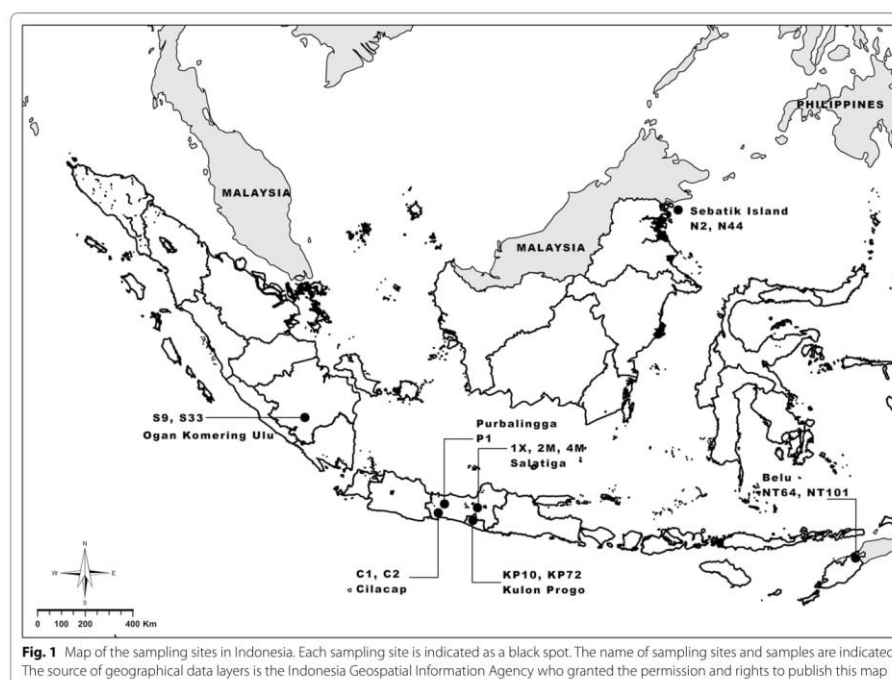
Yogyakarta. Representative field-collected specimens are deposited in the Systematics and Reference Laboratory, IVRCRD, Salatiga.

DNA extraction, amplification and sequencing

DNA was extracted from the legs of each mosquito using a DNeasy® Blood & Tissue Kit (Qiagen, Hilden, Germany) with modification based on the manufacturer's protocol. The amplification of ITS2 was performed with primers ITS2a (5′-TGT GAA CTG CAG GAC ACA T-3′) and ITS2b (5′-TAT GCT TAA ATT CAG GGG GT-3′) [39]. *cox*1 was amplified using the primers CI-N-2087 (5′-AAT TTC GGT CAG TTA ATA ATA TAG-3′) and TY-J-1460 (5′-TAC AAT TTA TCG CCT AAA CTT CAG CC-3′). PCR reactions were carried out using GoTaq® Green Master Mix (Promega, Madison, WI, USA). PCR thermocycling conditions for ITS2 were as follows: 94 °C for 10 min; followed by 40 cycles of denaturation at 94 °C for 1 min, annealing at 56 °C for 45 s and elongation at 72 °C for 1 min; followed by a final extention step at 72 °C for 10 min. For

amplification of the *cox*1 gene, the following conditions were used: initial denaturation at 94 °C for 1 min followed by five cycles of 94 °C for 30 s, 45 °C for 40 s and 72 °C for 1 min; this was then followed by 35 cycles of 94 °C for 30 s, 55 °C for 40 s and 72 °C for 1 min, and by a final extention step at 72 °C for 10 min [40]. The amplified PCR products were separated by 1.5% agarose gel electrophoresis and vizualized by SYBR® safe DNA gel stain (Invitrogen, Carlsbad, CA, USA). A 100-bp DNA ladder was used for calculating the size of the PCR products. Amplification products were purified using Applied Biosystems ExoSAP-IT™ (Thermo Fisher Scientific, Vilnius, Lithuania). Cycle sequencing was performed using the primers listed above and an Applied Biosystems BigDye™ Terminator v.3.1 Cycle Sequencing Kit (Life Technologies Cooperation, Austin, TX, USA). To remove unicorporated BigDye® terminators and salts, cycle sequencing products were purified using a BigDye® Xterminator Purification Kit (Life technologies, Bedford, MA, USA). Sequence data were obtained using a DNA sequencer (Applied

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Table 1 Sampling localities and specimens of *Anopheles* mosquitoes

Sample code	Location	Ecology	Altitude range (m)	Role as malaria vector	GenBank ID (ITS2)	GenBank ID (*cox1*)
P1	Purbalingga, Java	Wet rice field, plantation	250–329	Yes	MK656100	MK683475
C1	Cilacap, Java	Secondary forest, plantation, wet rice field	300–348	No	MK656095	MK683467
C2	Cilacap, Java	Secondary forest, plantation, wet rice field	300–348	No	MK656096	MK683468
KP10	Kulon Progo Java	Secondary forest, wet rice field	300–1000	Yes	MK659792	MK683471
KP72	Kulon Progo Java	Secondary forest, wet rice field	300–1000	Yes	MK659780	MK683472
1x	Insectary laboratory IVRCRD Salatiga (origin Kulon Progo)	Laboratory conditions	700	Yes	MK659773	MK683464
2M	Insectary laboratory IVRCRD Salatiga (origin Kulon Progo)	Laboratory conditions	700	Yes	MK675654	MK683465
4M	Insectary laboratory IVRCRD Salatiga (origin Kulon Progo)	Laboratory conditions	700	Yes	MK675653	MK683466
NT64	Belu, East Nusa Tenggara	Secondary forest, wet rice field	150–215	Yes	MK659796	MK683473
NT101	Belu, East Nusa Tenggara	Secondary forest, wet rice field	150–215	Yes	MK659794	MK683474
S9	Ogan Komering Ulu Sumatra	Coffee and rubber plantations	800–892	Yes	MK659795	MK683476
S33	Ogan Komering Ulu Sumatra	Coffee and rubber plantations	800–892	Yes	MK659793	MK683477
N2	Sebatik Island Kalimantan	Coconut, palm oil, coffee and cacao plantations	150–218	Yes	MK659798	MK683469
N44	Sebatik Island Kalimantan	Coconut, palm oil, coffee and cacao plantations	150–218	Yes	MK659797	MK683470

Biosystems® 3500 Genetic Analyzer) and analyzed using the Sequencing Analysis 6 program (Applied Biosystems).

Sequence analysis

Sequences were edited using Sequencing Analysis v.5.2 (Applied Biosystems). Sequences were aligned with MUSCLE using SeaView v.4.7 [41] and Mega X [42]. Phylogenetic trees were constructed with the maximum likelihood (ML) method and the Kimura-2 (K80) evolutionary model in Mega X. To assess the ML tree reliability, bootstraps were tested with 1000 replicates. To estimate the evolutionary divergence between sequences, genetic distances were analyzed by pairwise distance (p-distance) methods [43] in Mega X. Divergence time was calculated based on previously reported estimates giving 1 million years (Myr) for 2.3% difference [44, 45]. Sequences are deposited in GenBank under the following accession numbers: ITS2: N2 (MK659798), N44 (MK659797), S9 (MK659795), S33 (MK659793), NT64 (MK659796), NT101 (MK659794), KP10 (MK659792), KP72 (MK659780), 1x (MK659773), 2M (MK675654), 4M (MK675653), P1 (MK656100), C1 (MK656095) and C2 (MK656096); *cox1*: N2 (MK683469), N44 (MK683470), S9 (MK683476), S33 (MK683477), NT64 (MK683473), NT101 (MK683474), KP10 (MK683471), KP72 (MK683472), 1x (MK683464), 2M (MK683465),

4M (MK683466), P1 (MK683475), C1 (MK683467) and C2 (MK683468).

Results

ITS2 diversity and phylogeny of *Anopheles maculatus*

The comparative analysis of the ITS2 sequences of all *An. maculatus* samples and of available reference sequences from other members of the Maculatus Group and select other *Anopheles* species present in Indonesia indicates that two populations of *An. maculatus* are present in Indonesia. Samples of *An. maculatus* coming from Purbalingga (P1), Cilacap (C1, C2), Belu (NT64, NT101), Sebatik Island (N2, N44) and Ogan Komering Ulu (S9, S33) displayed 100% genetic similarity and were also 100% identical to *An. maculatus* sequences from the mainland Asian continent (Fig. 2, Additional file 1: Table S1). The GenBank *An. maculatus* sequences used as reference corresponded to mosquitoes isolated in India (JQ446438), Thailand (DQ518615), Vietnam (AY803351), Malaysia (DQ518619), Cambodia (DQ518618) and China (DQ518616). For the nuclear ribosomal ITS2 sequence, the similarity between all *An. maculatus* reference sequences and sequences from samples P1, C1, C2, NT64, NT101, N2, N44, S9 and S33 indicated a high conservation and genetic homogeneity regardless of distribution and geographical distance (Fig. 2). There was also no difference between samples from the continental

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Fig. 2 Phylogenetic analysis of the ITS2 sequences. Kulon Progo samples are identified with blue stars. The phylogenetic tree was constructed using the maximum likelihood (ML) method and the Kimura-2 (K80) evolutionary model in Mega X. To assess the ML tree reliability, bootstraps were tested at 1000 replicates

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Asian land mass and island groups. Conversely, the samples KP10, KP72, 1x, 2M and 4M isolated from Kulon Progo did not cluster with the continental *An. maculatus* sequences producing a separate, genetically distinct and homogeneous group more closely related to *An. dispar*. The sequences 1x, 2M and 4M, which correspond to a laboratory strain of *An. maculatus* collected decades ago in Kulon Progo, were identical to those samples collected for this work, i.e. KP10 and KP72, indicating strong genetic stability after years of continuous colonization.

This phylogenetic analysis indicates that although separated into two different genetic aggregates (groups), collectively the Indonesian samples belong to the Maculatus Group. With respect to genetic distances, the samples displayed two ranges of distances depending on the group considered (Additional file 1: Table S1). The Indonesian *An. maculatus* group, i.e. samples P1, C1, C2, NT64, NT101, S9, S33, N2 and N44, showed no genetic distance with the continental *An. maculatus* reference sequences. The ITS2 sequence appears entirely conserved. The genetic distance within the Maculatus Group ranged between 2.7–20.8%, while the genetic distance of the *An. maculatus* sequences from other group members ranged between 5–16.5% (Additional file 1: Table S1). The Kulon Progo samples (KP10, KP72, 1x, 2M and 4M) displayed no (0%) internal group distance and a 5.5–5.8%

distance with the other *An. maculatus* sequences. Comparison with other members, the Kulon Progo sequences displayed distances of 3%, 6.5%, 7.7–8%, 8%, 10.4–10.9%, 15.5–17% and 10.9% with *An. dispar*, *An. greeni*, *An. dravidicus*, *An. sawadwongporni*, *An. willmori*, *An. pseudowillmori* and *An. rampae*, respectively (Additional file 1: Table S1). The alignment of the Kulon Progo ITS2 sequences with *An. maculatus* (*s.s.*) from Indonesia, *An. maculatus* (*s.s.*) from mainland Asia, and *An. dispar* is provided in Additional file 2: Figure S1.

*cox*1 diversity and phylogeny of *Anopheles maculatus*

The comparative analysis of the mitochondrial *cox*1 sequences, indicative of the maternal lineage, showed that all the samples and reference sequences belonged to four genetically distinct and separated lineages, Lineage 1 being separated into two sublineages (Fig. 3a). Lineage 4 comprised only *Anopheles sinensis* (subgenus *Anopheles*, Hyrcanus Group) and was therefore used as outgroup for the rooted tree (Fig. 3b). Lineage 1a comprised *Anopheles stephensi*, *Anopheles flavirostris*, *An. dispar* (a Maculatus Group member), all the Indonesian samples including the Kulon Progo population, *An. gambiae* and *Anopheles barbirostris* (Clade I); while Lineage 1b included three out four species of the Leucosphyrus Complex, along with *Anopheles macarthuri*, a member of the Riparis

Fig. 3 Phylogenetic analysis of the *cox*1 sequences. **a** Midpoint rooted tree. **b** Tree rooted using *Anopheles sinensis* as outgroup. Kulon Progo samples are identified with stars. Phylogenetic trees were constructed with the maximum likelihood (ML) method and the Kimura-2 (K80) evolutionary model in Mega X. To assess the ML tree reliability, bootstraps were tested with 1000 replicates

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Subgroup, and *Anopheles cracens*, a member of the Dirus Complex, all five belonging to the Leucosphyrus Group [14]. Lineage 2 comprised only *An. balabacensis*, the fourth species of the Leucosphyrus Complex. Lineage 3 comprised all of the *An. maculatus* reference samples and members of the Maculatus Group available in GenBank, excluding *An. dispar* and the Indonesian *An. maculatus* samples. Lineage 3 and Lineage 4 branched directly on the root; whereas, Lineage 1a and Lineage 1b were further separated by a bootstrap of 99. The Indonesian *An. maculatus* sequences within Lineage 1a grouping displayed some internal genetic variability. All Kulon Progo samples examined were identical, while genetic distances of up to 2.6% were observed with *An. maculatus* (*s.s.*) from Indonesian (Additional file 3: Table S2). With respect to the other members of Lineage 1a, the closest species was *An. dispar* with a percentage of divergence ranging between 7.8–8.4% depending on the sample. The divergence of the Indonesian samples with *An. flavirostris* (Minimus Subgroup) ranged between 12.2–13.5%, while *An. stephensi* (Neocellia Series) ranged between 10.4–12.2% (Additional file 3: Table S2). The alignment of the *cox*1 sequences of the Kulon Progo samples, *An. maculatus* (*s.s.*) from Indonesia, *An. maculatus* (*s.s.*) from mainland Asia, and *An. dispar* is provided in Additional file 4: Figure S2.

Time of divergence

The time of divergence was calculated based on previously reported estimates of the variation of the *cox*1 gene in the genus *Anopheles* where 2.3% of divergence is estimated to correspond to 1 million years (Myrs) [42, 43]. The estimated time of divergence of *An. maculatus* (*s.s.*) from the Kulon Progo population and from *An. dispar* was estimated at between 26–26.2 Myrs, and between 30.2–30.9 Myrs, respectively, which corresponds to the Oligocene Epoch. The divergence of the Kulon Progo population from the other members of the Kulon Progo maternal lineage, i.e. *An. dispar*, *An. flavirostris* and *An. stephensi*, is dated 3.4 (Pliocene Epoch), 5.3 (Miocene Epoch) and 5.04 Myrs (Pliocene), respectively. The other Indonesian *An. maculatus* sequences displayed the same separation time with *An. dispar* as the Kulon Progo population with the exception of NT101 (Belu, East Nusa Tenggara), C1 (Cilacap, Central Java) and P1 (Purbalingga, Central Java), indicating separation around 3.13 and 3.65 Myrs ago (Pliocene), respectively. The separation of the Kulon Progo population from the other Indonesian *An. maculatus* samples was dated 0.65 (NT64, S9, S33), 0.43 (NT101, C1, P1) and 0.22 Myrs (C2, N2, N44), all corresponding to the latter part of the Pleistocene Epoch.

Discussion

Anopheles maculatus (*s.s.*) was previously believed to be the only member of the Maculatus Group present in Indonesia, a species regarded as an important vector of malaria in certain localities [6]. This Asian group is a diverse assemblage with at least nine described species, five of which fall into two subgroups [3, 10, 12]. Investigating the diversity of *An. maculatus* in Indonesia was therefore a prerequisite for a better understanding of the distribution, bionomics and variations in vector capacity over its wide geographical range. The investigation reported herein provides several conclusions. First, there is definitive evidence of at least two species within the Maculatus Group in Indonesia, confirming a recent report by Ali et al. [24], which raises the number of species to ten (none of which are nominal taxon) within the Maculatus Group. Secondly, *An. maculatus* (*s.s.*) appears genetically homogeneous throughout its geographical range in Asia. Thirdly, members of the group in Indonesia differ by maternal origin from all other members, including *An. maculatus* (*s.s.*) from mainland Asia, with the lone exception of *An. dispar*.

The Kulon Progo population has been suspected as a distinct species within the Maculatus Group based on unpublished work spanning several decades (MJB, personal comm). This population was recently proposed as a different species based on selected morphological characters and genetic (ITS2 and *cox*2) sequences [24]. The ITS2 phylogenetic analysis in the present study confirmed that the Kulon Progo population and all other known *An. maculatus* sequences analyzed in Indonesia are members of the Maculatus Group, yet they also comprise genetically distinct groups. ITS2 is not considered a good intraspecific marker due to its low evolution rate and high conservation; however, it is a good marker at the species level showing clear discrimination indicative of species separation [46–50]. The phylogenetic distance between the ITS2 sequences of the Kulon Progo population and other *An. maculatus* sample sequences both Indonesia and mainland Asia included in the analysis ranged between 5.5–5.8%. This is greater than the ITS2 distances displayed by other groups of closely related *Anopheles* species. For example, two sibling species in the *Anopheles farauti* complex (an assemblage of 8 species) differ by only 4.0% [51], while *An. greeni* and *An. dispar* (Maculatus Group) also differ by 4.0% [52]. In Africa, five species within the *An. gambiae* complex show intraspecific differences ranging between 0.4–1.6% [53], while two members of the *An. dirus* complex, *An. dirus* (*s.s.*) (formerly species A) and *An. baimaii* (species D), display 5.4% genetic distance [54]. The Kulon Progo population was shown to be morphologically distinct from *An. maculatus* (*s.s.*) while cross-mating experiments generated

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partially sterile hybrids [24]. The combined evidence confirms that the Kulon Progo population is a distinct species and one that likely extends throughout the greater Menoreh Hill region in central Java. Until a formal morphological description can be made, it is hereby proposed that the Kulon Progo population be designated an infrasubspecific entity, *An. maculatus* var. *menoreh*, in reference to its region of origin and to distinguish it from *An. maculatus* (s.s.).

The two Indonesian members of the Maculatus Group and *An. dispar* belong to the same mitochondrial lineage and differ from that of all other known members of the group outside Indonesia. Collectively, these data demonstrate the occurrence in Indonesia of an introgression of the *An. maculatus* (s.s.) chromosomal genome from continental to insular populations. A similar phenomenon of introgression has been demonstrated for *Anopheles sundaicus* complex in Southeast Asia [44]. Introgression is a key adaptive mechanism of *Anopheles* mosquitoes to exist in various environments [44, 55], and well described in the *Anopheles gambiae* complex [56, 57].

The Pleistocene Epoch (2.58 Myrs to 11,700 years ago) is believed to have played a key role in the distribution of *Anopheles* mosquitoes in Southeast Asia [46, 58–60]. The period was characterized by a series of glaciation and inter-glaciation periods, which generated dramatic climatic changes and large variations in sea level [61, 62]. During glaciation periods, islands west of the Wallace's Line were interconnected on the same land mass known as the Sunda Shelf [63–65]; whereas, during inter-glaciation events the rainforest environments expanded, thus providing more favorable habitats for *Anopheles* mosquitoes while island landmasses remained isolated. Sulawesi, the eastern Lesser Sunda and Maluku island chains, and western New Guinea Island were isolated and separated by sea from the western half of the Indonesian archipelago, while the Philippines followed a different biogeographical evolution. Palawan Island (western Philippines) was then connected to the Sunda Shelf but later separated and collided with the mobile belt of the Philippine archipelago. This geological history induced by shifts in climate is considered to have greatly influenced the current structural diversity of *Anopheles* populations in Southeast Asia and the evolution of present-day species complexes through successive genetic expansions and bottlenecks [66].

Based on the molecular evidence, the Kulon Progo population appears more closely related to *An. dispar*, a species that appears confined to the northern Philippines. The most parsimonious way to explain this geographical discrepancy is that their common ancestors gradually moved from continental Asia to the current island territories during the Oligocene, which corresponds to the calculated separation of the Kulon Progo lineage from the continental *An. maculatus* (s.s.) lineage (between 23 and 26.4 Myrs). A movement of *An. maculatus* from the continent appears to have occurred before 3.4 Myrs ago (between the late Oligocene and early Pliocene epochs), the calculated time of separation between *An. dispar* and the Kulon Progo population. This event led to introgression of the *An. maculatus* chromosomal genome into at least a portion of the maternal lineage identified as Lineage 1. During the Pliocene (3.4 Myrs ago), the ancestor of *An. dispar* was separated from the main introgressed population, likely the result of the tectonic shift of Palawan Island towards the current Philippine archipelago. During the Pleistocene, increased volcanism occurred in central and eastern Java but the Kulon Progo area was naturally spared from the surrounding destruction and served as a relic forest refuge [67]. This isolation event occurred between 0.22 and 0.65 Myrs, which corresponds to the calculated separation time between the Kulon Progo population and the other Indonesian archipelagic *An. maculatus* populations. During the late Pleistocene period (200,000 to 11,700 years ago), at least one other species invasion and introgression by continental *An. maculatus* appears to have occurred which generated the current Indonesian populations of *An. maculatus* (s.s.). This timescale is in agreement with that calculated for the movements of populations and introgression detected in *An. sundaicus* in Southeast Asia [44, 45].

Conclusions

Anopheles maculatus, along with *Anopheles balabacensis*, is the main malaria vector species occuring in the Kulon Progo area and the greater Menoreh region [33–37, 68–71]. The evidence presented here confirms that the Kulon Progo population is a distinct species and one that likely extends throughout the greater Menoreh Hill region in central Java. There are now two recognized members of the Maculatus Group present in Indonesia. However, a detailed morphological description of this novel species is required to establish a new nominal taxon. To distinguish it from *An. maculatus* (s.s.), in the interim it is hereby proposed an infrasubspecific entity ('variety'), *An. maculatus* var. *menoreh*.

Additional files

Additional file 1: Table S1. Pairwise genetic distance of ITS2 sequences. Genetic distances were calculated with the Kimura 2 parameters using Mega X.

Additional file 2: Figure S1. Alignment of ITS2 sequences. Alignment performed using Seaview v.4.7 with MUSCLE program for multialignment.

Abbreviations
ITS2: internal transcribed spacer 2; *cox*1: cytochrome oxidase subunit I; ML: maximum likelihood.

Availability of data and materials
Data supporting the conclusions of this article are included within the article and its additional files. Raw data are available from the corresponding author upon reasonable request. ITS2 sequences are deposited under accession numbers: N2 (MK659798), N44 (MK659797), S9 (MK659795), S33 (MK659793), NT64 (MK659796), NT101 (MK659794), KP10 (MK659792), KP72 (MK659780), 1x (MK659773), 2M (MK675654), 4M (MK675653), P1 (MK656100), C1 (MK656095) and C2 (MK656096). *cox*1 sequences are deposited under accession numbers: N2 (MK683469), N44 (MK683470), S9 (MK683476), S33 (MK683477), NT64 (MK683473), NT101 (MK683474), KP10 (MK683471), KP72 (MK683472), 1x (MK683464), 2M (MK683465), 4M (MK683466), P1 (MK683475), C1 (MK683467) and C2 (MK683468).

Ethics approval and consent to participate
This study involved the use of humans to collect adult mosquitoes in natural settings. Formal approval to conduct these activities was provided by the Ethical Commission Board of the NIHRD, Ministry of Health, Indonesia (no. KE.01.03/EC/077/2011, March 8, 2011).

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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Conclusions

This chapter presented the genetic homogeneity of *Anopheles maculatus*, one of the most important malaria vector in Indonesia. This taxon has been reported as a major malaria vector in the Menoreh Hills region, at the border of Central Java province and Jogjakarta province. It was also confirmed as an important malaria vector in southern Sumatra. Although *An. maculatus* is broadly distributed throughout the main islands of the Indonesian archipelago, excluding Maluku and Papua, this species has never been reported as a malaria vector in Kalimantan, Sulawesi, Bali, and the Lesser Sunda Islands. Previously, this species was considered the only member of the Maculatus group present in Indonesia.

We analyzed the diversity and phylogeny of *An. maculatus* samples collected in several locations in Java, Lesser Sunda Islands, Sumatra, and Kulon Progo (Menoreh hills region). In addition, samples from a 30-year-old laboratory colony originating from Kulon Progo were also included in this study. With the molecular-based species identification tools using the ITS2 (nuclear) and *cox1* (mitochondrial) markers, two species of the Maculatus group have now been identified in Indonesia. A novel species was confirmed as occurring in Kulon Progo. This novel species, more closely related to *An. dispar*, differs from all other known members of the Maculatus group, including *An. maculatus* (s.s.). The Kulon Progo population was temporally named as *An. maculatus* var. menoreh. This finding is important for identifying and implementing targeted and more effective malaria vector-control strategies. In this perspective, a better knowledge on this new species is now necessary to better define its geographic distribution and role as malaria vector.

Chapter 4. Genetic diversity of dengue vector, vector surveillance methods and entomological indices to assess risk of dengue transmission in Indonesia

Introduction

Dengue is the most important arthropod-borne viral disease and is one of the major public health concerns in Indonesia. In the past 50 years, the annual incidence rates (IR) of dengue hemorrhagic fever (DHF) have increased very sharply, i.e. from 0.05 cases per 100,000 and per year in 1968 to 78.85 cases per 100,000 and per year in 2017. Although case fatality rates (CFR) showed a declining trend over the last decades (from 41% in 1968 to 0.72% in 2017), incidence rates of dengue displays a rising pattern with increasing number of cases approximately every 5 years. The disease is caused by four dengue virus serotypes (DENV 1-4). Dengue virus is transmitted to humans through bites of mosquitoes belonging to the genus *Aedes* (subgenus *Stegomyia*), primarily by *Ae. aegypti* and by *Ae. albopictus* as a secondary vector (16,575–577).

Both species, *Ae. aegypti* and *Ae. albopictus,* have a high adaptive capacity to thrive in various man-made breeding habitats and can invade new continents *via* human movements and trade of goods as they did in most regions of Indonesia. The invasion and adaptation to new areas are closely related to their ecology and biology as well. As previously reported in several studies, molecular analyses revealed that genotyping of *Ae. aegypti* and *Ae. albopictus* is important to provide valuable information on population dynamics and the dispersal of *Ae. aegypti*, in particular for better understanding differences in vector competence and capacity to transmit dengue virus, ecological adaptations, and resistance to insecticides (578–582). However, information about the genetic diversity and structure among *Ae. aegypti* populations in Indonesia are still limited. In this study, we investigated the genetic diversity and structure of *Ae. aegypti* isolated from 40 districts/municipalities using cytochrome oxidase subunit I (*CoxI*) being a mitochondrial DNA (mtDNA) marker, with the aim to trace the distribution pattern of the dengue virus and to predict the risk of dengue transmission in Indonesia.

Aedes aegypti and *Ae. Albopictus* are present all Indonesian provinces and dengue epidemics also occur in almost all Indonesian provinces. Appropriate dengue vector surveillance methods should thus be implemented to assess the risk of dengue outbreaks. One of the most important challenges is to develop reliable, efficient, and effective sampling methods to collect the target dengue vector species. Although various dengue vector sampling methods have been documented, the lack of information on the optimal collection methods means that a study related to the field collection methods of adults and larvae of dengue vector needs to be implemented. Herein, we compared the existing vector surveillance method, e.g. larval collection, with other larval methods such as larval rearing and adult collection methods such as morning resting, and human landing collection, to investigate the best vector surveillance methods in relation to the presence of dengue virus.

Since there are neither drugs against dengue, nor effective vaccine, vector control is currently the only way to prevent and control dengue transmission. To monitor vector abundance for targeting and evaluation vector control, the World Health Organization (WHO) recommends vector surveillance based on larval surveys of container habitats. These indicators have been based on the traditional *Stegomyia* indices (House index-HI, Container index-CI, Breteau index-BI) (583). For many years, these larval indices remained the most used ones to measure vector infestation to prevent and predict the risk of dengue transmission. However, recent studies revealed that larval indices do not always reflect the abundance of adult mosquitoes and the risk of dengue transmission (583,584). Based on these observations, we developed a study to analyze the correlation between *Stegomyia* indices and the risk of dengue transmission by using data from a very large zone covering 78 sampling sites throughout Indonesia from Sumatra to Papua corresponding to different locations and ecosystems.

Articles displayed in Chapter 4

Article 4. Garjito TA, Widiarti, Hidajat MC, Handayani SW, Mujiyono M, Prihatin MT, Satoto TBT, Ubaidillah R, Sudomo M, Manguin S, Gavotte L, Frutos R. Replacement of populations of *Aedes aegypti* and *Aedes albopictus* in Indonesia. **(Submitted to PlosNTD)**

Article 5. Garjito TA, Susanti L, Mujiyono M, Prihatin MT, Susilo D, Nugroho SS, Mujiyanto M, Wigati W, Satoto TBT, Manguin S, Gavotte L, Frutos. R. Assessment of mosquito collection methods for dengue surveillance. **(Submitted to Parasite & Vectors)**

Article 6. Garjito TA, Hidajat MC, Kinansi RR, Setyaningsih R, Anggraeni YM, Mujiyanto M, Trapsilowati W, Jastal J, Ristiyanto R, Satoto TBT, Gavotte L, Manguin S, Frutos R. *Stegomyia* indices and risk of dengue transmission: a lack of correlation. **Frontiers in Public Health. 2020, 8:328.**

Article 4.

PLOS Neglected Tropical Diseases
Replacement of populations of Aedes aegypti and Aedes albopictus in Indonesia
--Manuscript Draft--

Manuscript Number:	
Full Title:	Replacement of populations of Aedes aegypti and Aedes albopictus in Indonesia
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Abstract:	Currently, Aedes aegypti, the principal vector of dengue virus in Indonesia, has spread throughout the archipelago. Invasion and high adaptability of Ae. aegypti to all of these areas is closely related to its ecology and biology. Between June 2016 and July 2017, larval and adult mosquito collections were conducted in 43 locations in 25 provinces of Indonesia using standardized sampling methods for dengue vector surveillance. The samples collected were analyzed for polymorphism and phylogenetic relationship using the mitochondrial cox 1 gene and the nuclear ribosomal Internal Transcribed Spacer 2 (ITS2). Ae. aegypti specimens were found to be homogenous all over Indonesia with all samples belonging to the same maternal lineage. Almost all Ae. aegypti samples collected in this study (89%) belonged to the same population. Populations of Ae. aegypti characterized few years ago were genetically different. The same situation was observed with Aedes albopictus for which the current population is different from the one described earlier. This indicates a recent and fast dynamic of population replacement for both Ae. aegypti and Ae. albopictus in Indonesia. This poses a threat for population-dependent inundative strategies of dengue vector control.
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Dear Editor,

We are very pleased to submit to PLoS Neglected Tropical Diseases a manuscript titled "Replacement of populations of *Aedes aegypti* and *Aedes albopictus* in Indonesia" and co-authored by Triwibowo Ambar Garjito, Widiarti, Muhammad Choirul Hidajat, Sri Wahyuni Handayani, Mujiyono, Mega Tyas Prihatin, Rosichon Ubaidillah, Mohammad Sudomo, Tri Baskoro Tunggul Satoto, Sylvie Manguin, Laurent Gavotte and Roger Frutos.

We investigated the genetic diversity, evolutionary relationship and distribution of *Aedes aegypti* and *Aedes albopictus* mosquitoes as part of national Indonesian programs on vector control and management. This study was conducted in 43 different locations and islands, from Western Sumatra (Aceh or NDA for Nangro Aceh Darussalam as it is currently known) to Eastern Indonesia (Papua) using the *cox*1 gene and ITS2 sequence. We discovered that for both species, a replacement of populations occurred throughout Indonesia in a rater short period of time. Not only the populations currently present are monophyletic and homogeneous but they differ from the populations collected in 2013 for *Ae. aegypti* and from 2013 to 2015 for *Ae. albopictus*. Our sample collection took place in 2016 and 2017. In a maximum of three years, these populations were completely replaced. This is not only important for understanding the dynamic of dengue and other *Aedes*-borne diseases but it has also consequences on the inundative strategies currently being assessed in Indonesia. These strategies, Wolbachia or sterile males, are population dependent and fast and massive replacements of populations might strongly affect their efficiency.

We really hope that this manuscript will meet the standards for publication in PLoS NTD

On behalf of all authors and with my best regards,

Triwibowo Ambar Garjito
Corresponding author

Replacement of populations of *Aedes aegypti* and *Aedes albopictus* in Indonesia

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Abstract

Currently, *Aedes aegypti,* the principal vector of dengue virus in Indonesia, has spread throughout the archipelago. Invasion and high adaptability of *Ae. aegypti* to all of these areas is closely related to its ecology and biology. Between June 2016 and July

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35 2017, larval and adult mosquito collections were conducted in 43 locations in 25
36 provinces of Indonesia using standardized sampling methods for dengue vector
37 surveillance. The samples collected were analyzed for polymorphism and phylogenetic
38 relationship using the mitochondrial *cox*1 gene and the nuclear ribosomal Internal
39 Transcribed Spacer 2 (ITS2). *Ae. aegypti* specimens were found to be homogenous all
40 over Indonesia with all samples belonging to the same maternal lineage. Almost all *Ae.*
41 *aegypti* samples collected in this study (89 %) belonged to the same population.
42 Populations of *Ae. aegypti* characterized few years ago were genetically different. The
43 same situation was observed with *Aedes albopictus* for which the current population is
44 different from the one described earlier. This indicates a recent and fast dynamic of
45 population replacement for both *Ae. aegypti* and *Ae. albopictus* in Indonesia. This poses
46 a threat for population-dependent inundative strategies of dengue vector control.

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49 **Author summary**

50   We investigated the genetic diversity, evolutionary relationship and distribution
51 of *Aedes aegypti* and *Aedes albopictus* mosquitoes collected in 43 different locations
52 and islands, from Western Sumatra (Aceh) to Eastern Indonesia (Papua) using the *cox*1
53 gene and ITS2 sequence. The results revealed that all *Ae. aegypti* samples belong to the
54 same maternal lineage. Two haplotypes in particular, H1 and H4, seem to correspond
55 to populations with a very high colonizing and demographic potential. When compared
56 to previous *Ae. aegypti* samples collected in 2013, this study shows that the current
57 population of *Ae. aegypti* has replaced previous populations throughout the country
58 over a short period of time. The very same replacement of populations occurred
59 between 2013 and 2016 with *Ae. albopictus*. Fast and massive replacements of
60 populations might strongly affect inundative dengue-vector control strategies, which
61 are population-dependent.

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68 Introduction

69   *Aedes aegypti* is known as a major vector of dengue viruses (family
70 *Flaviviridae*, genus *Flavivirus*, DENV) [1,2], yellow fever virus (family *Flaviviridae*,
71 genus *Flavivirus*, YF) [1,3,4], zika virus (family *Flaviviridae*, genus *Flavivirus*, ZIKV)
72 [5,6] and chikungunya virus (family *Togaviridae*, genus *Alphavirus*, CHIKV) [1,3].
73 This mosquito species originates from the forest of Africa and, since the 18th Century
74 has spread *via* transcontinental trade throughout tropical and subtropical regions [4,7-
75 10]. In Southeast Asia, *Ae. aegypti* was formally identified for the first time in Malaysia
76 and Thailand at the early 20th century [11,12]. *Ae. aegypti* was formally identified later
77 in Indonesia, in 1908 [13]. A local strain of *Ae. aegypti,* the Medan strain, was first
78 reported and successfully colonized in a laboratory in the 1930s [14]. Currently, the
79 species is reported to have spread throughout the archipelago [15,16].
80
81   Another dengue vector species mosquito is the Asian tiger mosquito, *Aedes*
82 *albopictus*. This species has for long been considered as a secondary vector of several
83 viruses [17,18,19,20]. *Ae. albopictus* originates in the forests of Southeast Asia, and has
84 spread worlwide since the 1970s [21]. According to the Global invasive species
85 database (http://www.issg.org/database/), this species has been recorded as one of the
86 worst invasive species in the world. Currently, *Ae. albopictus* can be found in Asia,
87 Africa, Europe, North and South America and many locations in the Pacific and Indian
88 oceans except Antartica [1,17]. As an invasive species, *Ae. albopictus* plays a potential
89 role to trigger a re-emergence of arboviruses transmission in many locations. Recently,
90 this species played an important role in Dengue, Chikungunya, and Zika outbreaks in
91 both endemic and invaded regions [22,23,24,25,26].
92
93   The invasion and adaptation to all of these areas are closely related to their
94 ecology and biology as well. *Ae aegypti* has high adaptability to urban and peridomestic
95 areas, where it breeds in the vicinity of human dwellings in a variety of artificial and
96 natural containers in urban and rural areas [1,15,27]. This species is also recognized as
97 the most anthropophilic mosquito and has the ability to blood-feed repeatedly on
98 humans almost on a daily basis [28]. This behavior may have contributed to the capacity
99 of *Ae. aegypti* to cause high epidemics of dengue fever in Indonesia. A total of 68,407
100 dengue cases (incidence: 78.85/100,000) with 493 deaths (case fatality rates (CFR):

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101   0.72%) were reported in 2017 [29,30]. *Ae. albopictus* displays a strong ecological
102   plasticity and could transmit DENV and has shown a remarkable capacity to adapt to
103   urban and sub-urbans under various climate conditions, displacing *Ae. aegypti*
104   population in some areas. *Ae. albopictus* has now become a significant vector of
105   CHIKV and DENV [17,31,32,33].

106

107   While an effective multivalent dengue vaccine is still under research and not yet
108   available, vector control and entomological surveillance are the only reliable means of
109   prevention and control of dengue fever [34-37]. Updated information on the genetic
110   diversity and evolutionary patterns among *Ae. aegypti* and *Ae. albopictus* populations
111   are needed to provide clues for better understanding the origin, the structuration and the
112   distribution of populations. Moreover, this is also a prerequisite to define differences in
113   vector competence and capacity to transmit dengue virus, in ecological adaptations, and
114   in resistance to insecticides [3,38,39]. However, the comprehensive information about
115   genetic diversity and structuration of populations of *Ae. aegypti* and *Ae. albopictus* in
116   Indonesia is currently insufficient.

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118   Therefore, we investigated the genetic diversity, evolutionary relationship and
119   distribution of *Ae. aegypti* and *Ae. albopictus* mosquitoes collected in different
120   locations and islands, from Western Sumatra (Aceh) to Eastern Indonesia (Papua) using
121   the mitochondrial *cox*1 or COI gene and the internal transcribed spacer 2 (ITS2) of the
122   ribosomal DNA.

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124

125   **Material and Methods**

126

127   **Study sites.** The study was conducted in 43 districts/municipalities in 25 dengue-
128   endemic provinces in Indonesia (Table 1, Figure 1). These provinces were Aceh, Riau,
129   Riau Islands, Jambi, Bangka-Belitung, Lampung, Banten, West Java, Central Java,
130   Yogyakarta, East Java, West Kalimantan, South Kalimantan, Central Kalimantan, East
131   Kalimantan, Bali, West Nusa Tenggara, East Nusa Tenggara, North Sulawesi, Central
132   Sulawesi, South Sulawesi, Southeast Sulawesi, Maluku, North Maluku, and West
133   Papua. Sampling of larva and adult mosquitoes were conducted as part of the 2nd year

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134   of the "Rikhus Vektora" project in July-August 2016 in 28 locations, the WHO project

135   SEINO (#1611945) in September-October 2016 in 6 locations, and subsequently in 9

136   locations as part of the 3rd year of "Rikhus Vektora" project in May-July 2017 (Figure

137   1).

138

139   **Collection and rearing of mosquitoes.** Larva, pupa and adult mosquitoes were

140   collected using standardized sampling methods for dengue vector surveillance [27-30].

141   In each house, larvae and pupae from different containers were put in different plastic

142   bags. All samples were then transported to a field laboratory. Larvae and pupae were

143   reared in the field laboratory for 3 days until the emergence of adult mosquitoes.

144   Mosquitoes were then morphologically identified, sorted according to locality and

145   preserved in 250 µl of RNAlater (Ambion-Thermo Fisher Scientific, Watham, USA).

146   They were then stored at -80°C until further analysis. Larvae which did not emerge

147   after 3 days were preserved the same way as adult mosquito samples for further

148   analysis.

149

150   **DNA extraction, amplification and sequencing.** Whole DNA from each mosquito

151   was individually extracted using a DNeasy® Blood & Tissue Kit (Qiagen, Hilden,

152   Germany) according to the manufacturer's standard protocol. The amplification of *cox*1

153   was conducted using the primers CI-N-2087 (5'-AAT TTC GGT CAG TTA ATA ATA

154   TAG-3') and TY-J-1460 (5'-TAC AAT TTA TCG CCT AAA CTT CAG CC-3') as

155   previously described [31]. The ITS2 sequence was amplified using the primers ITS2a

156   (5'-TGT GAA CTG CAG GAC ACA T-3') and ITS2b (5'-TAT GCT AAA ATT CAG

157   GGG GT-3'). PCR reactions were carried out using the GoTaq® Green Master Mix

158   (Promega, Madison, WI, USA). The conditions for PCR amplification of the *cox*1 gene

159   were as follows: 1 cycle at 94°C for 1 min for initial denaturation, followed by five

160   cycles at 94°C for 30 s, 45°C for 40 s and 72°C for 1 min. This was then followed by

161   35 cycles at 94°C for 30 s, 44°C for 40 s and 72°C for 1 min, and by a final extension

162   step at 72°C for 10 min [31]. PCR thermocycling conditions for ITS2 were as follows:

163   94°C for 10 min; followed by 40 cycles of denaturation at 94°C for 1 min, annealing at

164   56°C for 45 s and elongation at 72 °C for 1 min; followed by a final extension step at

165   72°C for 10 min. PCR products were electrophoresed in 1.5% agarose gel and

166   vizualized by SYBR® safe DNA gel stain (Invitrogen, Carlsbad, CA, USA) using a

167   Biorad Molecular Image Gel Doc XR (Biorad Laboratories Inc, California, USA). A

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168   100-bp DNA ladder was used for calculating the size of the PCR products. Amplicons

169   were purified using Applied Biosystems ExoSAP-IT™ (Thermo Fisher Scientific,

170   Vilnius, Lithuania). Cycle sequencing was performed using the primers listed above

171   and an Applied Biosystems BigDye™ Terminator v.3.1 Cycle Sequencing Kit (Life

172   Technologies Cooperation, Austin, TX, USA). To remove unincorporated BigDye®

173   terminators and salts, cycle sequencing products were purified using a BigDye®

174   Xterminator Purification Kit (Life technologies, Bedford, MA, USA). Sequence data

175   were obtained using a DNA sequencer (Applied Biosystems® 3500 Genetic Analyzer)

176   and analyzed using the Sequencing Analysis 6 program (Applied Biosystems). All

177   sequences have been deposited in Genbank under the accession numbers MW280620

178   to MW280792, MW280794 to MW280797 and MW280800 to MW280818 for *Ae.*

179   *aegypti cox*1 sequences. The accession numbers for *Ae. aegypti* ITS2 sequences range

180   from MW288143 to MW288145 and MW290431 to MW290468. The accession

181   numbers for *Ae. albopictus cox*1 sequences are MW280793, MW280798, MW280799

182   and MW283303 to MW283318. The accession numbers for *Ae. albopictus* ITS2

183   sequences are MW287155 to MW287157. The accession numbers of the *cox*1 and ITS2

184   sequences of the unknown species *Aedes* sp (sls25_Asp) are MW286812 and

185   MW293720, respectively.

186

187   **Polymorphism and phylogenetic analysis.** Sequences were analyzed for definition of

188   haplotypes using DnaSP software v.6.12.03 [45]. The relationship between haplotypes,

189   based on pairwise difference to generate a minimum spanning tree (MST) and

190   minimum spanning network (MSN), was calculated and modeled using Network

191   software and Hapstar v. 0.7. Multiple alignment and phylogenetic analysis were

192   performed using the SaeView package [46]. Phylogenetic trees were built using

193   maximum-likelihood (ML) with the general time reversible model with gama

194   distributed with 4 discrete categories (GTR+G). The clades support was assessed via

195   500 bootstrap replicates.

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198   **Results**

199

200   **Phylogenetic relationships of the collected sampled.** The samples collected in this
201   work fell into three different branches, both for *cox*1 and ITS2 (Supplementary Figure
202   1). These branches corresponded to *Ae. aegypti*, *Ae. albopictus* and another
203   undetermined *Aedes* species. The latter sample was therefore named sls25_Asp
204   (Supplementary Figure 1). The *cox*1 gene phylogeny showed the presence of two main
205   clusters in *Ae. aegypti*, Cluster Aae1 and Cluster Aae2, with Cluster Aae1 being
206   separated into two sub-clusters: Subcluster Aae1a and Subcluster Aae1b (Table 1,
207   Supplementary Figure 1). These clusters were separated by very low bootstraps values
208   indicating that the tree was not well structured and that the samples belonged to the
209   same population (Supplementary Figure 1). A similar feature was observed with the
210   *cox*1 gene from *Ae. albopictus*. However, three clusters could be identified with strong
211   bootstraps (100), Cluster Aal1, Cluster Aal2 and Cluster Aal3 (Table 2, Supplementary
212   Figure 1).

213

214   **Presence of a yet unidentified species**. The sample sls25_Asp from Maros in South
215   Sulawesi (site No 36 in Figure 1) was initially misidentified in the field as *Culex*
216   *quinquefasciatus*. This sample was branching apart from *Ae. aegypti* and *Ae. albopictus*
217   for the *cox*1 gene indicating that it was neither *Ae. aegypti* or *Ae. albopictus*
218   (Supplementary Figure 1). It was also different from *Cx. quinquefasciatus* used as
219   outgroup. The phylogeny of the ITS2 sequences showed a similar result. The sample
220   sls25_Asp was different from *Ae. aegypti* and *Ae. albopictus* (Supplementary Figure
221   2). The ITS2 sequence showed a best hit with an *Aedes polynesiensis* mosquito from
222   Fidji (AY822662) with a percentage of identity of 88.24 % whereas the *cox*1 sequence
223   displayed as best hit an *Ae. albopictus* sample from Vietnam (HQ398902) with 91.95 %
224   identity. However, there was no *cox*1 sequence for *Ae. polynesiensis* in Genbank and it
225   was thus impossible to confirm if the *cox*1 gene would also link sls25_Asp to *Ae.*
226   *polynesiensis.* A morphological analysis showed that the closest species, although still
227   with morphological differences, was *Aedes paullusi* (data not shown). There is no *cox*1
228   or ITS2 records for *Ae. paullusi* in databases. The *Ae. albopictus* and *Ae. polynesiensis*

7

229   hits for sls25_Asp *cox*1 and ITS2 sequences, respectively, might simply be default hits

230   due of the lack of relevant sequences in the databases.

231

232   **Phylogeny and polymorphism of *Aedes aegypti cox*1 gene.** The *cox*1 sequences from

233   samples collected for this work were compared to the only other source of *Ae. aegypti*

234   *cox*1 sequences from Indonesia, a 17-samples collection from 2013 in the North Coast

235   of Central Java [3]. These samples, identified in the tree by their accession numbers

236   from KP869121 to KP869126 and KP334259 to KP334269, make a completely

237   separate cluster (Figure 2). The genetic distance between the sequences from this work

238   and those reported by Yohan et al. [3] ranged from 0.4 % to 3.1 %. With a mutation

239   rate of the *cox*1 gene in insects ranging from 2.4 % Mya^{-1} to 3.5 % Mya^{-1} [47,48], the

240   time needed for the accumulation of these mutations is ranging from 114,285 years to

241   167,000 years for a mutation rate of 2.4 % Mya^{-1} and from 885,714 years to 1,291,667

242   years for a mutation rate of 3.1 % Mya^{-1}. When blasting the *cox*1 sequences from this

243   work against Genbank data, the 2013 sequences previously identified in Indonesia [3]

244   did not respond as best hits. However, best hits were obtained with the same score with

245   a series of 9 *cox*1 sequences of *Ae. aegypti* mosquitoes captured in Peru, Cambodia,

246   Puerto Rico, India, Georgia, England and Germany (Supplementary Table 1).

247   Subcluster Aae2a showed two best hits both from Kenya (Supplementary Table 1).

248   Subclusters Aae2b and Aae2d had only one corresponding best hit in Genbank from

249   Mozambique and Haiti, respectively (Supplementary Table 2 1). Subcluster Aae2c

250   showed five best hits with the same score from Egypt and Kenya (Supplementary Table

251   1). Interestingly, Subcluster Aae2e displayed four best hits with the same score but only

252   one was a wild-type mosquito captured in Haiti. The other three best hits corresponded

253   to the reference strains reared in laboratory conditions of Liverpool and RED

254   (Supplementary Table 1). Finally, two individual samples diverging from Cluster 1,

255   46_Aae (IS1) and 28-1-Aae (IS2) displayed each one a different best hit. IS1 showed a

256   best hit with a mosquito collected in Russia, whereas IS2 showed a best hit with *Aedes*

257   *aegypti formosus,* which is considered an ancestral feral population from Sub-Saharan

258   Africa [49,50] (Supplementary Table 1). The breakdown into individual haplotypes

259   showed that Cluster Aae1 comprised 176 samples out of 198 (89 %) and 39 haplotypes

260   out of 53 (73.6 %) with two of them, H1 and H4, being the most represented (Table 1,

261   Supplementary Table 1, Figure 3). The H1 haplotype comprised 57 samples (32.85 %)

262   whereas the haplotype H4 contained 61 samples (34.6 %) (Table 1). The Subcluster

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263   Aae1a comprised 16 haplotypes, including the haplotype H1, and 75 samples whereas
264   the Subcluster Aae1b contained the haplotype H4 and 22 other haplotypes for a total of
265   102 samples (Table 1).
266
267   **Phylogeographic distribution of *Aedes aegypti cox*1 lineages.** The Cluster Aae1 was
268   as expected present everywhere with the exception of East Aceh and North Lombok
269   (Supplementary Figure 3, Table 1). No correlation could be found between any cluster
270   and any location. When considering the geographic distribution of the haplotypes, a
271   lack of correlation was also observed (Supplementary Figure 4). Only a default
272   correlation could be observed, i.e. rare haplotypes from a region with few samples.
273   However, this is a sampling bias and is not significant.
274
275   **Phylogeny and polymorphism of *Aedes aegypti* ITS2.** The samples were distributed
276   into two clusters and 21 haplotypes (Table 1, Figure 4, Supplementary Table 3). Cluster
277   1 is divided into four sub-clusters (1a to 1d), which displayed limited variations (Figure
278   5). As a consequence, Cluster 1 gathered 42 samples and 23 haplotypes representing all
279   the sequence variations observed within this monophyletic group (Figure 5). The
280   different haplotypes were closely related with a maximum relative distance of 11.54 %
281   (Supplementary Table 3). Cluster 2 comprised only four samples, each one
282   corresponding to a different haplotype. They were very closely related with an overall
283   variation of 0.51 % (Supplementary Table 3).
284
285   **Phylogeographic distribution of *Aedes aegypti* ITS2 sequences.** The analysis of the
286   polymorphism of *Ae. aegypti* ITS2 haplotype in this study showed that cluster 1 was
287   the dominant one (Supplementary Figure 5). Subcluster 1a displayed the most extensive
288   distribution, covering Sumatra (East Aceh-Aceh, Pematang Raman-Jambi, New Week-
289   Riau, South Lampung-Lampung), Java (West Bandung-West Java, Semarang-Central
290   Java, Bantul-Yogyakarta, Malang-East Java), Kalimantan (Sambas-West Kalimantan,
291   Balikpapan-East Kalimantan), Bali (Karangasem), West Nusa Tenggara (Lombok) and
292   Sulawesi (Palu-Central Sulawesi, Maros-South Sulawesi) (Supplementary Figure 5).
293   Subcluster 1a showed a best hit with mosquitoes collected in Russia, Sri Lanka. The
294   other sub-clusters, namely 1b, 1c and 1d, showed a limited distribution. Subcluster 1b
295   was only found in the Sambas-West Kalimantan region, while subcluster 1c was
296   identified in two locations: Batam-Riau Islands, and Bantul-Yogyakarta. Subcluster 1d

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297   was found in two locations, i.e. Pematang Raman-Jambi and Palu-Central Sulawesi

298   (Supplementary Figure 5, Supplementary Table 3). Cluster 2 had a more limited

299   distribution. This cluster was found in Karangasem-Bali, Ambon-Maluku, Malang-East

300   Java and Batam-Riau islands (Supplementary Figure 5, Supplementary Table 3).

301

302   **Phylogeographic distribution, phylogeny and polymorphism of *Aedes albopictus***

303   ***cox*1 and ITS2.** The *cox*1 sequences of the *Ae. albopictus* samples collected in this

304   work were compared to those released by Maynard et al. [51] who collected samples in

305   Jakarta in 2012, in Waingapu (Sumba) in 2013 and in Timika (Papua) in 2015.

306   Sequences were also compared to those released by Battaglia et al. [52] who established

307   a worldwide classification of *Ae. albopictus cox*1 haplogroups [52]. The COI sequences

308   from Maynard et al. [51] matched perfectly the haplogroups defined by Battaglia et al.

309   [52] and were distributed within two different haplogroups, A2a and A1b1a. The

310   sequences obtained in this work did not correspond to any of the sequences reported by

311   Maynard et al. [51] and did not match any of the haplogroups defined by Battaglia et

312   al. [52] (Figure 6). Out of the three clusters identified within the *Ae. albopictus*

313   sequences reported in this work, Cluster Aal1 was closer, although clearly different, to

314   the haplogroups A2a, Cluster Aal3 was closer, although different also, to the

315   haplogroup A1ba1, and Cluster Aal2 was not close to any haplogroup. A total of 11

316   different haplotypes were found (Figure 7, Table 2). The genetic distance between the

317   *cox*1 sequences reported in this work and those from Maynard et al. [51] is ranging

318   from 0.4 % to 1.3 % depending on the sample. The time needed to accumulate the

319   number of mutations separating the samples from this work to those described by

320   Maynard et al. [51] is ranging from 114,285 years to 167,000 years for a mutation rate

321   of 2.4 % Mya^{-1} and from 371,428 years to 542,000 years for a mutation rate of 3.5 %

322   Mya^{-1}. Cluster Aal1 was found only in Central Kalimantan, whereas the other two

323   clusters were spread over different provinces (Supplementary Figure 6). However, the

324   sample size is too small to draw any significant conclusion on the phylogeography.

325   When blasted on databases, Cluster Aal1 and Cluster Aal2 both displayed best hits with

326   the same *Ae. albopictus* populations from The Philippines but with differing percentage

327   of identity ranging from 99.51 % to 99.85 % (Supplementary Table 2). Cluster Aal3

328   showed best hits with invasive populations of *Ae. albopictus* found in D.R. Congo,

329   China, Thailand, Greece, Brazil and USA with 99.65 % to 99.84 % of identity

330   (Supplementary Table 2). With respect to ITS2, the number of sequences available

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169

331   (only 3) was too small to draw a conclusion. Nevertheless, they corresponded to

332   invasive populations of *Ae. albopictus* found in Italy, Georgia, Israel or Sri Lanka

333   (Supplementary Table 4).

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Discussion

336 **Discussion**

337   Dengue, which is the fastest spreading arbovirus disease worldwide is also the
338 first ranking vector-borne disease in Indonesia and, thus, a national health priority. In
339 the absence of treatment and commercially-available vaccine, vector management
340 remains the only way to control the disease. However, in order to do so, the knowledge
341 of vectors population structure is an obligate prerequisite. The capacity of vectoring a
342 given pathogen is not correlated with the species but instead with the population. Some
343 populations of *Ae. aegypti* or *Ae. albopictus* are more prone to disseminate a given
344 pathogen than others [53,54]. Therefore, a species should be regarded as a
345 metapopulation or the combination of cross-fertile genetically distinct populations
346 displaying differing phenotypic traits [55]. The vector competence is one of these
347 phenotypic traits, which in *Aedes* and other mosquitoes, was shown to be related to
348 specific populations [56,57] and not to the species *per se*. Deciphering the structure of
349 the vectors populations is thus essential.
350

351   Although dengue is the transmissible disease ranking number 1 in Indonesia,
352 there have been very limited works on the analysis on the structure of the populations
353 of *Aedes* in Indonesia with only one study on *Ae. aegypti* [3] and one on *Ae. albopictus*
354 [51]. In both cases, the number of sampling sites was very limited. To these must be
355 added studies aiming at assessing the stability of populations in the framework of a
356 *Wolbachia* release program in Yogyakarta [58]. However, in this case the genetic
357 diversity was assessed with microsatellites and SNPs only on a sample from the city of
358 Yogyakarta in South Central Java [58]. This work is to our knowledge the first one
359 exploring the genetic diversity of *Ae. aegypti* and *Ae. albopictus* throughout Indonesia.
360

361   A first conclusion from this work is the homogeneity of the *Ae. aegypti*
362 populations found all over Indonesia. With respect to the COI sequences, all *Ae. aegypti*
363 samples belong to the same maternal lineage. Variations were observed and clusters
364 were described but they simply represent a polymorphism within a monophyletic
365 population. All clusters identified correspond to co-circulating variants. The main
366 different is that one cluster, Cluster Aae2, comprised samples displaying a larger
367 polymorphism. Cluster Aae1 and in particular the haplotypes H1 and H4 seemed to be
368 populations with a very high colonizing and demographic potential. These two

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369   haplotypes represent each about 30% of the samples collected all over Indonesia. They
370   represent indeed the very same population and the Cluster Aae1 makes up to 87% of
371   all samples and is present everywhere in Indonesia.

372

373   Another major outcome of this work is the report of what seems to be a dynamic
374   of population replacement in Indonesia for both *Ae. aegypti* and *Ae. albopictus*. The
375   populations of *Ae. aegypti* found all over Indonesia is not only different from the
376   populations characterized just few years before but is also highly homogeneous with
377   87 % of samples showing very limited polymorphism or no polymorphism at all.
378   Considering the size and structure of Indonesia, a huge archipelago spanning from the
379   Indian Ocean to the Pacific Ocean, this is unexpected. One would have instead expected
380   patched populations differing from one island to the other. What is observed is exactly
381   the contrary, the same population throughout the whole country, which seemed to have
382   settled in a short time. Previous sampling conducted in 2013 yielded genetically
383   different populations [3]. This difference could be considered as the consequence of a
384   sampling bias, mosquitoes having been captured in a specific area, i.e. Northern Coastal
385   Central Java. However, considering the extension of Cluster Aae1 throughout Indonesia
386   and its overwhelming presence among samples (87%), it is very unlikely that it would
387   have been missed in the 2013 sampling campaign. Furthermore, Cluster Aae1 was
388   found mostly present in this same Northern Coastal Central Java area. A more
389   parsimonious explanation is that this population invaded Indonesia after the 2013
390   sampling. This invasion was very fast, within a maximum of three years between 2013
391   date of the former sampling and 2016 date of our first sampling. The ITS2 marker
392   showed a similar trend and considering the high potential of nuclear DNA for
393   recombination and variation, finding the same cluster all over Indonesia confirms the
394   presence of a set of genetically closely related populations in Indonesia with one
395   specific population characterized by two very closely related, monophyletic haplotypes,
396   H1 and H4, being highly invasive most likely due to higher demographic and
397   adaptability potentials. This also suggests that assortative mating occurs, which restricts
398   greatly intraspecies breeding with preexisting populations.

399

400   *Ae. aegypti* has been shown to be highly susceptible to satyrization by *Ae.*
401   *albopictus* leading to the replacement of *Ae. aegypti* by *Ae. albopictus*, explaining thus
402   for part the invasive potential [59]. However, what is observed in Indonesia does not

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403 match this model despite the presence of populations of *Ae. albopictus* described as
404 invasive in other parts of the world. What is seen in Indonesia is instead a fast invasive
405 population of *Ae. aegypti* occupying all the archipelago and outcompeting *Ae.*
406 *albopictus*. The same phenomenon of population replacement in Indonesia is seen with
407 *Ae. albopictus*. In this case, there are two populations which, like with *Ae. aegypti,* do
408 not correspond to those previously described from 2012 to 2015 [51]. Unlike
409 populations described by Maynard et al. [51], they also do not correspond to the
410 haplogroups designed on samples from 2013 [52]. They are related but not the same.
411 Owing to the rate of mutation of the *cox1* gene in insects, 2.4 % Mya^{-1} to 3.5 % Mya^{-1}
412 depending on the model [47,48], the variations observed are not compatible with an
413 evolution of previous local populations and indicate rather a replacement by different
414 populations.

415

416   The domestication of *Ae. aegypti* and *Ae. albopictus* is a process closely linked
417 to the development of the human society and in particular to long distance mobility,
418 transportation of goods and international trade [17]. The current expansion of the
419 *Aedes*-borne diseases is by far a consequence of the global economy. *Ae. aegypti* and
420 *Ae. albopictus*, like all living organisms, are structured in metapopulations, which differ
421 slightly from each other and due to massive international transportation are distributed
422 all over the world within the areas suitable for the survival of these species. The
423 mobility of these populations from one place to another is a stochastic event, which
424 depends on the place of departure, the place of arrival, the genetic and physiological
425 traits of the populations involved, the economic situation and the commercial
426 exchanges and routes at a given time. The invasion by *Ae. albopictus* has been well
427 described as well as the replacement of *Ae. aegypti* by *Ae. albopictus* through
428 satyrization [59], but it is to our knowledge the first report of such a massive and fast
429 intraspecies replacement of existing populations in *Ae. aegypti* and *Ae. albopictus*.

430

431   This dynamic of replacement represents a threat for inundative strategies of
432 insect control such as sterile males release and *Wolbachia* establishment in existing
433 populations of *Ae. aegypti* [60,61]. These strategies are population-dependent and any
434 massive and fast replacement of populations will impair all efforts to establish the
435 inundative population. Only very limited trials of release of sterile males were
436 conducted in Indonesia and were not successful [62]. The establishment of *w*Mel

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437 *Wolbachia* in Indonesian populations of *Ae. aegypti* was conducted in pilot sites in four
438 locations in the city of Yogyakarta [61]. The stability and homogeneity of the local
439 population was assessed [61]. However, the test was conducted in a very small and
440 limited zone of 5 km² [61]. The establishment was successful but slow [61]. A wave of
441 massive invasion and fast replacement can easily and rapidly impair this long-term
442 strategy of vector control.
443
444

Conclusions

446 A general consequence of our results is that vector control should not be population-
447 based. Whatever the population, established or invasive, *Ae. aegypti* and *Ae. albopictus*
448 mosquitoes will have to breed in the human environment. Then, the best way to prevent
449 any population of vector from thriving is certainly to implement vector control as a very
450 local level, at maximum at the community level, essentially by eliminating breeding
451 sites, using very simple and affordable means of control such as containers and garbage
452 removal. The strategy of prevention of dengue transmission through community
453 participation currently recommended in Indonesia is most likely to be the most
454 successful of all. This approach named 3M for "Menutup" for covering water
455 containers, "Menguras" for cleaning water containers, and "Mengubur" for burying
456 discarded containers, is implemented under the responsibility of families in each
457 household with at least one person in charge of monitoring *Aedes* larvae in all water
458 storage [17,63]. This strategy shed the light on what is most needed for the successful
459 control of *Aedes*-borne diseases, not big science, big management or big strategies but
460 simply information, education, people awareness and community-based management.
461
462

Figures legends

464

Figure 1. Map of sampling sites

466

Figure 2. Phylogeny of the *Aedes aegypti cox*1 gene
468 The phylogenetic trees were built using maximum-likelihood (ML) with the general
469 time reversible model with gama distributed with 4 discrete categories (GTR+G). The

15

470   clade support was assessed via 500 bootstrap replicates. The tree was rooted using the

471   *Culex quinquefasciatus cox*1 gene (MK265737) as outgroup. The color code is that of

472   the *cox*1 subclusters shown in Table 1. "References" correspond to the *Ae. aegypti cox*1

473   sequences published by Yohan *et al*. in 2018 [3] from samples collected in 2013 which

474   accession numbers are .KP334259 to KP334269 and KP869121 to KP89126.

475

476   **Figure 3. Network of *Aedes aegypti cox*1 haplotypes**

477

478   **Figure 4. Network of *Aedes aegypti* ITS2 sequences**

479

480   **Figure 5. Phylogeny of the *Aedes aegypti* ITS2 sequences**

481   The phylogenetic tree was built using maximum-likelihood (ML) with the general time

482   reversible model with gama distributed with 4 discrete categories (GTR+G). The clade

483   support was assessed via 500 bootstrap replicates. The tree was rooted using the *Culex*

484   *quinquefasciatus* ITS2 sequence (HQ848572) as outgroup. The color code used is that

485   of the ITS2 subclusters given in Table 1.

486

487   **Figure 6. Phylogeny of *Aedes albopictus cox*1 genes**

488   The phylogenetic tree was built using maximum-likelihood (ML) with the general time

489   reversible model with gama distributed with 4 discrete categories (GTR+G). The clade

490   support was assessed via 500 bootstrap replicates. The tree was rooted using the *Culex*

491   *quinquefasciatus cox*1 gene (MK265737) as outgroup. The color code used is that of

492   the *cox*1 subclusters displayed in Table 2.

493

494   **Figure 7. Network of *Aedes albopictus cox*1 haplotypes**

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497

498   **Supporting Information**

499

500   **S1 Supplementary Figure 1. *cox*1 gene phylogeny of the collected samples**

501   The phylogenetic trees were built using maximum-likelihood (ML) with the general

502   time reversible model with gama distributed with 4 discrete categories (GTR+G). The

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503   clade support was assessed via 500 bootstrap replicates. The tree was rooted using the
504   *Culex quinquefasciatus cox*1 gene (MK265737) as outgroup.
505
506   **S2 Supplementary Figure 2. ITS2 phylogeny of the collected samples**
507   The phylogenetic tree was built using maximum-likelihood (ML) with the general time
508   reversible model with gama distributed with 4 discrete categories (GTR+G). The clade
509   support was assessed via 500 bootstrap replicates. The tree was rooted using the *Culex*
510   *quinquefasciatus* ITS2 sequence (HQ848572) as outgroup. The color code used is that
511   of the ITS2 subclusters of *Ae. aegypti* and *Ae. albopictus* displayed in Table 1 and Table
512   2, respectively.
513
514   **S3 Supplementary Figure 3. Geographic distribution of *Aedes aegypti cox*1 clusters**
515
516   **S4 Supplementary Figure 4. Geographic distribution of *Aedes aegypti cox*1**
517   **haplotypes**
518
519   **S5 Supplementary Figure 5. Phylogeographic distribution of *Aedes aegypti* ITS2**
520   **sequences**
521
522   **S6 Supplementary Figure 6. Geographic distribution of *Aedes albopictus cox*1**
523   **haplotypes**
524
525   **S7 Supplementary Table 1. Polymorphism of *Aedes aegypti cox*1 haplotypes from**
526   **Indonesia**
527
528   **S8 Supplementary Table 2. Polymorphism of *Aedes albopictus cox*1 haplotypes**
529   **from Indonesia**
530
531   **S9 Supplementary Table 3. Polymorphism of *Aedes aegypti* ITS2 haplotypes from**
532   **Indonesia**
533
534   **S10 Supplementary Table 4. Polymorphism of *Aedes albopictus* ITS2 haplotypes**
535   **from Indonesia**
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Ethics statement

Specific permissions were not required for field *Aedes* larval collections. Oral consent to inspect the *Aedes* breeding places in the household was obtained from the homeowners and local government authorities. Formal approval to conduct these activities was granted by the Ethical Commission Board of the NIHRD, Ministry of Health, Indonesia (No. LB.02.01/5.2/KE.003/2016, January 13, 2016; and No. LB.02.01/5.2/KE.020/2017, February 6, 2017).

Conflict of Interest

This work was supported exclusively by public funds. There is no conflict of interest.

Availability of data

All data are freely available. All sequences have been deposited in Genbank under the accession numbers MW280620 to MW280792, MW280794 to MW280797 and MW280800 to MW280818 for *Ae. aegypti cox*1 sequences. The accession numbers for *Ae. aegypti* ITS2 sequences range from MW288143 to MW288145 and MW290431 to MW290468. The accession numbers for *Ae. albopictus cox*1 sequences are MW280793, MW280798, MW280799 and MW283303 to MW283318. The accession numbers for *Ae. albopictus* ITS2 sequences are MW287155 to MW287157. The accession numbers of the *cox*1 and ITS2 sequences of the unknown species *Aedes* sp (sls25_Asp) are MW286812 and MW293720, respectively.

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798

25

Figure_1

1 WEST ACEH, NAD
2 PIDIE, NAD
3 EAST ACEH, NAD
4 PEKANBARU, RIAU
5 BATAM, RIAU ISLANDS
6 PEMATANG RAMAN, JAMBI
7 JAMBI, JAMBI
8 CENTRAL BANGKA, BANGKA-BELITUNG
9 BELITUNG, BANGKA-BELITUNG
10 SOUTH LAMPUNG, LAMPUNG
11 SERANG, BANTEN
12 PANDEGLANG, BANTEN
13 LEBAK, BANTEN
14 WEST BANDUNG, WEST JAVA
15 SUBANG, WEST JAVA
16 PANGANDARAN, WEST JAVA
17 SEMARANG CITY, CENTRAL JAVA
18 SEMARANG, CENTRAL JAVA

19 BANTUL, YOGYAKARTA
20 MALANG, EAST JAVA
21 DENPASAR, BALI
22 KARANGASEM, BALI
23 WEST LOMBOK, WEST NUSA TENGGARA
24 NORTH LOMBOK, WEST NUSA TENGGARA
25 CENTRAL SUMBA, EAST NUSA TENGGARA
26 BELU, EAST NUSA TENGGARA
27 SAMBAS, WEST KALIMANTAN
28 NORTH KAYONG, WEST KALIMANTAN

29 KETAPANG, WEST KALIMANTAN
30 PULANG PISAU, CENTRAL KALIMANTAN
31 BARITO KUALA, SOUTH KALIMANTAN
32 TANAH LAUT, SOUTH KALIMANTAN
33 KOTA BARU, SOUTH KALIMANTAN
34 BALIKPAPAN, EAST KALIMANTAN

35 BITUNG, NORTH SULAWESI
36 PALU, CENTRAL SULAWESI
37 MAROS, SOUTH SULAWESI
38 MUNA, SOUTHEAST SULAWESI
39 SOUTH HALMAHERA, NORTH MALUKU
40 AMBON, MALUKU

41 WEST-SOUTHEAST MALUKU, MALUKU
42 SOUTHEAST MALUKU, MALUKU
43 FAK-FAK, WEST PAPUA

185

Figure_2

Cqui - root

References

IS1

IS2

Aae1

Aae2a
Aae2e
Aae2c
Aae2d
Aae2b

89

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78

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93

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83

0.01

Figure_3

Legend:
SEMARANG CITY, CENTRAL JAVA
KARANGASEM, BALI
EAST ACEH, NAD
FAK-FAK, WEST PAPUA
BANTUL, YOGYAKARTA
BATAM, RIAU ISLANDS
SEMARANG, CENTRAL JAVA
PEKANBARU, RIAU
SAMBAS, WEST KALIMANTAN
MALANG, EAST JAVA
KETAPANG, WEST KALIMANTAN
AMBON, MALUKU
PANDEGLANG, BANTEN
SUBANG, WEST JAVA
SOUTH LAMPUNG, LAMPUNG
BATAM, RIAU ISLANDS
BALIKPAPAN, EAST KALIMANTAN
PALU, CENTRAL SULAWESI
CENTRAL BANGKA, BANGKA-BELITUNG
WEST-SOUTHEAST MALUKU, MALUKU
BARITO KUALA, SOUTH KALIMANTAN
PULANG PISAU, CENTRAL KALIMANTAN
LEBAK, BANTEN
SOUTH HALMAHERA, NORTH MALUKU
KOTA BARU, SOUTH KALIMANTAN
WEST BANDUNG, WEST JAVA
NORTH LOMBOK, WEST NUSA TENGGARA
PEMATANG RAMAN, JAMBI
MAROS, SOUTH SULAWESI
MUNA, SOUTHEAST SULAWESI
SERANG, BANTEN
PANGANDARAN, WEST JAVA
PIDIE, NAD
SOUTHEAST MALUKU, MALUKU
BITUNG, NORTH SULAWESI
WEST ACEH, NAD
WEST LOMBOK, WEST NUSA TENGGARA
NORTH KAYONG, WEST KALIMANTAN
BELU, EAST NUSA TENGGARA
CENTRAL SUMBA, EAST NUSA TENGGARA

+++++++++ Number and position of mutations separating haplotypes

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Figure_4

BATAM, RIAU ISLANDS
PEMATANG RAMAN, JAMBI
SAMBAS, WEST KALIMANTAN
KARANGASEM, BALI
PALU, CENTRAL SULAWESI
BALIKPAPAN, EAST KALIMANTAN
SEMARANG CITY, CENTRAL JAVA
MALANG, EAST JAVA
PEKANBARU, RIAU
WEST BANDUNG, WEST JAVA
BANTUL, YOGYAKARTA
EAST ACEH, ACEH
NORTH LOMBOK, WEST NUSA TENGGARA
MAROS, SOUTH SULAWESI

++++++++++ Number and position of mutations separating haplotypes

Figure_5

Figure_6

Figure_7

BATAM, RIAU ISLANDS
BELITUNG, BANGKA BELITUNG
DENPASAR, BALI
BALIKPAPAN, EAST KALIMANTAN
PULANG PISAU, CENTRAL KALIMANTAN
MAROS, SOUTH SULAWESI
JAMBI, JAMBI
SOUTH HALMAHERA, NORTH MALUKU
PIDIE, ACEH

++++++++++ Number and position of mutations separating haplotypes

Table_1

*cox*1 Cluster	*cox*1 Subcluster	*cox*1 Haplotype	ITS2 Cluster	ITS2 Haplo-type	Sample	Species	Location District/ municipality	Province	*cox*1 accession number	ITS2 accession number
Aae1	Aae1a	H1			13_Aae	*Ae. aegypti*	Serang	Banten	MW280620	
		H1			57_Aae	*Ae. aegypti*	Bantul	Yogyakarta	MW280631	
		H1			4_Aae	*Ae. aegypti*	Bantul	Yogyakarta	MW280639	
		H1	1a	H1	r12_Aae	*Ae. aegypti*	Pematang Raman	Jambi	MW280645	MW290457
		H1			r011a_Aae	*Ae. aegypti*	Pematang Raman	Jambi	MW280646	
		H1	1a	H21	ri007_Aae	*Ae. aegypti*	Pekanbaru	Riau	MW280647	MW290466
		H1			ri017_Aae	*Ae. aegypti*	Pekanbaru	Riau	MW280661	
		H1			71_Aae	*Ae. aegypti*	Bantul	Yogyakarta	MW280663	
		H1			79_Aae	*Ae. aegypti*	Sambas	West Kalimantan	MW280666	
		H1			97_Aae	*Ae. aegypti*	Sambas	West Kalimantan	MW280667	
		H1	1b	H5	83_Aae	*Ae. aegypti*	Sambas	West Kalimantan	MW280673	
		H1			80_Aae	*Ae. aegypti*	Sambas	West Kalimantan	MW280674	
		H1	1a	H1	ri021_Aae	*Ae. aegypti*	Pekanbaru	Riau	MW280678	MW290454
		H1			20_Aae	*Ae. aegypti*	Pangandaran	West Java	MW280683	
		H1			mlk36_Aae	*Ae. aegypti*	Ambon	Maluku	MW280686	
		H1			mlk65_Aae	*Ae. aegypti*	Ambon	Maluku	MW280687	
		H1			mlk79_Aae	*Ae. aegypti*	Ambon	Maluku	MW280688	
		H1			blp28_Aae	*Ae. aegypti*	Balikpapan	East Kalimantan	MW280698	
		H1	1a	H14	ri013_Aae	*Ae. aegypti*	Pekanbaru	Riau	MW280700	MW290446
		H1			31_Aae	*Ae. aegypti*	Pidie	NAD	MW280703	
		H1			57_1_Aae	*Ae. aegypti*	Central Bangka	Bangka Belitung	MW280708	
		H1	2	H16	b22_Aae	*Ae. aegypti*	Karangasem	Bali	MW280710	MW290455
		H1	1a	H1	b7_Aae	*Ae. aegypti*	Karangasem	Bali	MW280713	MW290449

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H1			jgj5_Aae	*Ae. aegypti*	Bantul	Yogyakarta	MW280716	
H1	1a	H1	jgj6_Aae	*Ae. aegypti*	Bantul	Yogyakarta	MW280717	MW290459
H1			blp1b_Aae	*Ae. aegypti*	Balikpapan	East Kalimantan	MW280724	
H1			jtg2b_Aae	*Ae. aegypti*	Semarang	Central Java	MW280739	
H1	1a	H1	mlg11_Aae	*Ae. aegypti*	Malang	East Java	MW280743	MW290453
H1	1a	H1	PL30_Aae	*Ae. aegypti*	Palu	Central Sulawesi	MW280755	MW290468
H1			ri006b_Aae	*Ae. aegypti*	Pekanbaru	Riau	MW280759	
H1			ri007_1_Aae	*Ae. aegypti*	Pekanbaru	Riau	MW280760	
H1			ri021_1_Aae	*Ae. aegypti*	Pekanbaru	Riau	MW280762	
H1	1	H1	sls21_Aae	*Ae. aegypti*	Maros	South Sulawesi	MW280763	MW290448
H1			JOG11_Aae	*Ae. aegypti*	Bantul	Yogyakarta	MW280765	
H1			58_Aae	*Ae. aegypti*	Central Bangka	Bangka Belitung	MW280769	
H1			b14_Aae	*Ae. aegypti*	Karangasem	Bali	MW280770	
H1			jgj7_Aae	*Ae. aegypti*	Bantul	Yogyakarta	MW280771	
H1			blp15_1_Aae	*Ae. aegypti*	Balikpapan	East Kalimantan	MW280773	
H1			r11_Aae	*Ae. aegypti*	Pematang Raman	Jambi	MW280778	
H1			blp-3_Aae	*Ae. aegypti*	Balikpapan	East Kalimantan	MW280779	
H1			8_1_Aae	*Ae. aegypti*	Southeast Maluku	Maluku	MW280784	
H1			11_1_Aae	*Ae. aegypti*	Bantul	Yogyakarta	MW280786	
H1			mlk40_Aae	*Ae. aegypti*	Ambon	Maluku	MW280790	
H1			pl126_Aae	*Ae. Aegypti*	Palu	Central Sulawesi	MW280796	
H1			19_1_Aae	*Ae. aegypti*	South Halmahera	North Maluku	MW280800	
H1			blp11_Aae	*Ae. aegypti*	Balikpapan	East Kalimantan	MW280801	
H1			ri002_Aae	*Ae. aegypti*	Pekanbaru	Riau	MW280802	
H1			ri003_Aae	*Ae. aegypti*	Pekanbaru	Riau	MW280803	
H1	1a	H1	ri013_1_Aae	*Ae. aegypti*	Pekanbaru	Riau	MW280805	
H1			r12_1_Aae	*Ae. aegypti*	Pematang Raman	Jambi	MW280807	

		H1				jgj1_Aae	*Ae. aegypti*	Bantul	Yogyakarta	MW280809	
		H1				jgj2_Aae	*Ae. aegypti*	Bantul	Yogyakarta	MW280810	
		H1	1a	H1	jog003_Aae	*Ae. aegypti*	Bantul	Yogyakarta	MW280812	MW290451
		H1	1a	H1	jog004_Aae	*Ae. aegypti*	Bantul	Yogyakarta	MW280813	MW290460
		H1			jog006_Aae	*Ae. aegypti*	Bantul	Yogyakarta	MW280814	
		H1	1c	H18	jog010_Aae	*Ae. aegypti*	Bantul	Yogyakarta	MW280815	MW290461
		H1			b4_Aae	*Ae. aegypti*	Karangasem	Bali	MW280712	
		H2			1B_Aae	*Ae. aegypti*	Semarang City	Central Java	MW280621	
		H8			9_18_Aae	*Ae. aegypti*	Fak-Fak	West Papua	MW280628	
		H10			2_Aae	*Ae. aegypti*	Bantul	Yogyakarta	MW280637	
		H10	1c	H12	btm_nl4_Aae	*Ae. aegypti*	Batam	Riau Islands	MW280693	MW290444
		H11			6_Aae	*Ae. aegypti*	Bantul	Yogyakarta	MW280640	
		H14			44_Aae	*Ae. aegypti*	Bantul	Yogyakarta	MW280651	MW288143
		H15			27_Aae	*Ae. aegypti*	Bantul	Yogyakarta	MW280654	
		H16			28_Aae	*Ae. aegypti*	Bantul	Yogyakarta	MW280655	
		H20			15_1_Aae	*Ae. aegypti*	Pandeglang	Banten	MW280680	
		H20			14_Aae	*Ae. aegypti*	Pandeglang	Banten	MW280679	
		H22			19_Aae	*Ae. aegypti*	Subang	West Java	MW280682	
		H32			sk2_Aae	*Ae. aegypti*	Barito Kuala	South Kalimantan	MW280718	
		H34			blp15_Aae	*Ae. aegypti*	Balikpapan	East Kalimantan	MW280723	
		H37	1a	H1	JTG228_Aae	*Ae. aegypti*	Semarang	Central Java	MW280737	
		H44			PL27_Aae	*Ae. aegypti*	Palu	Central Sulawesi	MW280752	MW288144
		H44			PL5_Aae	*Ae. aegypti*	Palu	Central Sulawesi	MW280757	
		H46			PL4_Aae	*Ae. aegypti*	Palu	Central Sulawesi	MW280756	
		H48			sls17_Aae	*Ae. aegypti*	Maros	South Sulawesi	MW280764	MW288145
	Aae1b	H4			6B_Aae	*Ae. aegypti*	Semarang City	Central Java	MW280623	
		H4			5_Aae	*Ae. aegypti*	Bantul	Yogyakarta	MW280627	

H4			25_Aae	*Ae. aegypti*	Bantul	Yogyakarta	MW280629	
H4			47_Aae	*Ae. aegypti*	Bantul	Yogyakarta	MW280630	
H4			23_Aae	*Ae. aegypti*	Bantul	Yogyakarta	MW280632	
H4			38_Aae	*Ae. aegypti*	Bantul	Yogyakarta	MW280633	
H4			52_Aae	*Ae. aegypti*	Bantul	Yogyakarta	MW280635	
H4			1_Aae	*Ae. aegypti*	Bantul	Yogyakarta	MW280636	
H4			3_Aae	*Ae. aegypti*	Bantul	Yogyakarta	MW280638	
H4			7_Aae	*Ae. aegypti*	Bantul	Yogyakarta	MW280641	
H4			24_Aae	*Ae. aegypti*	Bantul	Yogyakarta	MW280644	
H4			50_Aae	*Ae. aegypti*	Bantul	Yogyakarta	MW280649	
H4			41_Aae	*Ae. aegypti*	Bantul	Yogyakarta	MW280650	
H4			15_Aae	*Ae. aegypti*	Bantul	Yogyakarta	MW280652	
H4			26_Aae	*Ae. aegypti*	Bantul	Yogyakarta	MW280653	
H4			55_Aae	*Ae. aegypti*	Bantul	Yogyakarta	MW280656	
H4			60_Aae	*Ae. aegypti*	Bantul	Yogyakarta	MW280657	
H4			63_Aae	*Ae. aegypti*	Bantul	Yogyakarta	MW280658	
H4			69_Aae	*Ae. aegypti*	Bantul	Yogyakarta	MW280659	
H4			76_Aae	*Ae. aegypti*	Bantul	Yogyakarta	MW280660	
H4			53_Aae	*Ae. aegypti*	Bantul	Yogyakarta	MW280662	
H4			75_Aae	*Ae. aegypti*	Bantul	Yogyakarta	MW280664	
H4			67_Aae	*Ae. aegypti*	Bantul	Yogyakarta	MW280665	
H4			62_Aae	*Ae. aegypti*	Bantul	Yogyakarta	MW280668	
H4			68_Aae	*Ae. aegypti*	Bantul	Yogyakarta	MW280669	
H4			74_Aae	*Ae. aegypti*	Bantul	Yogyakarta	MW280671	
H4			pl1_Aae	*Ae. aegypti*	Palu	Central Sulawesi	MW280676	
H4			21_Aae	*Ae. aegypti*	Pangandaran	West Java	MW280684	
H4			25_1_Aae	*Ae. aegypti*	Bitung	North Sulawesi	MW280689	

H4	1a	H9	ba081_Aae	*Ae. aegypti*	Batam	Riau Islands	MW280691	MW290438
H4			pl1_1_Aae	*Ae. aegypti*	Palu	Central Sulawesi	MW280699	
H4			32_Aae	*Ae. aegypti*	West Aceh	NAD	MW280704	
H4			33_Aae	*Ae. aegypti*	West Aceh	NAD	MW280705	
H4			47_1_Aae	*Ae. aegypti*	West Lombok	West Nusa Tenggara	MW280706	
H4			52_1_Aae	*Ae. aegypti*	North kayong	West Kalimantan	MW280707	
H4			sk5_Aae	*Ae. aegypti*	Kota Baru	South Kalimantan	MW280720	
H4			sk6_Aae	*Ae. aegypti*	Tanah laut	South Kalimantan	MW280721	
H4			sk56_Aae	*Ae. aegypti*	Ketapang	West Kalimantan	MW280722	
H4			jb10_Aae	*Ae. aegypti*	West Bandung	West Java	MW280727	
H4	1a	H15	jb13_Aae	*Ae. aegypti*	West Bandung	West Java	MW280729	MW290450
H4			jb14_Aae	*Ae. aegypti*	West Bandung	West Java	MW280730	
H4			jb15_Aae	*Ae. aegypti*	West Bandung	West Java	MW280731	
H4			jb16_Aae	*Ae. aegypti*	West Bandung	West Java	MW280732	
H4			jb19_Aae	*Ae. aegypti*	West Bandung	West Java	MW280735	
H4			JTG10_Aae	*Ae. aegypti*	Semarang	Central Java	MW280736	
H4	1a	H19	mlg18_Aae	*Ae. aegypti*	Malang	East Java	MW280745	MW290462
H4	1a	H17	PL28_Aae	*Ae. aegypti*	Palu	Central Sulawesi	MW280753	MW290456
H4			43_Aae	*Ae. aegypti*	Belu	East Nusa Tenggara	MW280767	
H4			38_1_Aae	*Ae. aegypti*	Central Sumba	East Nusa Tenggara	MW280774	
H4			43_1_Aae	*Ae. aegypti*	Belu	East Nusa Tenggara	MW280776	
H4			3_1_Aae	*Ae. aegypti*	Southeast Maluku	Maluku	MW280782	
H4			6_1_Aae	*Ae. aegypti*	Southeast Maluku	Maluku	MW280783	
H4			10_1_Aae	*Ae. aegypti*	Bantul	Yogyakarta	MW280785	
H4			13_11_1_Aae	*Ae. aegypti*	South Halmahera	North Maluku	MW280787	
H4			15_1_1_Aae	*Ae. aegypti*	Muna	Southeast Sulawesi	MW280788	
H4			mlg9_Aae	*Ae. aegypti*	Malang	East Java	MW280750	

	H4			mlk73_Aae	*Ae. aegypti*	Ambon	Maluku	MW280792	
	H4			pl1_1_1_Aae	*Ae. aegypti*	Palu	Central Sulawesi	MW280795	
	H4			3843_Aae	*Ae. aegypti*	Central Sumba	East Nusa Tenggara	MW280816	
	H4			37_Aae	*Ae. aegypti*	West Southeast Maluku	Maluku	MW280817	
	H6			8B_Aae	*Ae. aegypti*	Semarang City	Central Java	MW280625	
	H9			jgj8_Aae	*Ae. aegypti*	Bantul	Yogyakarta	MW280634	
	H12			JTG27_Aae	*Ae. aegypti*	Semarang	Central Java	MW280738	
	H12			mlg12_Aae	*Ae. aegypti*	Malang	East Java	MW280744	
	H12			mlg21_Aae	*Ae. aegypti*	Malang	East Java	MW280748	
	H12			8_Aae	*Ae. aegypti*	Bantul	Yogyakarta	MW280642	
	H12			jgj3_Aae	*Ae. aegypti*	Bantul	Yogyakarta	MW280811	
	H13			ri011_Aae	*Ae. aegypti*	Pekanbaru	Riau	MW280804	
	H13			ri024_Aae	*Ae. aegypti*	Pekanbaru	Riau	MW280806	
	H13	1a	H1	ri004_Aae	*Ae. aegypti*	Pekanbaru	Riau	MW280643	MW290463
	H13	1a	H1	ri016_Aae	*Ae. aegypti*	Pekanbaru	Riau	MW280648	MW290458
	H13	1a	H1	ri014_Aae	*Ae. aegypti*	Pekanbaru	Riau	MW280677	MW290447
	H13			ktg_H05_Aae	*Ae. aegypti*	Pulang Pisau	Central Kalimantan	MW280741	
	H13			b8_Aae	*Ae. aegypti*	Karangasem	Bali	MW280714	
	H17			82_Aae	*Ae. aegypti*	Sambas	West Kalimantan	MW280670	
	H18			81_Aae	*Ae. aegypti*	Sambas	West Kalimantan	MW280672	
	H18			2_1_Aae	*Ae. aegypti*	Lebak	Banten	MW280781	
	H18	2	H20	mlk48_Aae	*Ae. aegypti*	Ambon	Maluku	MW280791	MW290465
	H18			mlk654_Aae	*Ae. aegypti*	Ambon	Maluku	MW280794	
	H18			mlk54_Aae	*Ae. aegypti*	Ambon	Maluku	MW280772	
	H19			56_Aae	*Ae. aegypti*	Bantul	Yogyakarta	MW280675	
	H21			18_Aae	*Ae. aegypti*	Subang	West Java	MW280681	
	H23			66_Aae	*Ae. aegypti*	South Lampung	Lampung	MW280685	

		H27	1a	H1	65_Aae	*Ae. aegypti*	South Lampung	Lampung	MW280696	
		H29	1a	H1	ri010_Aae	*Ae. aegypti*	Pekanbaru	Riau	MW280701	MW290464
		H29			ri008_Aae	*Ae. aegypti*	Pekanbaru	Riau	MW280761	
		H31			15_18_1_Aae	*Ae. aegypti*	West Southeast Maluku	Maluku	MW280818	
		H31			15_18_Aae	*Ae. aegypti*	South Halmahera	North Maluku	MW280715	
		H33			sk4_Aae	*Ae. aegypti*	Kota Baru	South Kalimantan	MW280719	
		H35			blp23_Aae	*Ae. aegypti*	Balikpapan	East Kalimantan	MW280726	
		H36			jb11_Aae	*Ae. aegypti*	West Bandung	West Java	MW280728	
		H36			jb17_Aae	*Ae. aegypti*	West Bandung	West Java	MW280733	
		H36			jb18_Aae	*Ae. aegypti*	West Bandung	West Java	MW280734	
		H39	2	H13	mlg10_Aae	*Ae. aegypti*	Malang	East Java	MW280742	MW290445
		H40			mlg19_Aae	*Ae. aegypti*	Malang	East Java	MW280746	
		H41	1a	H1	mlg20_Aae	*Ae. aegypti*	Malang	East Java	MW280747	
		H42	1a	H1	mlg3_Aae	*Ae. aegypti*	Malang	East Java	MW280749	MW290452
		H47	1a	H1	ri004b_Aae	*Ae. aegypti*	Pekanbaru	Riau	MW280758	
		H50			9_18_1_Aae	*Ae. aegypti*	Fak-Fak	West Papua	MW280768	
		H51			sls32_Aae	*Ae. aegypti*	Maros	South Sulawesi	MW280775	
		H51			16_1_Aae	*Ae. aegypti*	Muna	Southeast Sulawesi	MW280789	
Aae2	Aae2a	H7	1a	H11	10B_Aae	*Ae. aegypti*	Semarang City	Central Java	MW280626	MW290443
	Aae2a	H7			29_Aae	*Ae. aegypti*	East Aceh	NAD	MW280702	
	Aae2a	H43	1d	H8	PL2_Aae	*Ae. aegypti*	Palu	Central Sulawesi	MW280751	MW290437
	Aae2a	H45	1a	H7	PL29_Aae	*Ae. aegypti*	Palu	Central Sulawesi	MW280754	MW290436
	Aae2b	H5	1a	H1	7B_Aae	*Ae. aegypti*	Semarang City	Central Java	MW280624	MW290442
	Aae2b	H30	1a	H6	b006_Aae	*Ae. aegypti*	Karangasem	Bali	MW280709	MW290435
	Aae2b	H38	1a	H1	jtg44_Aae	*Ae. aegypti*	Semarang City	Central Java	MW280740	MW290467
	Aae2c	H24	1a	H1	ba01_Aae	*Ae. aegypti*	Batam	Riau Islands	MW280690	MW290433
	Aae2c	H25	2	H2	ba16l_Aae	*Ae. aegypti*	Batam	Riau Islands	MW280692	

	Aae2d	H52	1d	H4	r10_Aae	*Ae. aegypti*	Pematang Raman	Jambi	MW280777	
	Aae2d	H53	1a	H3	r13_Aae	*Ae. aegypti*	Pematang Raman	Jambi	MW280808	MW290434
	Aae2e	H3	1a	H1	4B_Aae	*Ae. aegypti*	Semarang	Central Java	MW280622	MW290441
	Aae2e	H3			b3_Aae	*Ae. aegypti*	Karangasem	Bali	MW280622	
	Aae2e	H26	1a	H1	tb131_Aae	*Ae. aegypti*	Batam	Riau Islands	MW280797	MW290439
	Aae2e	H26			tb71l_Aae	*Ae. aegypti*	Batam	Riau Islands	MW280695	
	Aae2e	H26	1a	H10	blp2_Aae	*Ae. aegypti*	Balikpapan	East Kalimantan	MW280725	MW290440
	Aae2e	H26			55_1_Aae	*Ae. aegypti*	Ketapang	West Kalimantan	MW280780	
Individual samples	IS1	H49	1a	H1	46_Aae	*Ae. aegypti*	North Lombok	West Nusa Tenggara	MW280766	MW290432
	IS2	H28	1a	H1	28_1_Aae	*Ae. aegypti*	East Aceh	NAD	MW280697	MW290431

NAD: Nangro Aceh Darussalam (new denominaitn of the former Province of Aceh)

Table_2

COI cluster	COI Haplotype	Sample	Species	Location		cox1 accession number	ITS2 accession number
				District/municipality	Province		
Aal1	H2	61_Aal	*Ae. albopictus*	Belitung	Bangka Belitung	MW283306	
Aal1	H3	b023_Aal	*Ae. albopictus*	Denpasar	Bali	MW283307	
Aal1	H3	b24_Aal	*Ae. albopictus*	Denpasar	Bali	MW283308	
Aal1	H6	blp1_Aal	*Ae. albopictus*	Balikpapan	East Kalimantan	MW283309	
Aal1	H9	sls30_Aal	*Ae. albopictus*	Maros	South Sulawesi	MW283313	
Aal1	H5	sls95_Aal	*Ae. albopictus*	Maros	South Sulawesi	MW283315	
Aal1	H3	ktg06_Aal	*Ae. albopictus*	Pulang Pisau	Central Kalimantan	MW280798	
Aal1	H3	ktgp09_Aal	*Ae. albopictus*	Pulang Pisau	Central Kalimantan	MW280793	
Aal1	H4	ktgp26_Aal	*Ae. albopictus*	Pulang Pisau	Central Kalimantan	MW283303	
Aal1	H5	sls16_Aal	*Ae. albopictus*	Maros	South Sulawesi	MW283304	
Aal1	H7	blp20_Aal	*Ae. albopictus*	Balikpapan	East Kalimantan	MW283310	
Aal2	H8	ktg08_Aal	*Ae. albopictus*	Pulang Pisau	Central Kalimantan	MW283311	MW287155
Aal2	H8	ktgt14_Aal	*Ae. albopictus*	Pulang Pisau	Central Kalimantan	MW283312	
Aal2	H8	ktgp28	*Ae. albopictus*	Pulang Pisau	Central Kalimantan	MW280799	
Aal3	H10	r14_Aal	*Ae. albopictus*	Jambi	Jambi	MW283314	MW287156
Aal3	H10	30_Aal	*Ae. albopictus*	Pidie	Aceh	MW283318	
Aal3	H10	r15_Aal	*Ae. albopictus*	Jambi	Jambi	MW283317	
Aal3	H1	TB66l_Aal	*Ae. albopictus*	Batam	Riau Islands	MW283305	MW287157
Aal3	H11	22_30_Aal	*Ae. albopictus*	South Halmahera	North Maluku	MW283316	

Supplementary fig_1
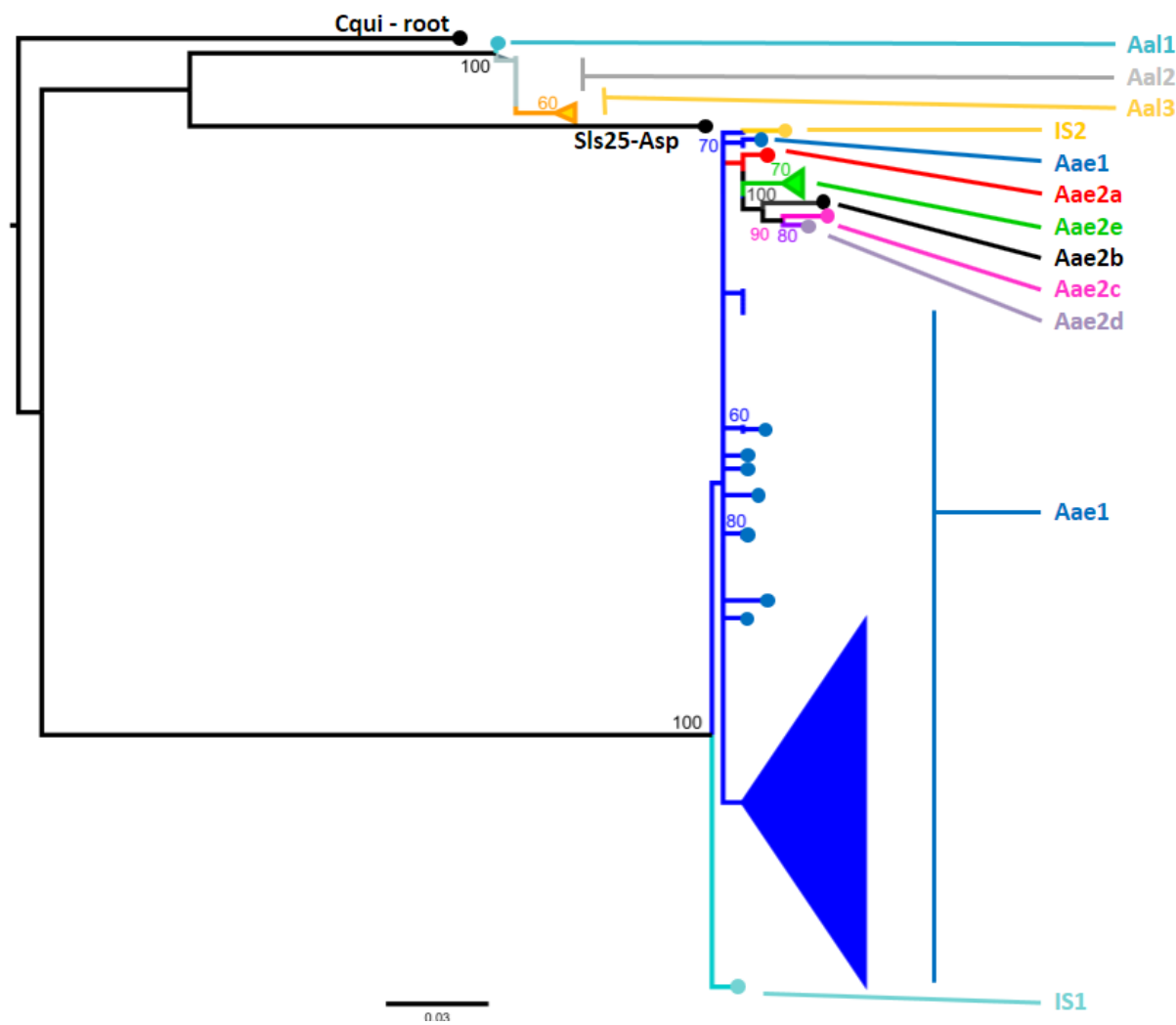
Supplementary fig_2

Supplementary fig_3
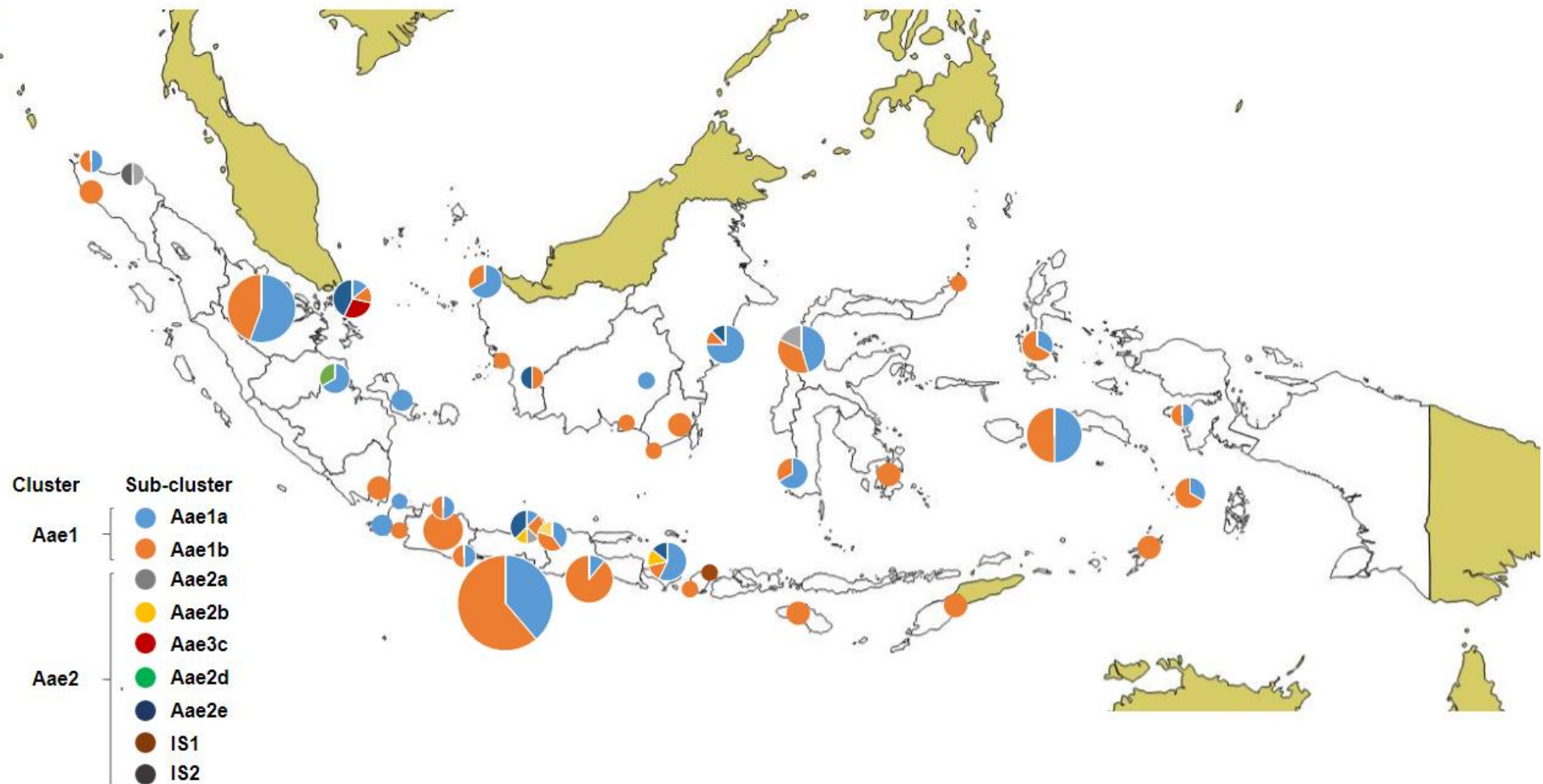
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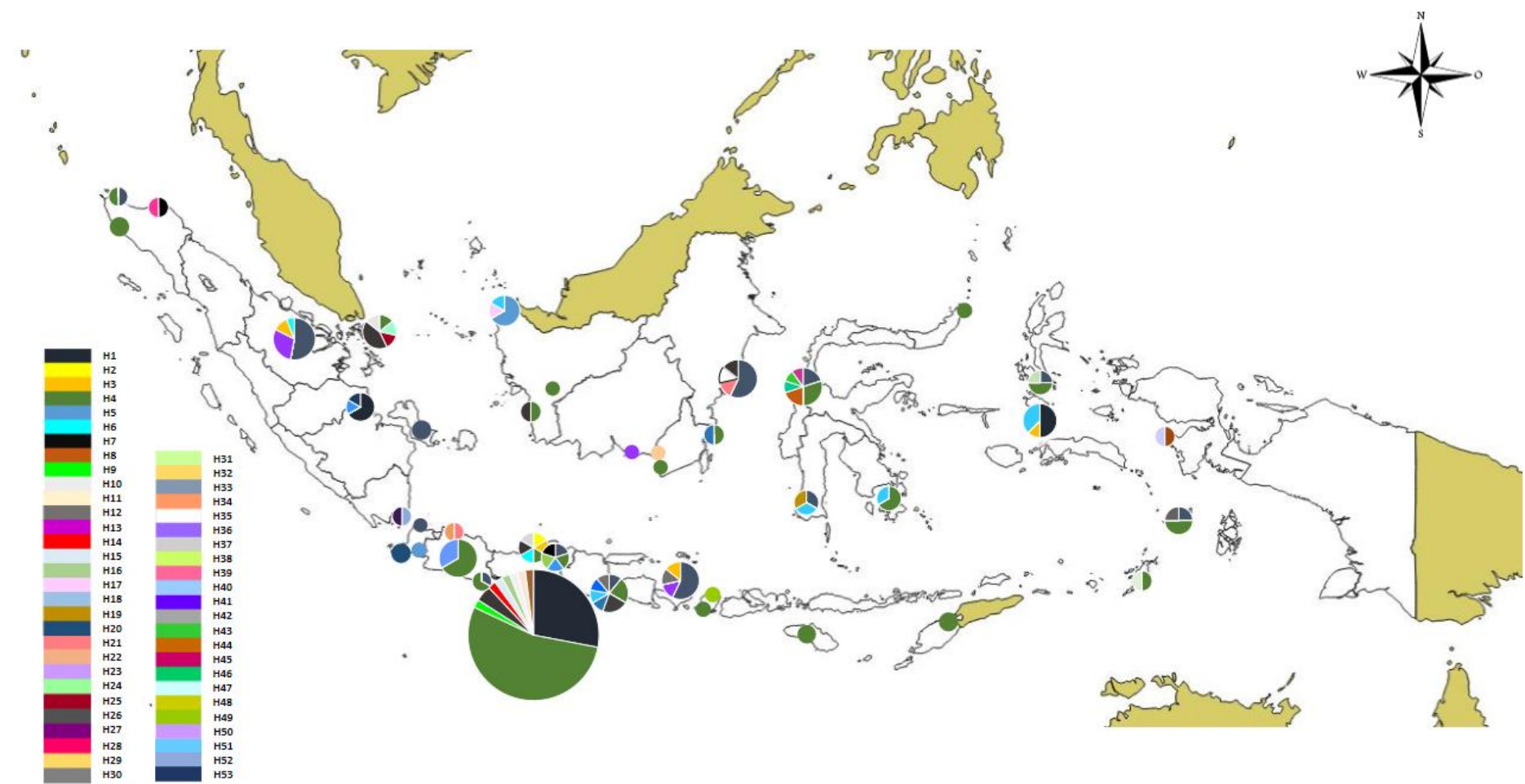
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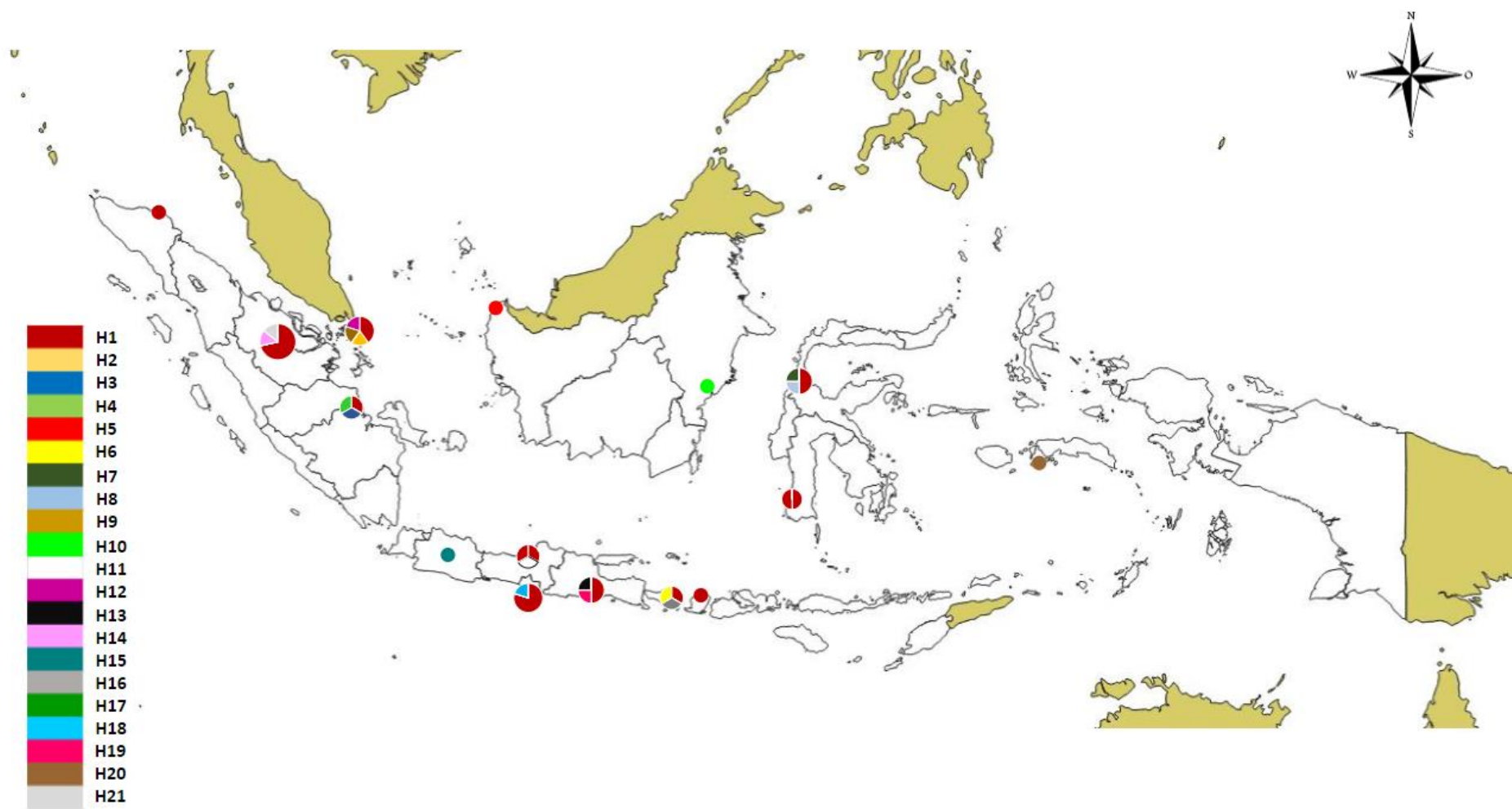
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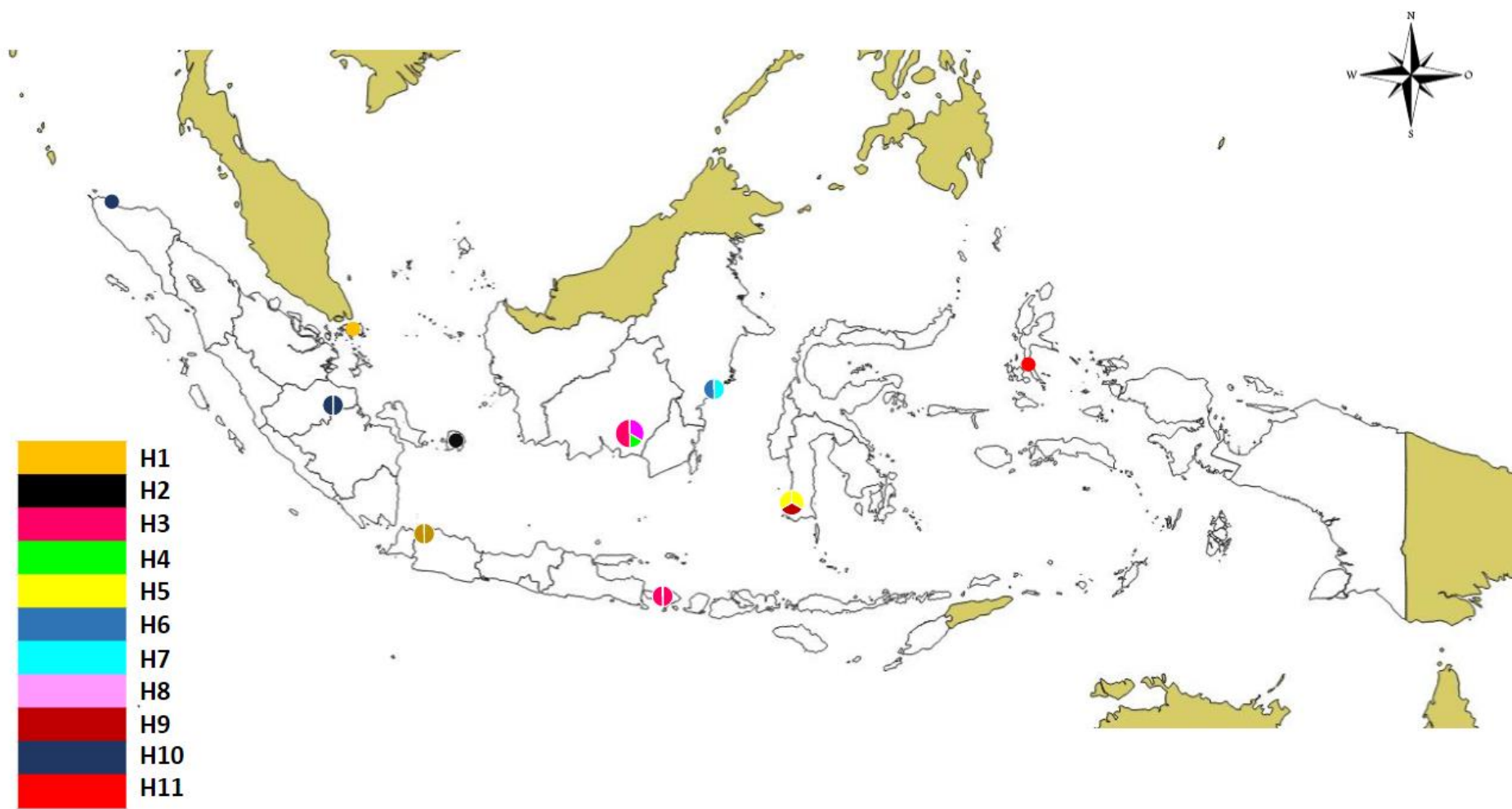
Supplementary Table_1

Supplementary Table 1. Polymorphism of *Aedes aegypti cox*1 haplotypes from Indonesia

Cluster	Cluster best hit [a]	Location	Haplotype	% identity [b]
1	MN299016	Peru	H1	99.67 %
	MN299014	Cambodia	H2	99.18 %
	MN299008	Puerto Rico	H4	99.51 %
	MG198586	Georgia	H6	99.34 %
	MF999266	India	H8	99.18 %
	MF043259	England	H9	99.34 %
	KY022527	Germany	H10	99.34 %
	KY022526	Germany	H11	99.34 %
	AF425846	NA	H12	99.51 %
			H13	99.51 %
			H14	98.85 %
			H15	99.34 %
			H16	99.01 %
			H17	99.34 %
			H18	99.51 %
			H19	99.34 %
			H20	99.34 %
			H21	99.34 %
			H22	99.34 %
			H23	99.34 %
			H27	99.51 %
			H29	99.51 %
			H31	99.34 %
			H32	99.34 %
			H33	99.51 %
			H34	99.34 %
			H35	99.51 %
			H36	99.51 %
			H37	99.18 %
			H39	99.51 %
			H40	99.18 %
			H41	99.34 %
			H42	99.34 %
			H44	99.51 %
			H46	99.34 %
			H47	99.51 %
			H48	99.34 %
			H50	99.18 %
			H51	99.51 %
2a	MK300222	Kenya	H7	99.51 %

	MK300216	Kenya	H43	99.34 %
			H45	99.18 %
2b	MN299002	Mozambique	H5	98.69 %
			H30	98.69 %
			H38	99.18 %
2c	MT328866	Egypt	H24	99.18 %
	MK300229	Kenya	H25	99.34 %
	MK300226	Kenya		
	MK300223	Kenya		
	MK300217	Kenya		
2d	MN298993	Haiti	H52	99.51 %
			H53	99.67 %
2e	MN298997	Haiti	H3	99.67 %
	AY432106	Strain Liverpool	H26	99.84 %
	AY432648	Strain Liverpool		
	AF390098	Strain RED		
IS1	AY056597	Sub-Saharan Africa [c]	H49	99.18 %
IS2	MH251910	Russia	H28	98.85 %

a) All sequences displaying the same best hit score were reported with their respective accession number

b) The percentage of identity of a given haplotype is the same for each best hit sequence

c) The best hit corresponds to the form *Ae. aegypti formosus* which is considered to be the ancestral form of *Ae. aegypti*.

Supplementary table_2

Supplementary Table 2. Polymorphism of *Aedes albopictus cox*1 haplotypes from Indonesia

Haplogroup	Cluster best hit [a]	Location	Haplotype	% identity [b]
A2a	KX809764	Philippines	H1	99.67 %
	KX809761	Philippines	H2	99.84 %
	KX383935	Philippines	H3	99.51 %
			H4	99.67 %
A1b1	MN299017	D. R. Congo	H5	99.84%
	KU738429	China	H6	99.67 %
	KU738428	China		
	KU738427	China		
	KU738426	China		
	KU738425	China		
	KU738424	China		
	KX383928	Thailand		
	KX383927	Greece		
	KX383926	Greece		
	KX383925	Thailand		
	KX383924	Brazil		
	KC690951	USA		
	KC690941	USA		
	KC690940	USA		

a) All sequences displaying the same best hit score were reported with their respective accession number

b) The percentage of identity of a given haplotype is the same for each best hit sequence

Supplementary table_3

Supplementary Table 3. Polymorphism of *Aedes aegypti* ITS2 haplotypes from Indonesia

Cluster	Cluster best hit [a]	Location	Haplotype	% identity [b]
1a	MH142327	Russia	H1	99.65 %
	KY382418	Sri Lanka	H2	99.30 %
	HE820724	Russia	H3	99.30 %
			H6	98.25%
			H7	98.25%
			H8	98.23%
			H4	97.55%
			H5	97.54%
			H9	97.20%
			H10	94.68%
1b	MH142327	Russia	H11	99.51%
	MH142320	Russia		
	MH142318	Russia		
	KY328418	Sri Lanka		
	KF471584	Rockefeller Strain		
	KF471587	Rockefeller Strain		
	KF471579	New Caledonia (France)		
	KP259840	India		
	HE820724	Russia		
1c	KF471584	Rockefeller Strain	H12	91.29%
	MH142327	Russia		91.23%[c]
	KY382418	Sri Lanka		91.23%[c]
	HE820724	Russia		91.23%[c]
1d	KF471584	Rockefeller strain	H13	88.24%
	MH142327	Russia		88.11%[c]
	KY382418	Sri Lanka		88.11%[c]
	HE820724	Russia		88.11%[c]
1e	KF471579	New Caledonia (France)	H14	94.93 %
	KF471584	Rockefeller Strain	H15	92.61%
1f	KU497614	NA	H16	99.65%
	KU497614	NA	H17	99.65%
	KU497614	NA	H18	99.65%
	KU497614	NA	H19	96.14%

a) All sequences displaying the same best hit score were reported with their respective accession number

b) The percentage of identity of a given haplotype is the same for each best hit sequence

c) Second best hit score

Supplementary table_4

Supplementary Table 4. Polymorphism of *Aedes albopictus* ITS2 haplotypes from Indonesia

Sample	Best hit [a]	Location	Haplotype	% identity [b]
ktg08_Aal	MN062760	Israel	H1	98.82 %
	MN062758	Israel		
	MN062754	Israel		
	MN062753	Israel		
	MN062749	Israel		
	MN062743	Israel		
	MN062742	Israel		
	KY382421	Sri Lanka		
	KF471600	Italy		
	KF471594	Italy		
	KF471591	Italy		
	JX679394	Italy		
	JX679391	Italy		
	JX679390	Italy		
	JX679387	Italy		
r14_Aal	MH142323	Georgia	H2	100 %
	JX679389	Italy		
TB66L_Aal	MH142322	Georgia	H3	99.41 %
	KF471598	Italy		
	KF471595	Italy		
	JX679395	Italy		

a) All sequences displaying the same best hit score were reported with their respective accession number

b) The percentage of identity of a given haplotype is the same for each best hit sequence

Article 5.

Parasites & Vectors
Assessment of mosquito collection methods for dengue surveillance
--Manuscript Draft--

Manuscript Number:	PARV-D-20-01055
Full Title:	Assessment of mosquito collection methods for dengue surveillance
Article Type:	Research
Section/Category:	Dipteran Vectors and Associated Disease

Abstract:	Background: Several methods exist to collect and assess the abundance of dengue vector mosquitoes, i.e. morning adult collection using an aspirator, pupal collection, various ovitraps, whole night collection using human landing methods, and larval collection. This diversity of methods might be a source of variability and lack of statistical significance when trying to correlate mosquito density and risk of dengue outbreak. There is also a lack of published data regarding the effectiveness of these methods
	Methods: A mosquito survey was conducted in 39 locations corresponding to 15 dengue endemic provinces in Indonesia. The larval surveys were performed by collecting at least a single Aedes larva from each container, and then reared up until hatching. Three adult mosquito sampling methods were also used, including morning resting collection, human landing collection, animal baited trap. All field samples were tested for dengue. Factor Analysis of Mixed Data (FAMD) was conducted to analyze the effectiveness of the collection methods against mosquito species and dengue incidence.
	Results: A total of 44,675 mosquitoes were collected. The single larva method was the most efficient method. Out of a total of 89 dengue-positive pools, the most frequently encountered virus was DENV2, which made up half of the positive samples, followed by DENV3 and DENV1, respectively. FAMD showed that no correlation could be found between any methods and the presence of dengue virus in mosquitoes. Moreover, no correlation could be found between either any methods or the incidence.
	Conclusions: There was no concistency in the efficacy of a given method and the incidence of dengue in the human population. There was no correlation between any of the parameters considered, i.e. method, incidence of dengue, location and presence of dengue virus in mosquitoes. This indicates that entomological factors are not reliable indicators.
	Keywords: Aedes aegypti, Aedes albopictus, dengue, collection methods, dengue incidence, Indonesia

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Are you submitting to an Article Collection?	No

1   **Assessment of mosquito collection methods for dengue surveillance**

2

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1

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34

Abstract

Background: Several methods exist to collect and assess the abundance of dengue vector mosquitoes, i.e. morning adult collection using an aspirator, pupal collection, various ovitraps, whole night collection using human landing methods, and larval collection. This diversity of methods might be a source of variability and lack of statistical significance when trying to correlate mosquito density and risk of dengue outbreak. There is also a lack of published data regarding the effectiveness of these methods

Methods: A mosquito survey was conducted in 39 locations corresponding to 15 dengue endemic provinces in Indonesia. The larval surveys were performed by collecting at least a single *Aedes* larva from each container, and then reared up until hatching. Three adult mosquito sampling methods were also used, including morning resting collection, human landing collection, animal baited trap. All field samples were tested for dengue. Factor Analysis of Mixed Data (FAMD) was conducted to analyze the effectiveness of the collection methods against mosquito species and dengue incidence.

Results: A total of 44,675 mosquitoes were collected. The single larva method was the most efficient method. Out of a total of 89 dengue-positive pools, the most frequently encountered virus was DENV2, which made up half of the positive samples, followed by DENV3 and DENV1, respectively. FAMD showed that no correlation could be found between any methods and the presence of dengue virus in mosquitoes. Moreover, no correlation could be found between either any methods or the incidence.

Conclusions: There was no concistency in the efficacy of a given method and the incidence of dengue in the human population. There was no correlation between any of the parameters considered, i.e. method, incidence of dengue, location and presence of dengue virus in mosquitoes. This indicates that entomological factors are not reliable indicators.

Keywords: *Aedes aegypti, Aedes albopictus,* dengue, collection methods, dengue incidence, Indonesia

2

67

Background

69

70 Dengue is the most rapidly spreading arboviral disease worldwide [1]. Recent studies

71 estimate that 55 to 100 million dengue cases are reported annually with 3.9 billion

72 people at risk [2,3]. Indonesia is an hyperendemic dengue country, i.e. all four serotypes

73 are circulating, with the highest number of dengue cases in South-East Asia [4,5].

74 Dengue incidence in Indonesia has increased significantly over the last four decades

75 from 0.05 per 100,000 in 1968 to 78.8 per 100,000 in 2016 [6]. The Dengue virus is

76 transmitted to humans by the bite of infected *Aedes aegypti* mosquitoes, the main

77 vector, and *Ae. albopictus,* the secondary vector. These species are anthropophilic, i.e.

78 they live in human environments and breed in various sites, such as water containers,

79 flowerpots, birdbaths, disposed water-holding vessels, waste disposal areas, small

80 containers, discarded tyres, natural holes in vegetation, etc. [7–10]. Both are present in

81 urban and sub-urban areas. With no treatment and while an effective vaccine is still

82 under study, vector control remains the only effective way to prevent and control

83 dengue.

84   Vector surveillance methods have remained mostly unchanged for more than

85 three decades [11]. Larval survey is the most widely adopted dengue vector surveillance

86 method to locate larval habitats and to measure the abundance of *Ae. aegypti* and *Ae.*

87 *albopictus* [12,13]. The *Stegomyia* indices, i.e. House index (HI), Container Index (CI)

88 and Breteau index (BI) to which a specific Free Larval Index (FLI) is added in

89 Indonesia, are used for calculating mosquito abundance and for predicting the risk of

90 dengue transmission [11]. However, previous studies have demonstrated the lack of

91 correlation between *Stegomyia* indices and the risk of dengue outbreak [12,14-18],

92 while a correlation was found between human population density and incidence of

93 dengue [18]. Several methods exist to collect mosquitoes, i.e. morning adult collection

94 using an aspirator, pupal collection, various ovitraps, whole night collection using

95 human landing methods, and larval collection. This diversity of methods might be a

96 source of variability and lack of statistical significance when trying to correlate

97 mosquito density and risk of dengue outbreak. Furthermore, there is a lack of published

98 data regarding the effectiveness of these methods [12,19-22]. Therefore, we conducted

99 a comparative analysis to assess their relative effectiveness.

3

Methods

Study Sites

The study was conducted in 39 locations corresponding to 39 districts/municipalities in 15 dengue endemics provinces in Indonesia (Figure 1). These provinces include Aceh, West Sumatra, Lampung, Bangka-Belitung, West Kalimantan, South Kalimantan, North Sulawesi, East Java, South-East Sulawesi, Maluku, West Nusa Tenggara, North Maluku. This study is part of the Indonesia national project, Rikhus Vektora that started in 2016

Study design

A mosquito survey was performed in all study sites from July to August 2016, during the rainy season. Single larval methods were performed randomly in at least 100 households in each study site during the study. *Aedes* larvae were collected and then reared in a field laboratory by using plastic trays with tap water and fish food for 3-4 days. Adult collection of *Aedes* mosquitoes were performed in the morning (morning resting) on mosquitoes resting inside houses using manual aspirators. Adult mosquitoes were also collected outside using standard procedures for all night human-landing collection methods from 6.00 pm to 6.00 am. Each adult collection method was performed in every study site. All methodologies used in this study have been previously described [22]. Field data collections for larva and adult *Aedes* mosquitoes were performed by trained collectors in collaboration with local volunteers, local authorities and staff from district/municipality dengue control programs.

Single larva and rearing methods

The larval surveys were performed by collecting at least a single *Aedes* larva from each container. All larvae then were taken to the field laboratory located in the study sites and reared up to the fourth days until adult hatching. Emerged adult mosquitoes were then killed in a freezer (-20ºC) or by using ethyl acetate for 5 to 10 minutes and immediately stored in 1.5 ml vial tubes with RNAlater (Qiagen, Hilden, Germany) by pools of 25 mosquitoes and kept refrigerated at 4°C prior to further analysis. Reared larvae and pupae that did not hatch to adult stage up to the fourth days were preserved under the same conditions for further analysis.

4

133 *Adult mosquito collection methods*

134 Three adult mosquito sampling methods were conducted simultaneously in all study

135 sites, including: (1) morning resting collection, (2) human landing collection, (3) animal

136 baited trap.

137 (1). Morning resting collections were made by eight collectors using hand nets and

138 aspirators. Collections were conducted from 7.00 am to 9.00 am and included any

139 resting locations within the house. All adults mosquitoes were placed into labelled

140 paper caps and taken to the field laboratory for further analysis.

141 (2). Human landing collection were performed by eight local volunteers as collectors

142 in three selected houses of each study sites for sampling adult mosquitoes using mouth

143 aspirators. They were all trained before collecting mosquitoes. Three teams of two

144 people sampled outdoors (up to 5 meters from the house) and indoors (inside the house).

145 Each collector sat on chairs with exposing their legs for 50 of 60 minutes per hour.

146 Sampling was conducted all night from 6.00 pm to 6.00 am. The team changed roles

147 regularly every 2 hours with a 2 hour-break. Although, the targeted *Aedes* mosquitoes

148 are diurnal, the Indonesia law does not allow human landing collections during day

149 time. Therefore, collections had to be conducted at night. This introduces a strong bias

150 in the sampling but since it is what surveillance teams do in accordance with the law,

151 this method was nevertheless performed. Mosquitoes that have been collected per hour

152 were then taken to the field laboratory for species identification and further analysis.

153 (3). Animal baited trap was conducted by using tame animal placed inside a net all

154 night. Mosquito collections were carried out for 15 minutes per hour inside the nets by

155 3 collectors. Collected mosquitoes were then similarly preserved as for the human

156 landing collection method.

157

158   All mosquitoes from these three collecting methods identified as *Ae. aegypti*

159 and *Ae. albopictus* were then killed with ethyl acetate, pooled up to 25 mosquitoes in

160 labelled 1.5 ml vial tubes with RNAlater (Qiagen, Hilden, Germany) and preserved

161 based on the same cold chain management than the one above used for larvae.

162

163 **Detection of Dengue virus from mosquitoes**

164 The *Ae. aegypti* and *Ae. albopictus* mosquito pools were homogenized in 1.5ml tubes

165 containing 200 µl PBS 1x by using pellet pestles. RNA was extracted using

5

166 QIAamp®Viral RNA Mini Kit (Qiagen®, Courtaboeuf, France). RNAs were extracted

167 from 200-µl homogenized samples following the manufacturer's instructions.

168   All RNA extracted samples were analyzed for Dengue detection using Lanciotti's

169 protocol [23]. The nested RT-PCR for Dengue was performed using SimpliAmp

170 Thermal Cycler Applied Biosystems™ (ThermoFisher Scientific®, United States).

171 Amplification of Dengue RNA was carried out with following specific primers : D1

172 (5'-TCA ATA TGC TGA AAC GCG CGA GAA ACC G-3'), D2 (5'-TTG CAC CAA

173 CAG TCA ATG TCT TCA GGT TC-3'), TS1   (5'-CGT CTC AGT GAT CCG

174 GGG G-3'), TS2 (5'-CGC CAC AAG GGC CAT GAA CAG-3'), TS3 (5'-TAA CAT

175 CAT CAT GAG ACA GAG C- 3'), and TS4 (5'-CTC TGT TGT CTT AAA CAA GAG

176 A-3').

177   The first amplification of Dengue virus was performed using Superscript III

178 one-step RT-PCR kit (Invitrogen, Carlsbad, CA). The cycling conditions consisted of

179 initial 95°C denaturation step for 2 minutes, followed by 40 cycles of 95°C denaturation

180 for 30 seconds, 60°C annealing for 1 minute, and 72°C extension for 1 minute 30

181 seconds, and a final extension step 72°C for 10 minutes. Samples were then stored at

182 4°C. First step PCR products were run on 2% agarose gel under 120 V current for 1

183 hour and followed by visualization using SYBR® safe DNA gel stain (Invitrogen,

184 Carlsbad, CA, USA) under UV condition in GelDoc system and check for presence of

185 the 511 bp control band corresponding to dengue virus (DENV) positive. Subsequent

186 serotyping was conducted by using 1st step PCR product with thermal cycle setting as

187 follow : initial denaturation step at 95°C for 2 minutes, followed by 10 cycles of

188 denaturation step at 95°C for 30 seconds, 60°C annealing for 1 minute, and an extension

189 step at 72°C for 1 minute and 30 seconds. The final extension step was conducted at

190 72°C for 10 minutes. Subsequently, samples were stored at 4°C. Amplification product

191 on 2% agarose gel were then carried out under 80V current for 1 hour and check under

192 UV condition. Multiplex serotyping reaction is expected to produce single-specific

193 band with the size of 482bp for DEN-1 , 119bp for DENV-2, 290bp for DENV-3, and

194 389bp for DENV-4. All of field samples were tested for the presence of dengue virus

195 after being pooled by 25 individuals of the same species.

196

197 **Statistical analyses**

198 A first Factor Analysis of Mixed Data (FAMD) [24] was conducted using the incidence

199 data, the number of mosquitoes and the number of positive pools for each dengue

6

serotype as quantitative parameters, and mosquito species, methods of collection and provinces as qualitative parameters. The effectiveness of the collection methods (qualitative data) against mosquito species (quantitative data) was assessed using a second FAMD. These analyses were performed using the R software with FactoMineR [25].

Results

Mosquito sampling

A total of 44,675 mosquitoes were collected from 39 locations (Figure 1, Supplementary Table 1). Out of these 44,675 mosquitoes collected, 32,525 (72.8%) were *Ae. aegypti* and 10,300 (23.1%) were *Ae. albopictus*, while 1,850 (4.1%) were undetermined. When considering the method of capture, the highest number of captured individuals was, as expected, obtained when targeting larvae. The single larva method was the most efficient in terms of number of individuals collected. A total of 36,500 larvae were collected with this method out which 27,475 were *Ae. aegypti*, 7,775 were *Ae. albopictus* and 1,250 were not identified. The rearing method, although less efficient also yielded large numbers of individuals. Out of 6,450 larvae collected and reared, 4,325 were *Ae. aegypti,* 1,575 were *Ae. albopictus* and 550 were not identified. With both larval methods a bias was observed in favor of *Ae. aegypti* which represented 75.27% and 37.05% of all samples for the single lava and rearing methods, respectively. Very different results were obtained with the adult capture method. From the three methods used, human landing was the most efficient even though a bias is introduced by the legal obligation to perform this approach by night. Out of 1,325 adult mosquitoes captured 325 were *Ae. aegypti*, 975 were *Ae. albopictus* and 25 were not identified. The higher proportion of *Ae. albopictus* might be related to the fact this species is more crepuscular than *Ae. aegypti*. The animal baited trap method yielded only 25 mosquitoes, all being *Ae. albopictus*. The ratio between *Ae. aegypti* and *Ae. albopictus* was reversed with a bias this time in favor of *Ae. albopictus*. It represented 73.58% and 53.33% for the human landing and morning resting methods, respectively. The animal baited trap method yielded only *Ae. albopictus*, but considering the very low number of mosquitoes captured, i.e. 25, this is not significant.

7

Distribution of dengue virus

A total of 89 pools were positive for dengue virus. The most frequently encountered virus was DENV2 (n=44), which made up half of the positive samples. DENV3 and DENV1 followed with 20 and 17 positive pools, respectively. DENV4 was detected in only one pool. Combinations were also detected. 8 pools contained a combination of DENV1 and DENV2, whereas the combination of DENV1 and DENV3 was found in only one pool. Another single pool contained the triple combination DENV1-DENV2-DENV3. With respect to the geographic distribution, a strong imbalance was observed. A large part of the detected dengue viruses, i.e. 56 (63%), were found in mosquitoes collected in the province of Aceh. All four dengue virus serotypes and all positive combinations were found in this province. The other provinces where positive pools were detected are: West Sumatra (n=5), Lampung (n=6), Bangka-Belitung (n=4), West Kalimantan (n=2), South Kalimantan (n=1), North Sulawesi (n=2), East Java (n=7) and Maluku (n=6). A strong imbalance was also observed when considering the nature of the positive samples. Mosquito larvae were the almost exclusive source of virus, i.e. 93.3% (n=83), with 70.8% (n=63) found with the single larva method and 22.5% (n=20) for the rearing method. Only 6 pools (6.7%) of adult mosquitoes were found positive with the human landing method totalizing 2 pools (2.3%), while 4 pools (4.4%) were found positive in mosquitoes collected with the morning resting method. An imbalanced result was also found regarding the mosquito species with 76.4% (n=68) of the positive pools corresponding to *Ae. aegypti* and 23.6% (n=21) corresponding to *Ae. albopictus*.

Correlation assessment

A Factor Analysis of Mixed Data (FAMD) was performed to determine the potential correlation between the various parameters considered: mosquito species, province, number of mosquitoes, collection method, dengue virus and dengue incidence (Figure 2a). The only correlation which could be found was between the province and the incidence (Figure 2a). However, the global level of explanation was low (20%) indicating a lack of correlation between any of the parameters with the exception of province and incidence of dengue. A similar result was found when comparing the different collection methods with the mosquito species. The only, but rather weak, correlation which could be found was the preferred association of the larval methods

8

266 with *Ae. aegypti* and the adult methods with *Ae. albopictus* (Figure 2b, Supplementary

267 Table 1).

268

Discussion

270   In the absence of commercialized vaccines and of any medical treatment, the

271 management of dengue relies only on mosquito control and on prevention. Finding

272 efficient and reliable descriptors for assessing the risk of dengue outbreaks is thus a

273 priority in all dengue-endemic countries. The main tools currently in use for assessing

274 this risk of dengue outbreak is the *Stegomiya* indices [15,26] which rely on the

275 calculation of the relative density of mosquito larvae present in containers and in

276 households through the Container Index (CI), the House Index (HI) and the Breteau

277 Index (BI) [26,27]. However, these indices were shown to have no correlation with

278 dengue infection rates and are thus not reliable descriptors [18,28-30].

279   The development of entomological indices other than the *Stegomiya* indices

280 could then be envisioned. However, as entomological indices, they will still be based

281 on the capture of mosquitoes regardless of the calculation model applied. This work is

282 to our knowledge the most extensive one with 44,675 mosquitoes collected in 39

283 different sampling locations over Indonesia in a short period of two months, allowing

284 thus a robust statistical analysis. The main conclusion of this work is that it is not the

285 *Stegomiya* indices only, but any kind of entomological indices that might be at best of

286 very limited use. Not only no correlation could be found between any methods and the

287 presence of dengue virus in mosquitoes but no correlation could be found between

288 either any methods or the incidence. Finally, there was no consistency in the efficiency

289 of a given method for detecting dengue. The single-larva and rearing methods yielded

290 63% of all dengue-positive samples in the sole province of Aceh. However, the

291 incidence of dengue in Aceh is not the highest among all provinces and is rather in the

292 average. Provinces displaying the highest incidence such as Bangka-Belitung, South

293 Kalimantan or North Sulawesi did not yield any dengue-positive larvae. The only single

294 positive pool in these provinces was found in South Kalimantan among morning resting

295 adults. This lack of correlation between incidence and dengue infection rate in

296 mosquitoes is also a drawback for methods associating the capture of adults and the

297 direct detection of dengue virus in the sampled mosquitoes [31-33].

9

298   The use of *Stegomiya* indices and the monitoring and collections of mosquitoes

299   are today the main means of assessing the risk of dengue outbreaks and efficiency of

300   mosquito control. Owing to the lack of correlation of the *Stegomiya* indices with the

301   risk of dengue outbreaks and dengue incidence [18], the lack of consistency of the

302   various collection methods, and the very low level of dengue detection in mosquitoes,

303   the monitoring of mosquitoes to assess the risk of dengue outbreaks should be

304   reconsidered. The risk with these methods is mostly that of misleading interpretation,

305   and misguided decisions and allocation of resources. The only factor found positively

306   correlated with the incidence of dengue was the human population density [18].

307

Conclusions

309   The lack of correlation between dengue incidence and entomological factors

310   demonstrated in this work and in a separate study on the *Stegomiya* indices [18]

311   indicates that they are not reliable. Since the only correlation was found with a societal

312   factor, i.e. the human population density [18], efforts should be devoted to the

313   development of novel societal indices to achieve an efficient management of the risk of

314   dengue outbreaks. It is even more important to communicate on this issue because

315   dengue endemic countries worldwide, as well as WHO, still base their

316   recommendations and dengue management procedures on entomological indices.

317

318

Additional Data

Supplementary Table 1. Sampling localities and characteristics of samples

Abbreviations

HI: House Index, CI: Container Index, BI: Breteau Index, FAMD: Factor Analysis of Mixed Data

Ethics approval

This study involved the use of humans to collect adult mosquitoes in natural settings. Formal approval to conduct these activities was provided by the Ethical Commission Board of the NIHRD, Ministry of Health, Indonesia (No. LB.02.01/5.2/KE.003/2016 and No. LB.02.01/5.2/KE.020/2017).

Consent for publication

Not applicable

Availability of data and materials

Data supporting the conclusions of this article are included within the article.

11

353

Competing interest

The authors declare that they have no competing interests.

356

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363

Authors Contributions

TAG, LS, TBT, WI, MTP conceived and designed the field studies. MO, SSN, DS prepared samples. TAG and MTP ran molecular analyses and laboratory experiments. TAG, LG and RF analyzed the data. MA prepared the map. TAG and RF wrote the manuscript. SM, LG and RF provided critiques and significant revisions to the manuscript.

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470 **Legends to figures**

471

472 **Figure 1. Map of the sampling sites throughout Indonesia**

473

474 **Figure 2. Multivariate analysis of parameters**

475 **2a. Global Factor Analysis of Mixed Data**

476 **2b. FAMD assessment of the effectiveness of the collection methods**

477

Multivariate analysis of parameters

2a
Global Factor Analysis of Mixed Data
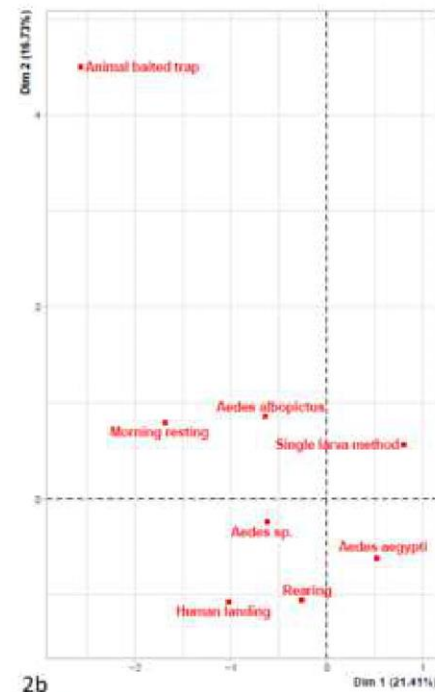
2b
FAMD assessment of the effectiveness of the collection methods

231

Figure 1

Figure 2

2a

2b
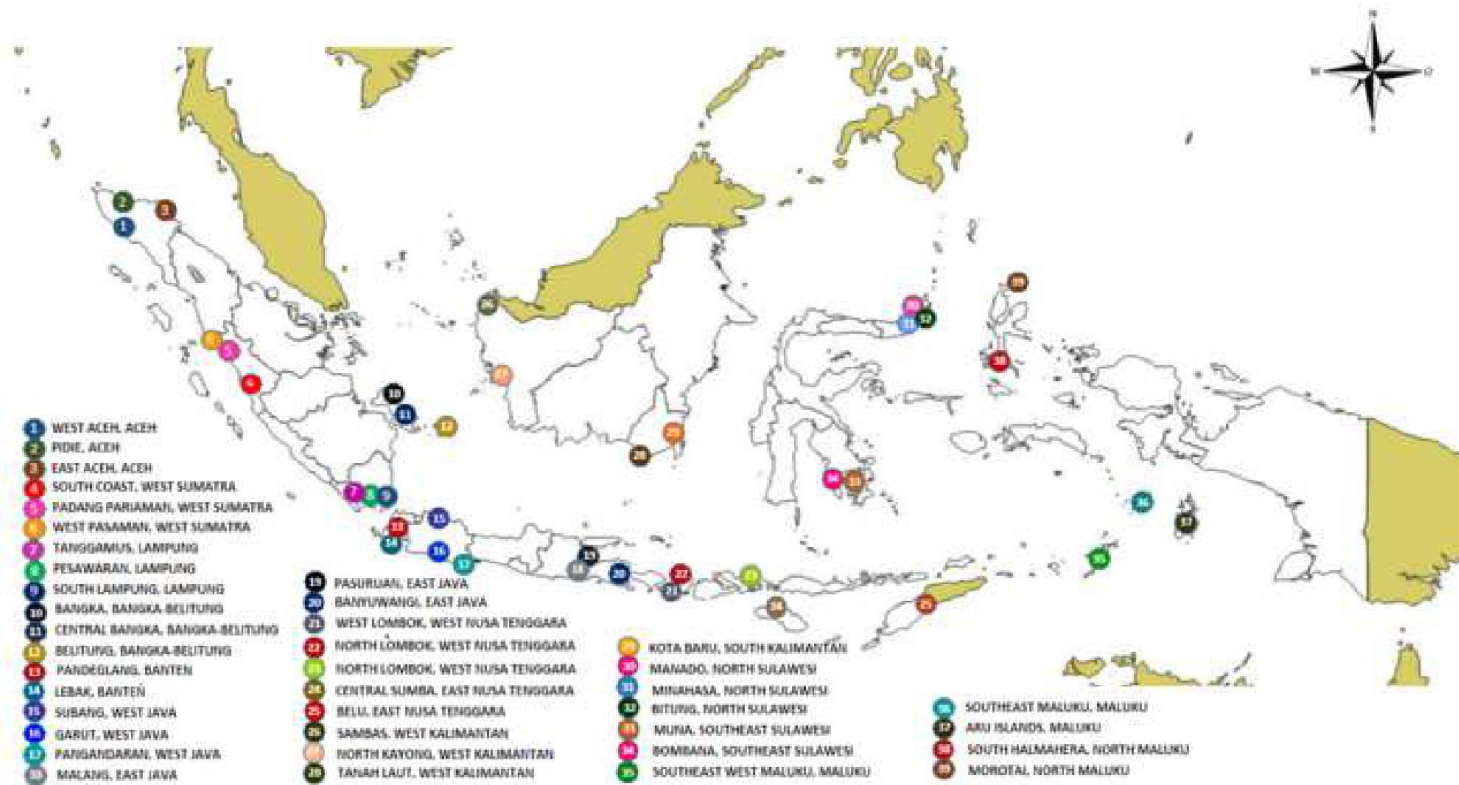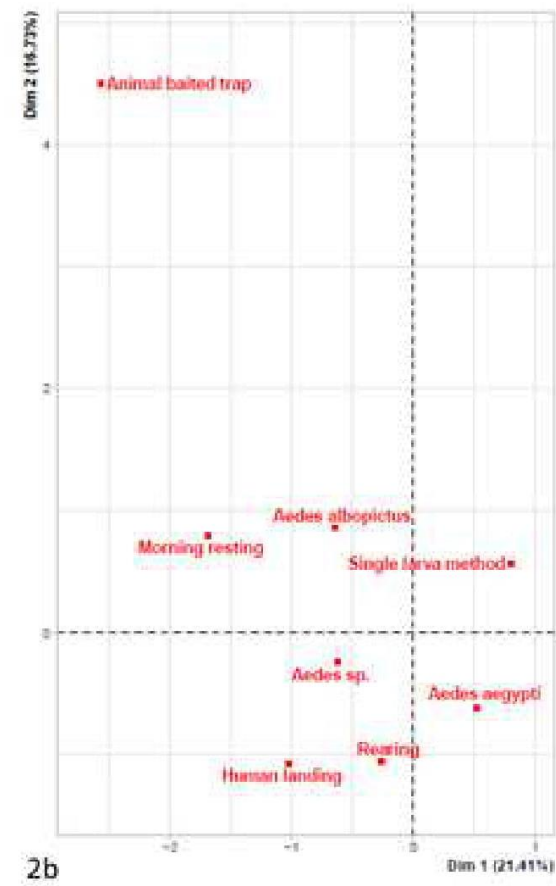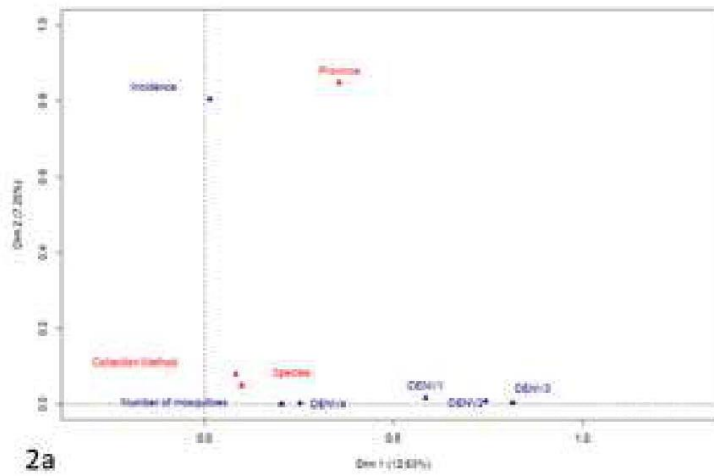
Supplementary table_1

Province	District	Year	Species	Type of collection	Collection Method	Incidence per 100000	Number of pools	Number of mosquitoes	Number of positive pools								Sample type
									DENV-1	DENV-2	DENV-3	DENV-4	DENV-1 + DENV-2	DENV-1 + DENV-3	DENV-2 + DENV-3	DENV-1 + DENV-2 + DENV-3	
Aceh	East Aceh	2016	*Aedes aegypti*	Larva collection	Single larva method	37.93	67	1675	1	5	2	0	2	1	0	1	Larvae
Aceh	East Aceh	2016	*Aedes aegypti*	Larva collection	Rearing	37.93	11	275	0	3	1	0	0	0	0	0	Adults
Aceh	East Aceh	2016	*Aedes albopictus*	Larva collection	Rearing	37.93	2	50	0	0	0	0	0	0	0	0	Adults
Aceh	East Aceh	2016	*Aedes aegypti*	Adult collection	Human landing	37.93	2	50	0	0	0	0	0	0	0	0	Adults
Aceh	East Aceh	2016	*Aedes albopictus*	Adult collection	Human landing	37.93	10	500	0	0	0	0	0	0	0	0	Adults
Aceh	West Aceh	2016	*Aedes aegypti*	Larva collection	Single larva method	33.85	56	1400	1	5	3	1	0	0	0	0	Larvae
Aceh	West Aceh	2016	*Aedes albopictus*	Larva collection	Single larva method	33.85	31	775	3	2	1	0	0	0	0	0	Larvae
Aceh	West Aceh	2016	*Aedes aegypti*	Adult collection	Human landing	33.85	1	25	0	0	0	0	0	0	0	0	Adults
Aceh	West Aceh	2016	*Aedes albopictus*	Adult collection	Human landing	33.85	1	25	0	0	0	0	0	0	0	0	Adults
Aceh	Pidie	2016	*Aedes aegypti*	Larva collection	Single larva method	44.37	52	1300	4	7	5	0	0	0	0	0	Larvae
Aceh	Pidie	2016	*Aedes albopictus*	Larva collection	Single larva method	44.37	9	225	0	2	2	0	0	0	0	0	Larvae
Aceh	Pidie	2016	*Aedes albopictus*	Larva collection	Single larva method	44.37	21	525	0	2	0	0	0	0	0	0	Larvae
Aceh	Pidie	2016	*Aedes aegypti*	Larva collection	Rearing	44.37	3	75	0	2	0	0	1	0	0	0	Adults
Aceh	Pidie	2016	*Aedes aegypti*	Adult collection	Human landing	44.37	4	100	0	0	0	0	0	0	0	0	Adults
Aceh	Pidie	2016	*Aedes albopictus*	Adult collection	Human landing	44.37	5	125	0	0	0	0	0	0	0	0	Adults
West Sumatra	South coast	2016	*Aedes aegypti*	Larva collection	Single larva method	62.58	79	1975	1	0	0	0	0	0	0	0	Larvae

West Sumatra	South coast	2016	*Aedes aegypti*	Adult collection	Morning resting	62.58	3	75	0	0	0	0	0	0	0	0	Adults
West Sumatra	South coast	2016	*Aedes aegypti*	Adult collection	Human landing	62.58	1	25	0	0	0	0	0	0	0	0	Adults
West Sumatra	South coast	2016	*Aedes aegypti*	Larva collection	Rearing	62.58	1	25	0	0	0	0	0	0	0	0	Adults
West Sumatra	South coast	2016	*Aedes albopictus*	Adult collection	Morning resting	62.58	3	75	0	0	0	0	0	0	0	0	Adults
West Sumatra	South coast	2016	*Aedes albopictus*	Adult collection	Human landing	62.58	1	25	0	0	0	0	0	0	0	0	Adults
West Sumatra	South coast	2016	*Aedes albopictus*	Larva collection	Single larva method	62.58	5	125	0	0	0	0	0	0	0	0	Larvae
West Sumatra	Padang pariaman	2016	*Aedes aegypti*	Larva collection	Single larva method	46.74	19	475	0	**1**	0	0	0	0	0	0	Larvae
West Sumatra	Padang pariaman	2016	*Aedes albopictus*	Larva collection	Single larva method	46.74	33	825	0	0	0	0	0	0	0	0	Larvae
West Sumatra	West Pasaman	2016	*Aedes aegypti*	Larva collection	Single larva method	39.96	2	50	0	0	0	0	0	0	0	0	Larvae
West Sumatra	West Pasaman	2016	*Aedes albopictus*	Larva collection	Single larva method	39.96	1	25	0	0	0	0	0	0	0	0	Larvae
West Sumatra	West Pasaman	2016	*Aedes aegypti*	Larva collection	Rearing	39.96	1	25	0	0	**1**	0	0	0	0	0	Adults
West Sumatra	West Pasaman	2016	*Aedes aegypti*	Adult collection	Human landing	39.96	1	25	0	0	0	0	**1**	0	0	0	Adults
West Sumatra	West Pasaman	2016	*Aedes albopictus*	Adult collection	Morning resting	39.96	1	25	0	0	**1**	0	0	0	0	0	Adults
Lampung	South Lampung	2016	*Aedes aegypti*	Larva collection	Single larva method	44.5	4	100	**2**	**1**	0	0	0	0	0	0	Larvae
Lampung	South Lampung	2016	*Aedes albopictus*	Adult collection	Morning resting	44.5	1	25	0	0	0	0	0	0	0	0	Adults
Lampung	South Lampung	2016	*Aedes albopictus*	Larva collection	Single larva method	44.5	15	375	0	**2**	0	0	0	0	0	0	Larvae
Lampung	Tanggamus	2016	*Aedes albopictus*	Larva collection	Single larva method	66.34	8	200	0	0	0	0	0	0	0	0	Larvae
Lampung	Tanggamus	2016	*Aedes albopictus*	Adult collection	Morning resting	66.34	1	25	0	0	0	0	0	0	0	0	Adults

Lampung	Pesawaran	2016	*Aedes aegypti*	Larva collection	Single larva method	89.05	15	375	0	**1**	0	0	0	0	0	0	Larvae
Bangka-belitung	Bangka	2016	*Aedes albopictus*	Adult collection	Human landing	21.70	1	25	0	0	0	0	0	0	0	0	Larvae
Bangka-belitung	Bangka	2016	*Aedes albopictus*	Larva collection	Rearing	21.70	14	350	0	0	0	0	0	0	0	0	Adults
Bangka-belitung	Bangka	2016	*Aedes aegypti*	Larva collection	Single larva method	21.70	40	1000	0	0	0	0	0	0	0	0	Larvae
Bangka-belitung	Bangka	2016	*Aedes aegypti*	Larva collection	Rearing	21.70	21	525	0	**3**	0	0	0	0	0	0	Adults
Bangka-belitung	Belitung	2016	*Aedes albopictus*	Larva collection	Rearing	103.40	2	50	0	0	0	0	0	0	0	0	Adults
Bangka-belitung	Belitung	2016	*Aedes aegypti*	Larva collection	Rearing	103.40	6	150	0	0	0	0	0	0	0	0	Adults
Bangka-belitung	Belitung	2016	*Aedes albopictus*	Larva collection	Rearing	103.40	6	150	0	0	0	0	0	0	0	0	Adults
Bangka-belitung	Central Bangka	2016	*Aedes aegypti*	Larva collection	Rearing	35.20	22	550	0	0	0	0	0	0	0	0	Adults
Bangka-belitung	Central Bangka	2016	*Aedes aegypti*	Larva collection	Single larva method	35.20	39	975	0	0	0	0	0	0	0	0	Larvae
Bangka-belitung	Central Bangka	2016	*Aedes albopictus*	Larva collection	Rearing	35.20	3	75	0	0	0	0	0	0	0	0	Adults
Bangka-belitung	Central Bangka	2016	*Aedes albopictus*	Larva collection	Single larva method	35.20	25	625	0	0	0	0	0	0	0	0	Larvae
Bangka-belitung	Central Bangka	2016	*Aedes albopictus*	Adult collection	Morning resting	35.20	1	25	0	**1**	0	0	0	0	0	0	Adults
West Kalimantan	Sambas	2016	*Aedes aegypti*	Larva collection	Single larva method	3.99	12	300	0	0	0	0	0	0	0	0	Larvae
West Kalimantan	Sambas	2016	*Aedes albopictus*	Larva collection	Single larva method	3.99	4	100	0	0	0	0	0	0	0	0	Larvae
West Kalimantan	Sambas	2016	*Aedes aegypti*	Adult collection	Human landing	3.99	1	25	0	**1**	0	0	0	0	0	0	Adults
West Kalimantan	North Kayong	2016	*Aedes aegypti*	Larva collection	Single larva method	9.32	10	250	0	0	0	0	0	0	0	0	Larvae
West Kalimantan	North Kayong	2016	*Aedes albopictus*	Larva collection	Single larva method	9.32	10	250	0	0	0	0	0	0	0	0	Larvae
West Kalimantan	North Kayong	2016	*Aedes aegypti*	Larva collection	Rearing	9.32	1	25	0	**1**	0	0	0	0	0	0	Adults

South Kalimantan	Tanah laut	2016	*Aedes aegypti*	Larva collection	Single larva method	120.56	65	1625	0	0	0	0	0	0	0	0	Larvae
South Kalimantan	Tanah laut	2016	*Aedes albopictus*	Larva collection	Single larva method	120.56	14	350	0	0	0	0	0	0	0	0	Larvae
South Kalimantan	Tanah laut	2016	*Aedes albopictus*	Adult collection	Morning resting	120.56	1	25	0	0	0	0	0	0	0	0	Adults
South Kalimantan	Tanah laut	2016	*Aedes aegypti*	Adult collection	Morning resting	120.56	1	25	0	**1**	0	0	0	0	0	0	Adults
South Kalimantan	Kota Baru	2016	*Aedes aegypti*	Larva collection	Single larva method	82.87	124	3100	0	0	0	0	0	0	0	0	Larvae
South Kalimantan	Kota Baru	2016	*Aedes albopictus*	Larva collection	Single larva method	82.87	15	375	0	0	0	0	0	0	0	0	Larvae
North Sulawesi	Minahasa	2016	Aedes albopictus	Larva collection	Single larva method	68.94	6	150	0	0	0	0	0	0	0	0	Larvae
North Sulawesi	Minahasa	2016	Aedes aegypti	Larva collection	Single larva method	68.94	29	725	0	0	**2**	0	0	0	0	0	Larvae
North Sulawesi	Manado	2016	Aedes aegypti	Larva collection	Single larva method	132.51	8	200	0	0	0	0	0	0	0	0	Larvae
North Sulawesi	Manado	2016	Aedes albopictus	Larva collection	Single larva method	132.51	13	325	0	0	0	0	0	0	0	0	Larvae
North Sulawesi	Manado	2016	Aedes albopictus	Adult collection	Human landing	132.51	2	50	0	0	0	0	0	0	0	0	Adults
North Sulawesi	Manado	2016	Aedes aegypti	Adult collection	Human landing	132.51	1	25	0	0	0	0	0	0	0	0	Adults
North Sulawesi	Manado	2016	Aedes albopictus	Larva collection	Rearing	132.51	8	200	0	0	0	0	0	0	0	0	Adults
North Sulawesi	Manado	2016	Aedes aegypti	Larva collection	Rearing	132.51	2	50	0	0	0	0	0	0	0	0	Adults
North Sulawesi	Bitung	2016	Aedes aegypti	Larva collection	Single larva method	57.89	3	75	0	0	0	0	0	0	0	0	Larvae
East Java	Malang	2016	*Aedes sp.*	Larva collection	Single larva method	54.18	33	825	0	0	0	0	0	0	0	0	Larvae
East Java	Malang	2016	*Aedes sp.*	Adult collection	Human landing	54.18	1	25	0	0	0	0	0	0	0	0	Adults
East Java	Malang	2016	*Aedes sp*	Adult collection	Morning resting	54.18	1	25	0	0	0	0	0	0	0	0	Adults

East Java	Malang	2016	*Aedes albopictus*	Adult collection	Animal baited trap	54.18	1	25	0	0	0	0	0	0	0	0	Adults
East Java	Banyuwangi	2016	*Aedes albopictus*	Larva collection	Single larva method	20.81	1	25	**1**	0	0	0	0	0	0	0	Larvae
East Java	Banyuwangi	2016	*Aedes aegypti*	Larva collection	Single larva method	20.81	1	25	**1**	0	0	0	0	0	0	0	Larvae
East Java	Pasuruan	2016	*Aedes aegypti*	Larva collection	Rearing	165.67	6	150	**1**	0	0	0	**4**	0	0	0	Adults
Southeast Sulawesi	Muna	2016	*Aedes aegypti*	Larva collection	Single larva method	90.70	78	1950	0	0	**2**	0	0	0	0	0	Larvae
Southeast Sulawesi	Muna	2016	*Aedes albopictus*	Larva collection	Single larva method	90.70	10	250	0	0	0	0	0	0	0	0	Larvae
Southeast Sulawesi	Muna	2016	*Aedes aegypti*	Adult collection	Human landing	90.70	8	200	0	0	0	0	0	0	0	0	Adults
Southeast Sulawesi	Muna	2016	*Aedes aegypti*	Larva collection	Rearing	90.70	15	375	0	0	0	0	0	0	0	0	Adults
Southeast Sulawesi	Bombana	2016	*Aedes aegypti*	Adult collection	Human Landing	49.30	1	25	0	0	0	0	0	0	0	0	Adults
Maluku	Southeast Maluku	2016	*Aedes aegypti*	Larva collection	Single larva method	NA	133	3325	0	0	0	0	0	0	0	0	Larvae
Maluku	Southeast Maluku	2016	*Aedes albopictus*	Larva collection	Single larva method	NA	12	300	0	0	0	0	0	0	0	0	Larvae
Maluku	Southeast Maluku	2016	*Aedes aegypti*	Larva collection	Rearing	NA	3	75	**1**	**2**	0	0	0	0	0	0	Adults
Maluku	Southeast Maluku	2016	*Aedes aegypti*	Adult collection	Morning resting	NA	1	25	**1**	0	0	0	0	0	0	0	Adults
Maluku	Southeast west Maluku	2016	*Aedes aegypti*	Adult collection	Morning resting	NA	1	25	0	**1**	0	0	0	0	0	0	Adults
Maluku	Southeast west Maluku	2016	*Aedes aegypti*	Adult collection	Human landing	NA	1	25	0	**1**	0	0	0	0	0	0	Adults
Maluku	Aru islands	2016	*Aedes aegypti*	Larva collection	Rearing	NA	34	850	0	0	0	0	0	0	0	0	Adults
Maluku	Aru islands	2016	*Aedes albopictus*	Larva collection	Rearing	NA	2	50	0	0	0	0	0	0	0	0	Adults
West Nusa Tenggara	West Lombok	2016	*Aedes sp.*	Larva collection	Rearing	40.96	22	550	0	0	0	0	0	0	0	0	Adults

West Nusa Tenggara	Bima	2016	*Aedes aegypti*	Larva collection	Single larva method	35.68	16	400	0	0	0	0	0	0	0	0	Larvae
West Nusa Tenggara	North Lombok	2016	*Aedes aegypti*	Larva collection	Single larva method	108.86	19	475	0	0	0	0	0	0	0	0	Larvae
North Maluku	South Halmahera	2016	*Aedes albopictus*	Larva collection	Single larva method	NA	28	700	0	0	0	0	0	0	0	0	Larvae
North Maluku	South Halmahera	2016	*Aedes aegypti*	Adult collection	Human landing	NA	1	50	0	0	0	0	0	0	0	0	Adults
North Maluku	South Halmahera	2016	*Aedes aegypti*	Larva collection	Rearing	NA	23	575	0	0	0	0	0	0	0	0	Adults
North Maluku	South Halmahera	2016	*Aedes aegypti*	Larva collection	Single larva method	NA	42	1025	0	0	0	0	0	0	0	0	Larvae
North Maluku	Morotai	2016	Aedes albopictus	Larva collection	Single larva method	NA	12	300	0	0	0	0	0	0	0	0	Larvae
North Maluku	Morotai	2016	*Aedes aegypti*	Larva collection	Single larva method	NA	3	50	0	0	0	0	0	0	0	0	Larvae
West Java	Garut	2016	*Aedes aegypti*	Larva collection	Single larva method	20.08	17	425	0	0	0	0	0	0	0	0	Larvae
West Java	Garut	2016	*Aedes albopictus*	Larva collection	Single larva method	20.08	1	25	0	0	0	0	0	0	0	0	Larvae
West Java	Subang	2016	*Aedes aegypti*	Larva collection	Single larva method	45.21	9	225	0	0	0	0	0	0	0	0	Larvae
West Java	Subang	2016	*Aedes albopictus*	Larva collection	Single larva method	45.21	2	50	0	0	0	0	0	0	0	0	Larvae
West Java	Pangandaran	2016	*Aedes aegypti*	Larva collection	Single larva method	36.40	5	125	0	0	0	0	0	0	0	0	Larvae
West Java	Pangandaran	2016	*Aedes albopictus*	Larva collection	Single larva method	36.40	8	200	0	0	0	0	0	0	0	0	Larvae
Banten	Pandeglang	2016	*Aedes aegypti*	Larva collection	Single larva method	75.13	3	75	0	0	0	0	0	0	0	0	Larvae
Banten	Pandeglang	2016	*Aedes albopictus*	Larva collection	Single larva method	75.13	1	25	0	0	0	0	0	0	0	0	Larvae
Banten	Lebak	2016	*Aedes aegypti*	Larva collection	Rearing	35.10	4	100	0	0	0	0	0	0	0	0	Adults
Banten	Lebak	2016	*Aedes aegypti*	Larva collection	Single larva method	35.10	5	125	0	0	0	0	0	0	0	0	Larvae

Banten	Lebak	2016	*Aedes albopictus*	Larva collection	Rearing	35.10	26	650	0	0	0	0	0	0	0	0	Adults
Banten	Lebak	2016	*Aedes albopictus*	Larva collection	Single larva method	35.10	25	625	0	0	0	0	0	0	0	0	Larvae
East Nusa Tenggara	Belu	2016	*Aedes sp.*	Larva collection	Single larva method	16.3	17	425	0	0	0	0	0	0	0	0	Larvae
East Nusa Tenggara	Belu	2016	*Aedes aegypti*	Larva collection	Rearing	16.3	19	475	0	0	0	0	0	0	0	0	Adults
East Nusa Tenggara	Ende	2016	*Aedes aegypti*	Larva collection	Single larva method	42.9	53	1325	0	0	0	0	0	0	0	0	Larvae
East Nusa Tenggara	Ende	2016	*Aedes aegypti*	Larva collection	Rearing	42.9	1	25	0	0	0	0	0	0	0	0	Adults
East Nusa Tenggara	Sumba	2016	*Aedes aegypti*	Larva collection	Single larva method	0	93	2325	0	0	0	0	0	0	0	0	Larvae
							1778	44675	17	44	20	1	8	1	0	1	

Dear Editor,

We are very pleased to submit to Parasites & Vectors a manuscript titled "Assessment of mosquito collection methods for dengue surveillance" and coauthored by Triwibowo Ambar Garjito, Lulus Susanti, Mujiyono Mujiyono, Mega Tyas Prihatin, Dwi Susilo, Sidiq Setyo Nugroho, Mujiyanto Mujiyanto, Raden Ajeng Wigati, Tri Baskoro Tunggul Satoto, Sylvie Manguin, Laurent Gavotte, and Roger Frutos.

In the absence of commercialized vaccines and of any medical treatment, the management of dengue relies only on mosquito control and on prevention. Finding efficient and reliable descriptors for assessing the risk of dengue outbreaks is thus a priority in all dengue-endemic countries. The main tools currently in use for assessing this risk of dengue outbreak is the *Stegomiya* indices. It was previously demonstrated that there was no correlation between the *Stegomiya* indices and the incidence of dengue. The development of entomological indices other than the *Stegomiya* indices could then be envisioned. However, as entomological indices, they will still be based on the capture of mosquitoes regardless of the calculation model applied. This work, which is to our knowledge the most extensive one with 44,675 mosquitoes collected in 39 different sampling locations over Indonesia in a short period of two months, was done to assess the different methods of mosquito collection under real conditions. The main conclusion of this work is that no correlation could be found between any methods, the incidence of dengue and the presence of dengue in mosquitoes. The development of descriptors other than entomological ones should be considered.

We hope that this manuscript will meet all standards for publication in Parasites & Vectors.

With best regards

On behalf of all authors

Triwibowo Ambar Garjito

Article 6.

Stegomyia Indices and Risk of Dengue Transmission: A Lack of Correlation

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Dengue is present in 128 countries worldwide and is still expanding. There is currently no treatment or universally approved vaccine available. Therefore, prevention and control of mosquito vectors remain the most efficient ways of managing the risk of dengue outbreaks. The *Stegomyia* indices have been developed as quantitative indicators of the risk of dengue outbreaks. However, conflictual data are circulating about their reliability. We report in this article the first extensive study on *Stegomyia* indices, covering 78 locations of differing environmental and socio-economic conditions, climate, and population density across Indonesia, from West Sumatra to Papua. A total of 65,876 mosquito larvae and pupae were collected for the study. A correlation was found between incidence and human population density. No correlation was found between the incidence of dengue and the *Stegomyia* indices.

Keywords: *Stegomyia* indices, dengue, incidence, mosquito-borne disease, *Aedes aegypti*, *Aedes albopictus*

INTRODUCTION

Dengue is one of the most widespread mosquito-borne arbovirus disease worldwide. Dengue viruses are present in 128 countries worldwide with major public health, social and economic consequences (1–7). Dengue is a complex disease with a wide spectrum of clinical symptoms, ranging from asymptomatic to fatal, which is often unrecognized or misdiagnosed and confused with other fever-causing tropical diseases (8). The World Health Organization (WHO) estimates that about 390 million dengue infections occur annually, with 96 million clinical manifestation and 500,000 hospitalization (9). At least 2.5% of these hospitalizations result in death and almost half of the global world population is at risk of dengue infection (9). Southeast Asia is the most impacted region and displays the highest incidence of dengue worldwide with all four dengue serotypes circulating in most countries (1, 10).

Indonesia displays the highest dengue burden in Southeast Asia (11). First described in Jakarta and Surabaya in 1968, dengue expanded in all provinces and has become a major national health priority. The incidence of dengue has increased significantly over the past 47 years from 0.05/100,000 in 1968 to 50.75/100,000 in 2015 (12, 13). Indonesia is a hyperendemic country with

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all four dengue virus serotypes (DENV1 to DENV4) circulating. In 2015, the dengue endemic areas included 412 districts/municipalities out of a total of 497 (82.9%). Dengue is spreading in all human dwellings from large urban areas to small rural villages (11–15).

Dengue viruses (DENV) are mainly transmitted to humans by two species of *Aedes* mosquitoes, i.e., *Aedes aegypti* and *Aedes albopictus*. *Ae. aegypti* is the main dengue vector, highly anthropophilic, and well-adapted to urban life. It feeds mostly at daytime with a multiple host blood meal-seeking behavior, but can also bite at night depending on light conditions. *Ae. aegypti* breeds in a variety of artificial habitats with clear stagnant water (16). The secondary vector, *Ae. albopictus*, also known as Tiger mosquito, bites at daytime too but hosts also include animals such as amphibians, reptiles, birds and mammals. *Ae. albopictus* breeds in a wide variety of artificial and natural habitats such as tires, bamboo stumps, tree holes, etc. (17). In Indonesia, large-scale migrations from rural to urban areas over the past three decades have created slum settlements with inadequate water and sanitation facilities and poor waste management, leading to the emergence of many new breeding sites for both *Ae. aegypti* and *Ae. albopictus* (13, 14). The Indonesian climate with favorable tropical rainfall, temperature and humidity also facilitates the development of additional *Aedes* breeding sites (16). This situation has strongly increased the risk of dengue transmission in suburban areas.

The risk of dengue transmission is influenced by various factors, including trade of goods and human mobility, population density, urbanization, climate, presence of invasive populations of *Aedes* vectors and pathogens, virus evolution, density of competent vectors, and ineffective vector control strategies (18, 19). While an efficient vaccine is still under research, entomological surveillance and vector control remain the only ways to prevent and control dengue transmission (19–21). Therefore, WHO recommends a routine vector surveillance to provide a quantifiable measurement of dengue vector fluctuations and their geographical distribution for assessing the risk of outbreaks and to determine vector control interventions (2, 22). These indicators have been based on the traditional *Stegomyia* indices (HI, House Index; CI, Container Index; BI, Breteau Index) (23) to which a national Free Larva Index (FLI) was added in Indonesia. These larval and pupal indices remain the most used parameters to measure vector infestation since the capture of adult mosquitoes is labor-intensive and requires access to private premises (19, 24).

Initially, the *Stegomyia* indices were proposed to prevent and predict the risk of yellow fever transmission and critical thresholds have never been determined for dengue transmission (22, 25). A House Index (HI) threshold of 1% or less, or a Breteau Index (BI) threshold of five or less have been considered to prevent dengue transmission because of similarities in the epidemiology of dengue and yellow fever viruses (18, 26, 27). Furthermore, the Pan American Health Organization (PAHO) has divided the risk factors for dengue transmission into three levels: low (HI<0.1%), medium (0.1%<HI<5%), and high (HI>5%) (28). However, the reliability and sensitivity of the *Stegomyia* indices have been questioned (2, 19, 25, 29–31).

Until now, although several studies have been published on the reliability of the *Stegomyia* indices, no comprehensive analyses have yet been conducted. Articles were either reviews covering a broad range of regions and cases or technical articles providing quantitative data but limited to specific areas (2, 19, 25, 27, 28, 32–46). We therefore developed this study to analyze the relationship between *Stegomyia* indices and actual dengue situations over a very large zone covering 78 sampling sites throughout Indonesia from Sumatra to Papua corresponding to different locations (urban/rural) and ecosystems (coastal/non-coastal). We report here a complete analysis on the two main vectors, *Ae. aegypti* and *Ae. albopictus*.

MATERIALS AND METHODS

Study Area

The study was conducted in 78 locations corresponding to 78 districts/municipalities in 26 dengue-endemic provinces in Indonesia (**Figure 1**, **Table 2**). These provinces were: Aceh, Riau, Riau Islands, West Sumatra, Jambi, Bangka Belitung, Lampung, Banten, West Java, Yogyakarta, Central Java, East Java, West Kalimantan, South Kalimantan, Central Kalimantan, East Kalimantan, Southeast Sulawesi, South Sulawesi, North Sulawesi, Central Sulawesi, Bali, West Nusa Tenggara, East Nusa Tenggara, Maluku, North Maluku, and West Papua. The mosquito collection was implemented as part of the "Rikhus Vektora" project in July–August 2016 in 48 districts/cities, the WHO project SEINO 1611945 in September–October 2016 in 12 additional city locations, and finally in 18 locations in May–July 2017 as part of the Rikhus Vektora project (**Figure 1**).

Study Design

The sampling plan was built using entomological data, dengue cases, socio-demographic and spatial data. Collections were undertaken at three time periods, July-August 2016 in 48 locations, September-October 2016 in 12 additional locations, and in May-July 2017 in 18 locations. These sampling periods correspond to rainy seasons in the respective locations. Each sampling periods was determined after the actual start of the rainy season and was initiated at least 1 month after the beginning of the rainy season. At least 100 households were taken at random in each sampling location to assess the presence of *Aedes* breeding sites. Three separate assessments were conducted at the same time. *Ae. aegypti* larvae and pupae, *Ae. albopictus* larvae and pupae, and *Ae. aegypti* + *Ae. albopictus* larvae and pupae were separately recorded in each sampling location. The *Stegomyia* indices were calculated for each sampling location for the three categories using the following formulas (23, 47, 48):

Container Index (CI): number of infected containers × 100/total number of containers

House Index (HI): number of infected houses × 100/total number of houses

Breteau Index (BI): number of positive containers/number of houses explored × 100

These indices were completed by a legal Indonesian index, the Free Larva Index (FLI) calculated according to the following formula:
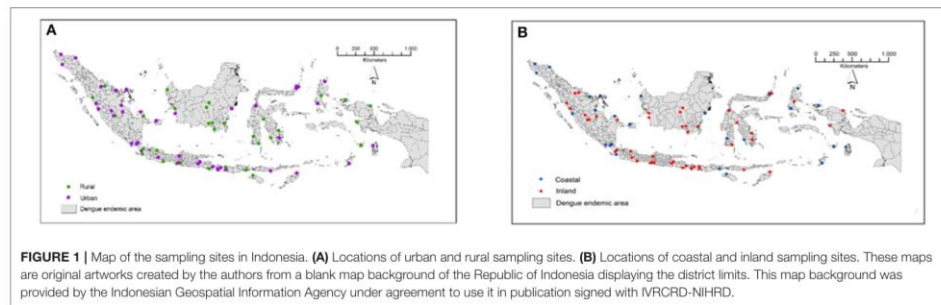
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FIGURE 1 | Map of the sampling sites in Indonesia. **(A)** Locations of urban and rural sampling sites. **(B)** Locations of coastal and inland sampling sites. These maps are original artworks created by the authors from a blank map background of the Republic of Indonesia displaying the district limits. This map background was provided by the Indonesian Geospatial Information Agency under agreement to use it in publication signed with IVRCRD-NIHRD.

FLI: number of houses without larva $\times$ 100/total number houses

The Free Larva Index (FLI) is the reverse of the House Index (HI) making these two indices strongly negatively correlated.

Entomological Data Collection

Artificial and natural water-holding containers, which were potential *Aedes* breeding sites, were sampled using standardized sampling methods (23, 47–49). All pupae and larvae from positive containers were collected in separate small ziplock plastic bags. Afterwards, all samples were transported to field laboratories and counted. Due to difficulties to identify species at the larval and pupal stages, all larvae and pupae from each container were transferred to separate individual adult cages. Collected *Aedes* larvae and pupae were placed in rearing jars filled with 150 mL of freshwater and were covered with fine gauze. All larvae were fed with fish food (TetraBits, Germany). Larvae and pupae were reared until the emergence of adults for species identification.

Sociodemographic Data Collection

The incidence, number of new dengue cases per total population for the time of the study, was considered for each community health center. Sampling locations were discriminated according to their status; i.e., urban or rural, as defined by the Ministry of Health, Republic of Indonesia, and according to the ecosystem, i.e., coastal or inland. Urban areas were defined as areas without major agricultural activity and displaying concentrations of centralized government services, social services, and economic activities. Rural areas were defined as areas having major agricultural activity, including the management of natural resources and displaying local government services, social services, and economic activities. The official discrimination between urban and rural areas is based on facilities, services, and equipment offered and not on a population density threshold. Coastal areas were terrestrial environments under marine influence whereas inland areas were far enough from the seashore to no longer be under marine influence. The number of dengue cases was taken from the national health data profile for district/city level in the time of study. The density of population (**Table 1**) in the zone of action of the health centers at the time of

study were taken from the centralized database of health centers from the Ministry of Health, Republic of Indonesia.

Data Analysis

A principal component analysis (PCA) was conducted using the incidence, the human population density and the four *Stegomyia* indices (HI, BI, CI, and FLI). The PCA analysis was performed on the totality of the 50 sampling locations where dengue cases have been reported by health centers. Three sets of analyses were performed separately for *Ae. aegypti*, *Ae. albopictus* and for the sum of *Ae. aegypti* and *Ae. albopictus* mosquitoes. The normality of the data distribution was assessed using the Kolmogorov-Smirnov normality test (50). Potential correlations between incidence and each index, and between incidence and average human densities were assessed using the Kendall τ (tau) coefficient test for rank correlation (51). This statistical test determines whether there is an ordinal association between two measured parameters. Under the null hypothesis of independence of the two datasets tested, the Kendall tau (τ) coefficient is expected to be equal to 0. Thus, a $p > 0.05$ indicates an acceptance of the null hypothesis and therefore an absence of correlation between the two datasets. The Kendall τ (tau) coefficient test for rank correlation was performed for all sites (78 sites), and only for sites were dengue cases have been recorded (50 sites). The influence of locations and ecosystems on incidence and mosquito densities was tested by Kruskal-Wallis test followed by a Siegel and Castellan *post-hoc* test for the datasets not displaying a normal distribution, and by ANOVA followed by a Bonferroni *post-hoc* test for datasets characterized by a normal distribution. All analyses were performed using Statistica v10.

RESULTS

Sampling and Data Collection

Mosquitoes were collected in a total of 78 locations out of which 46 were classified as urban and 32 as rural (**Figure 1**, **Table 2**). A total of 65,876 mosquito larvae (including 55,389 *Ae. aegypti* and 10,487 *Ae. albopictus*), were collected in the 78 sampling sites (**Table 2**). With the exception of Warsadim in West Papua

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TABLE 1 | Population density in the sampling sites.

Province	Village	Health center	Location	Ecosystem	Incidence	Population density (number of persons/km^2)
Aceh	Ujong Baroh	Johan Pahlawan	Urban	Coastal	0	1028.52
Aceh	Blok Benke	Kota Sigli	Urban	Coastal	67	2148.97
Aceh	Keude Aceh	Idi Rayeuk	Urban	Coastal	0	479.71
Riau	Selat Panjang Selatan	Alah Air	Urban	Coastal	122	669.52
Riau	Boncah Mahang	Sebangar	Urban	Inland	54	501.34
Riau	Bukit Kayu Kapur	Bukit Kayu Kapur	Rural	Inland	62	247.66
Riau Islands	Buliang	Batuaji	Urban	Inland	41	2917.02
Riau Islands	Tiban indah	Sekupang	Urban	Coastal	45	744.95
West Sumatra	Pakandangan	Enam Lingkung	Urban	Inland	100	485.43
West Sumatra	Aua Kuniang	Lembah Binuang	Rural	Inland	12	105.70
West Sumatra	Salido	Salido	Urban	Coastal	0	103.13
Jambi	Kenali Besar	Kenali Besar	Urban	Inland	91	1711.55
Jambi	Pinang Merah	Kenali Besar	Urban	Inland	91	1711.55
Jambi	Lubuk Kepayang	Air Hitam	Rural	Inland	0	24.12
Jambi	Jaya Setia	Muaro Bungo	Urban	Inland	210	1141.70
Jambi	Tungkal Harapan	Tungkal II	Urban	Coastal	142	1172.59
Bangka Belitung	Kuto Panji	Belinyu	Urban	Coastal	6	82.26
Bangka Belitung	Mangkol	Benteng	Rural	Inland	24	436.76
Bangka Belitung	Air Saga	Air Saga	Urban	Coastal	29	1033.30
Lampung	Jati Baru	Tanjung Bintang	Urban	Coastal	5	648.43
Lampung	Teluk Pandan	Hanura	Urban	Coastal	23	448.82
Lampung	Pasar Madang	Kota Agung	Urban	Coastal	60	545.46
Banten	Cipeucang	Binuangeun	Rural	Coastal	0	401.94
Banten	Cigondang	Labuan	Urban	Inland	0	3585.31
Banten	Ciomas	Padarincang	Rural	Inland	5	642.19
West Java	Tambak Dahan	Tambak Dahan	Rural	Inland	13	827.33
West Java	Mekargalih	Tarogong	Urban	Coastal	0	1630.22
West Java	Ciliang	Parigi	Rural	Inland	0	454.20
Yogyakarta	Kedungpoh	Nglipar II	Rural	Inland	152	401.93
Yogyakarta	Bugel	Panjatan II	Rural	Inland	151	727.26
Yogyakarta	Bangunharjo	Sewon II	Urban	Inland	360	1953.24
Central Java	Sendang Mulyo	Kedung Mundu	Urban	Inland	64	9272.12
Central Java	Sendang Guwo	Kedung Mundu	Urban	Inland	64	9272.12
East Java	Seneporejo	Silir Agung	Rural	Inland	35	920.06
East Java	Sumber Dawesari	Grati	Urban	Inland	24	1523.13
East Java	Jero	Tumpang	Urban	Inland	217	1101.93
West Kalimantan	Tengah	Kedondong	Urban	Inland	0	223.53
West Kalimantan	Pangkalan Buton	Sukadana	Rural	Inland	6	183.96
West Kalimantan	Twi Mentibar	Selakau	Rural	Coastal	0	90.74
South Kalimantan	Pabahanan	Pabahanan	Rural	Inland	31	101.27
South Kalimantan	Sungai Kupang	Sungai Kupang	Rural	Inland	14	834.65
South Kalimantan	Sumber Rahayu	Wanaraya	Rural	Inland	124	70.56
Central Kalimantan	Tampang Tumbang Anjir	Anjir	Rural	Inland	0	32.28
Central Kalimantan	Tumbang Masao	Tumbang Kunyi	Rural	Inland	0	2.87
Central Kalimantan	Kantan Muara	Pangkoh	Rural	Inland	0	39.71
East Kalimantan	Sepinggan Baru 31	Sepinggan Baru	Urban	Coastal	562	2699.96
East Kalimantan	Sepinggan Baru 59	Sepinggan Baru	Urban	Coastal	562	2699.96
South East Sulawesi	Bajo Indah	Soropia	Rural	Inland	0	1355.43
South East Sulawesi	Laea	Poleyang Selatan	Rural	Coastal	431	77.51
South East Sulawesi	Raha 3	Katobu	Urban	Inland	0	2245.73

(Continued)

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TABLE 1 | Continued

Province	Village	Health center	Location	Ecosystem	Incidence	Population density (number of persons/km²)
South Sulawesi	Lestari	Tomoni	Rural	Inland	458	101.93
South Sulawesi	Palambarae	Bontonyeleng	Rural	Inland	72	536.27
South Sulawesi	Bawasalo	Segeri	Rural	Coastal	722	560.74
North Sulawesi	Bahu	Bahu	Urban	Inland	170	1576.64
North Sulawesi	Manembo Nembo Atas	Sagerat	Urban	Inland	35	905.92
North Sulawesi	Leilem	Sonder	Urban	Coastal	0	318.76
Central Sulawesi	Balaroa	Sangurara	Urban	Inland	200	3935.79
Central Sulawesi	Ujuna	Kamonji	Urban	Inland	191	5131.52
Bali	Kaliakah	Negara	Urban	Inland	325	518.09
Bali	Padang Kerta	Karangasem	Urban	Inland	1,087	1116.93
Bali	Buduk	Mengwi	Urban	Inland	1,036	2111.19
Bali	Sesetan	Denpasar Selatan I	Urban	Coastal	924	5265.03
Bali	Panjer	Denpasar Selatan I	Urban	Inland	924	5265.03
West Nusa Tenggara	Kramajaya	Narmada	Urban	Inland	17	817.78
West Nusa Tenggara	Pela	Monta	Rural	Coastal	0	149.72
West Nusa Tenggara	Medana	Tanjung	Rural	Inland	0	416.65
East Nusa Tenggara	Bairafu	Umanen	Urban	Inland	4	1486.56
East Nusa Tenggara	Nanganesa	Ngalupolo	Urban	Inland	0	140.72
East Nusa Tenggara	Wendewa Utara	Mamboro	Rural	Coastal	0	43.34
Maluku	Sifnana	Saumlaki	Urban	Coastal	0	262.43
Maluku	Siwalima	Siwalima	Urban	Coastal	0	173.50
Maluku	Faan	Watdek	Rural	Coastal	0	79.37
North Maluku	Labuha	Labuha	Urban	Coastal	0	143.68
North Maluku	Norweda	Weda	Rural	Inland	0	39.25
North Maluku	Nakamura	Daruba	Urban	Coastal	0	66.44
West Papua	Wagom Utara	Sekban	Rural	Inland	0	163.39
West Papua	Prafi Mulia	Prafi	Rural	Inland	6	50.90
West Papua	Warsadim	Warsadim	Rural	Coastal	0	3.55

where only *Ae. malayanensis* was found, either *Ae. aegypti* or *Ae. albopictus* or both were found in all other sampling sites. Apart from Warsadim, only one site, did not host any *Ae. aegypti*, i.e., Bugel in the Province of Yogyakarta, whereas 26 sites were free of *Ae. albopictus*. The combination of *Ae. aegypti* and *Ae. albopictus* was found in 50 sampling sites (**Table 2**). Out of the 78 health centers analyzed, 28 did not display any case of dengue during the time of the study (**Table 2**). For the 50 locations displaying dengue cases, the incidence ranged from 4 in Bairafu (East Nusa Tenggara) to 1,087 in Padang Kerta (Bali) (**Table 2**).

Data Normality

The D-statistic from Kolmogorov-Smirnov normality test for dengue incidence indicates that the data do not follow a normal distribution ($p = 0.002$; **Figure 2**). Similarly, the number of mosquito larvae caught does not follow a normal distribution for *Ae. aegypti* ($p = 0.0492$), as well as for *Ae. albopictus* ($p = 0.0023$). The sum of all *Ae. aegypti* and *Ae. albopictus* larvae was the only dataset following a normal distribution ($p = 0.0751$).

Correlation Between Dengue Infection Rates and Human Density

The PCA analysis indicated a clear correlation between dengue incidence and the human population density registered for each location (**Figure 3**). This correlation was confirmed by the Kendall rank correlation coefficients test ($\tau = 0.242$; $p = 0.0125$), indicating that the dengue incidence increased along with the human population density.

Correlation Between Dengue Infection Rates and Larvae Indices

Tests on the value of the coefficient τ (Kendall rank correlation coefficients test) for the incidence of each sampling location vs. each of the indices at the same location were systematically higher than the limit *p*-value of 0.05 indicating that the test was significant. Only places clinical dengue cases have been recorded were considered in the analysis. The null hypothesis of independence of the data was therefore accepted indicating that there was no correlation between the incidences, any of the indices (CI, HI, BI and FLI) and the number of mosquitoes in all of the 50 epidemic locations analyzed (**Table 3**). This lack of

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TABLE 2 | Entomological indices from *Aedes* larvae and pupae survey at 78 sampling sites in Indonesia.

Province	Village	Health center[a]	Location	Ecosystem	Incidence	*Aedes aegypti*					*Aedes albopictus*					*Aedes aegypti* + *Aedes albopictus*				
						Number of *Ae. aegypti*	HI	BI	CI	FLI	Number of *Ae. albopictus*	HI	B I	CI	FLI	Number of *Ae. aegypti* + *Ae. albopictus*	HI	BI	CI	FLI
Aceh	Ujong Baroh	Johan Pahlawan	Urban	Coastal	0	402	37	51	20.82	63	254	22	24	9.79	78	656	50	75	30.61	50
Aceh	Blok Benke	Kota Sigli	Urban	Coastal	67	882	31	36	15.93	69	0	0	0	0	100	882	31	36	15.93	69
Aceh	Keude Aceh	Idi Rayeuk	Urban	Coastal	0	1,315	57	74	35.41	43	74	1	1	0.48	99	1,389	58	75	35.88	42
Riau	Selat Panjang Selatan	Alah Air	Urban	Coastal	122	157	18	28	6.39	82	187	32	46	10.5	68	344	49	74	16.89	51
Riau	Boncah Mahang	Sebangar	Urban	Inland	54	74	10	11	2.56	90	311	19	27	6.29	81	385	29	38	8.86	71
Riau	Bukit Kayu Kapur	Bukit Kayu Kapur	Rural	Inland	62	146	32	42	13.13	68	475	22	26	8.13	78	621	54	68	21.25	46
Riau Islands	Buliang	Batuaji	Urban	Inland	41	1,275	15	15	2	85	0	0	0	0	100	1,275	15	15	2	85
Riau Islands	Tiban indah	Sekupang	Urban	Coastal	45	750	11	11	4.49	89	0	0	0	0	100	750	11	11	4.49	89
West Sumatra	Pakandangan	Enam Lingkung	Urban	Inland	100	909	18	21	5.66	82	1,045	38	51	13.75	62	1,954	49	72	19.41	51
West Sumatra	Aua Kuniang	Lembah Binuang	Rural	Inland	12	171	2	158	1.89	98	74	8	10	6.33	92	245	10	13	9.23	90
West Sumatra	Salido	Salido	Urban	Coastal	0	2,419	34	42	18.5	66	78	3	4	1.76	97	2,497	35	46	15.42	65
Jambi	Kenali Besar	Kenali Besar	Urban	Inland	91	900	34	51	16.45	66	0	0	0	0	100	900	34	51	16.45	66
Jambi	Pinang Merah	Kenali Besar	Urban	Inland	91	275	34	51	13.18	66	0	0	0	0	100	275	34	51	13.18	66
Jambi	Lubuk Kepayang	Air Hitam	Rural	Inland	0	80	25	34	11.15	75	185	13	19	6.23	87	265	38	53	17.38	62
Jambi	Jaya Setia	Muaro Bungo	Urban	Inland	210	234	44	68	15.77	56	0	0	0	0	100	234	44	68	15.77	56
Jambi	Tungkal Harapan	Tungkal II	Urban	Coastal	142	862	90	315	41.39	10	0	0	0	0	100	862	90	315	41.39	10
Bangka Belitung	Kuto Panji	Belinyu	Urban	Coastal	6	1,270	31	36	13.23	69	212	10	11	4.04	90	1,482	37	47	17.28	63
Bangka Belitung	Mangkol	Benteng	Rural	Inland	24	1,291	33	39	11.75	67	1,357	32	42	12.65	68	2,648	59	81	24.39	41
Bangka Belitung	Air Saga	Air Saga	Urban	Coastal	29	122	30	32	10.45	70	214	14	7.52	23	86	336	42	55	17.97	58
Lampung	Jati Baru	Tanjung Bintang	Urban	Coastal	5	20	4	4	1.98	96	134	10	12	5.94	90	154	14	16	7.92	86
Lampung	Teluk Pandan	Hanura	Urban	Coastal	23	490	46	53	22.94	54	68	3	3	1.29	97	558	47	56	24.24	53
Lampung	Pasar Madang	Kota Agung	Urban	Coastal	60	619	16	16	6.75	84	272	21	21	8.86	79	891	30	37	15.61	70
Banten	Cipeucang	Binuangeun	Rural	Coastal	0	541	39	50	25.64	61	18	4	4	2.05	96	559	42	54	27.69	58
Banten	Cigondang	Labuan	Urban	Inland	0	122	47	58	23.02	53	45	3	3	1.19	97	167	48	61	24.21	52
Banten	Ciomas	Padarincang	Rural	Inland	5	80	40	50	20.41	60	0	0	0	0	100	80	40	50	20.41	60
West Java	Tambak Dahan	Tambak Dahan	Rural	Inland	13	595	18	18	8.65	82	27	2	2	0.96	98	622	20	20	9.62	80
West Java	Mekargalih	Tarogong	Urban	Coastal	0	1,041	29	35	14.34	71	0	0	0	0	100	1,041	29	35	14.34	71
West Java	Ciliang	Parigi	Rural	Inland	0	28	4	4	1.78	96	175	10	10	4.44	90	203	12	14	6.22	88
Yogyakarta	Kedungpoh	Nglipar II	Rural	Inland	152	5	5	8	2.15	95	349	36	49	13.17	64	354	41	57	15.32	59
Yogyakarta	Bugel	Panjatan II	Rural	Inland	151	0	0	0	0	100	82	23	27	9.82	77	82	23	27	9.82	77
Yogyakarta	Bangunharjo	Sewon II	Urban	Inland	360	160	26	52	14.36	74	0	0	0	0	100	160	26	52	14.36	74

(Continued)

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TABLE 2 | Continued

Province	Village	Health center[a]	Location	Ecosystem	Incidence	Aedes aegypti					Aedes albopictus					Aedes aegypti + Aedes albopictus				
						Number of Ae. aegypti	HI	BI	CI	FLI	Number of Ae. albopictus	HI	B I	CI	FLI	Number of Ae. aegypti + Ae. albopictus	HI	BI	CI	FLI
North Sulawesi	Bahu	Bahu	Urban	Inland	170	407	13	13	7.1	87	0	0	0	0	100	407	13	13	7.1	87
North Sulawesi	Manembo Nembo Atas	Sagerat	Urban	Inland	35	224	23	28	10.18	77	30	25	29	10.18	75	254	44	57	20.73	56
North Sulawesi	Leilem	Sonder	Urban	Coastal	0	423	26	40	13.65	74	152	7	10	3.41	93	575	32	50	17.06	68
Central Sulawesi	Balaroa	Sangurara	Urban	Inland	200	950	32	52	10.55	68	0	0	0	0	100	950	32	52	10.55	68
Central Sulawesi	Ujuna	Kamonji	Urban	Inland	191	1,025	26	30	7.73	74	0	0	0	0	100	1,025	26	30	7.73	74
Bali	Kaliakah	Negara	Urban	Inland	325	68	12	17	6.29	88	37	6	8	2.96	94	105	19	25	9.26	81
Bali	Padang Kerta	Karangasem	Urban	Inland	1,087	37	15	18	8.05	85	44	20	22	9.32	80	81	27	41	17.37	73
Bali	Buduk	Mengwi	Urban	Inland	1,036	98	25	42	16.54	75	80	20	20	7.87	80	178	45	62	24.41	55
Bali	Sesetan	Denpasar Selatan I	Urban	Coastal	924	825	23	30	11.81	77	0	0	0	0	100	825	23	30	11.81	77
Bali	Panjer	Denpasar Selatan I	Urban	Inland	924	625	30	36	11.8	70	0	0	0	0	100	625	30	36	11.8	70
West Nusa Tenggara	Kramajaya	Narmada	Urban	Inland	17	126	9	9	5.59	91	55	2	2	1.24	98	181	11	11	6.83	89
West Nusa Tenggara	Pela	Monta	Rural	Coastal	0	534	26	29	11.79	74	0	0	0	0	100	534	26	29	11.79	74
West Nusa Tenggara	Medana	Tanjung	Rural	Inland	0	55	20	20	10.26	80	0	0	0	0	100	55	20	20	10.26	80
East Nusa Tenggara	Bairafu	Umanen	Urban	Inland	4	174	41	45	26.47	59	0	0	0	0	100	174	41	45	26.47	59
East Nusa Tenggara	Nanganesa	Ngalupolo	Urban	Inland	0	2,352	52	66	33.33	48	5	2	2	1.01	98	2,357	52	68	34.34	48
East Nusa Tenggara	Wendewa Utara	Mamboro	Rural	Coastal	0	2,882	63	88	45.59	37	10	1	1	0.52	99	2,892	64	89	46.11	36
Maluku	Sifnana	Saumlaki	Urban	Coastal	0	333	72	72	26.28	28	0	0	0	0	100	333	72	72	26.28	28
Maluku	Siwalima	Siwalima	Urban	Coastal	0	2,078	60	83	36.24	40	66	3	3	1.31	97	2,144	60	86	37.55	40
Maluku	Faan	Watdek	Rural	Coastal	0	5,650	81	157	35.84	19	1,095	18	31	7.08	82	6,745	91	188	42.92	9
North Maluku	Labuha	Labuha	Urban	Coastal	0	2,160	30	44	15.02	70	859	10	28	9.56	90	3,019	33	72	24.57	67
North Maluku	Norweda	Weda	Rural	Inland	0	140	4	4	1.92	96	52	1	1	0.48	99	192	5	5	2.4	95
North Maluku	Nakamura	Daruba	Urban	Coastal	0	19	2	2	1.05	98	188	24	28	14.66	76	207	26	30	15.71	74
West Papua	Wagom Utara	Sekban	Rural	Inland	0	583	77	187	33.33	23	28	20	22	3.92	80	611	77	209	37.25	23
West Papua	Prafi Mulia	Prafi	Rural	Inland	6	170	54	80	15.59	46	0	0	0	0	100	170	54	80	15.59	46
West Papua	Warsadim	Warsadim	Rural	Coastal	0	0[b]	0	0	0	100	0[b]	0	0	0	100	0[b]	0	0	0	100

HI, House Index; CI, Container Index; BI, Breteau Index; FLI, Free Larva Index.
[a]*Health Centers are Community Health Centers (CHC) or Puskesmas in Indonesian. They are government-mandated community health clinics providing healthcare for population on sub-district. These clinics are present in every sub-districts.*
[b]*All mosquitoes collected were Aedes malayanensis.*

TABLE 2 | Continued

Province	Village	Health center[a]	Location	Ecosystem	Incidence	Aedes aegypti					Aedes albopictus					Aedes aegypti + Aedes albopictus				
						Number of Ae. aegypti	HI	BI	CI	FLI	Number of Ae. albopictus	HI	BI	CI	FLI	Number of Ae. aegypti + Ae. albopictus	HI	BI	CI	FLI
North Sulawesi	Bahu	Bahu	Urban	Inland	170	407	13	13	7.1	87	0	0	0	0	100	407	13	13	7.1	87
North Sulawesi	Manembo Nembo Atas	Sagerat	Urban	Inland	35	224	23	28	10.18	77	30	25	29	10.18	75	254	44	57	20.73	56
North Sulawesi	Leilem	Sonder	Urban	Coastal	0	423	26	40	13.65	74	152	7	10	3.41	93	575	32	50	17.06	68
Central Sulawesi	Balaroa	Sangurara	Urban	Inland	200	950	32	52	10.55	68	0	0	0	0	100	950	32	52	10.55	68
Central Sulawesi	Ujuna	Kamonji	Urban	Inland	191	1,025	26	30	7.73	74	0	0	0	0	100	1,025	26	30	7.73	74
Bali	Kaliakah	Negara	Urban	Inland	325	68	12	17	6.29	88	37	6	8	2.96	94	105	19	25	9.26	81
Bali	Padang Kerta	Karangasem	Urban	Inland	1,087	37	15	18	8.05	85	44	20	22	9.32	80	81	27	41	17.37	73
Bali	Buduk	Mengwi	Urban	Inland	1,036	98	25	42	16.54	75	80	20	20	7.87	80	178	45	62	24.41	55
Bali	Sesetan	Denpasar Selatan I	Urban	Coastal	924	825	23	30	11.81	77	0	0	0	0	100	825	23	30	11.81	77
Bali	Panjer	Denpasar Selatan I	Urban	Inland	924	625	30	36	11.8	70	0	0	0	0	100	625	30	36	11.8	70
West Nusa Tenggara	Kramajaya	Narmada	Urban	Inland	17	126	9	9	5.59	91	55	2	2	1.24	98	181	11	11	6.83	89
West Nusa Tenggara	Pela	Monta	Rural	Coastal	0	534	26	29	11.79	74	0	0	0	0	100	534	26	29	11.79	74
West Nusa Tenggara	Medana	Tanjung	Rural	Inland	0	55	20	20	10.26	80	0	0	0	0	100	55	20	20	10.26	80
East Nusa Tenggara	Bairafu	Umanen	Urban	Inland	4	174	41	45	26.47	59	0	0	0	0	100	174	41	45	26.47	59
East Nusa Tenggara	Nanganesa	Ngalupolo	Urban	Inland	0	2,352	52	66	33.33	48	5	2	2	1.01	98	2,357	52	68	34.34	48
East Nusa Tenggara	Wendewa Utara	Mamboro	Rural	Coastal	0	2,882	63	88	45.59	37	10	1	1	0.52	99	2,892	64	89	46.11	36
Maluku	Sifnana	Saumlaki	Urban	Coastal	0	333	72	72	26.28	28	0	0	0	0	100	333	72	72	26.28	28
Maluku	Siwalima	Siwalima	Urban	Coastal	0	2,078	60	83	36.24	40	66	3	3	1.31	97	2,144	60	86	37.55	40
Maluku	Faan	Watdek	Rural	Coastal	0	5,650	81	157	35.84	19	1,095	18	31	7.08	82	6,745	91	188	42.92	9
North Maluku	Labuha	Labuha	Urban	Coastal	0	2,160	30	44	15.02	70	859	10	28	9.56	90	3,019	33	72	24.57	67
North Maluku	Norweda	Weda	Rural	Inland	0	140	4	4	1.92	96	52	1	1	0.48	99	192	5	5	2.4	95
North Maluku	Nakamura	Daruba	Urban	Coastal	0	19	2	2	1.05	98	188	24	28	14.66	76	207	26	30	15.71	74
West Papua	Wagom Utara	Sekban	Rural	Inland	0	583	77	187	33.33	23	28	20	22	3.92	80	611	77	209	37.25	23
West Papua	Prafi Mulia	Prafi	Rural	Inland	6	170	54	80	15.59	46	0	0	0	0	100	170	54	80	15.59	46
West Papua	Warsadim	Warsadim	Rural	Coastal	0	0[b]	0	0	0	100	0[b]	0	0	0	100	0[b]	0	0	0	100

HI, House Index; CI, Container Index; BI, Breteau Index; FLI, Free Larva Index.

[a]Health Centers are Community Health Centers (CHC) or Puskesmas in Indonesian. They are government-mandated community health clinics providing healthcare for population on sub-district. These clinics are present in every sub-districts.
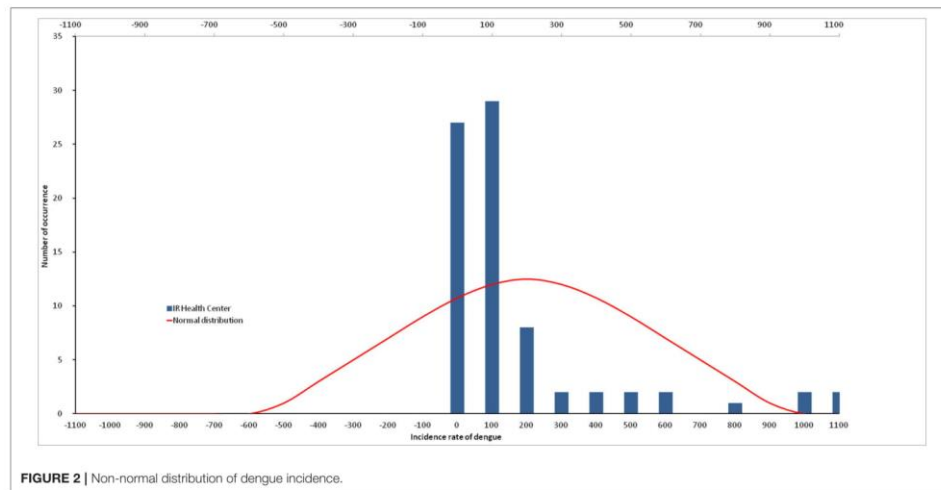
[b]All mosquitoes collected were Aedes malayanensis.

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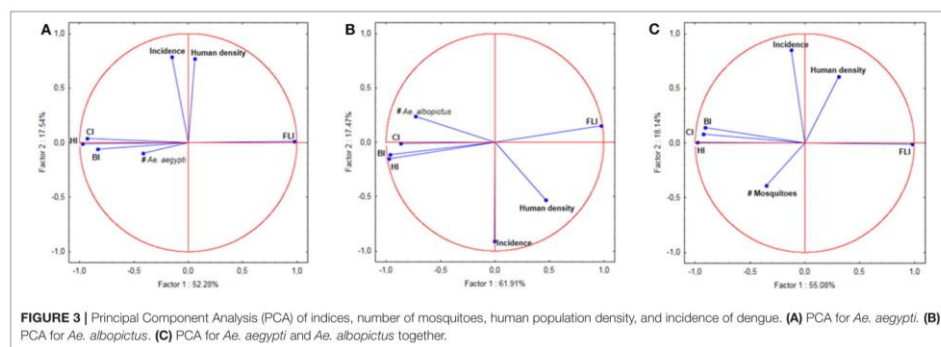**FIGURE 2** | Non-normal distribution of dengue incidence.

FIGURE 3 | Principal Component Analysis (PCA) of indices, number of mosquitoes, human population density, and incidence of dengue. **(A)** PCA for *Ae. aegypti*. **(B)** PCA for *Ae. albopictus*. **(C)** PCA for *Ae. aegypti* and *Ae. albopictus* together.

correlation was observed for *Ae. aegypti* alone, for *Ae. albopictus* alone and for the sum of *Ae. aegypti* and *Ae. albopictus* (**Table 3**). The Principal Component Analysis (PCA) displayed a very high level of explanation for the datasets tested (**Figure 3**). For *Ae. aegypti* alone, the PCA explained 69.82% of the data spread (axis 1: 52.28% and axis 2: 17.54%) (**Figure 3A**). For *Ae. albopictus* alone, the PCA explained 79.38% of the data spread (axis 1: 61.91% and axis 2: 17.47%) (**Figure 3B**). For both species, i.e., *Ae. aegypti* and *Ae. albopictus* considered together, the level of explanation of the data spread given by the PCA analysis was 73.22% (axis 1: 55.08% and axis 2: 18.14%) (**Figure 3C**). For each PCA, the same observations can be made, namely: (i) a strong autocorrelation of the different indices with each other, (ii) a correlation between the indices and the total number of mosquitoes, (iii) a correlation between dengue incidence and average human density, and finally (iv) a complete lack of correlation between dengue incidence in a study site and the *Stegomyia* indices shown by the orthogonal position observed in all PCA analyses between indices and incidence.

Influence of Locations and Ecosystems

The incidence was not significantly correlated with the different environments considered: urban vs. rural (**Figure 1A**) and coastal vs. inland (**Figure 1B**) (Kruskal-Wallis: $H = 7.72$; $p = 0.0523$). Mosquito distributions were significantly different (tested by Kruskal-Wallis non-parametric statistical test) for each type of environment for both *Ae. aegypti* ($H = 8.43$; $p = 0.038$) and *Ae. albopictus* ($H = 7.96$; $p = 0.0468$). Differences (Siegel and

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TABLE 3 | Tau (τ) and *p*-values obtained for incidence and entomological indices by Kendall rank correlation coefficients test.

Species	House Index	Breteau Index	Container Index	Free Larva Index
ALL LOCATIONS CONSIDERED				
Ae. aegypti	$\tau = -0.101$	$\tau = -0.062$	$\tau = -0.134$	$\tau = 0.101$
	$p = 0.1926$	$p = 0.4248$	$p = 0.0821$	$p = 0.1926$
Ae. albopictus	$\tau = -0.039$	$\tau = -0.056$	$\tau = -0.057$	$\tau = 0.039$
	$p = 0.6107$	$p = 0.4659$	$p = 0.4633$	$p = 0.6107$
Ae. aegypti and	$\tau = -0.085$	$\tau = -0.039$	$\tau = -0.144$	$\tau = 0.085$
Ae. albopictus	$p = 0.2731$	$p = 0.6107$	$p = 0.0506$	$p = 0.2731$
LOCATIONS WITH NO DENGUE CASES EXCLUDED				
Ae. aegypti	$\tau = 0.037$	$\tau = 0.065$	$\tau = 0.043$	$\tau = -0.037$
	$p = 0.7066$	$p = 0.5034$	$p = 0.6575$	$p = 0.7066$
Ae. albopictus	$\tau = -0.014$	$\tau = -0.023$	$\tau = -0.043$	$\tau = 0.014$
	$p = 0.8869$	$p = 0.8184$	$p = 0.6575$	$p = 0.8869$
Ae. aegypti and	$\tau = 0.043$	$\tau = 0.131$	$\tau = 0.016$	$\tau = -0.043$
Ae. albopictus	$p = 0.6575$	$p = 0.1808$	$p = 0.8737$	$p = 0.6575$

Castellan *post-hoc* test) were marginal and only appeared between urban/inland and urban/coastal for *Ae. aegypti* ($p = 0.037$) and between rural/inland and rural/coastal for *Ae. albopictus* ($p = 0.0404$). For the combination of both species, which is the only dataset in this work following a normal distribution, the ANOVA test indicated no difference between environments ($F = 2.045$; $p = 0.1149$).

DISCUSSION

Following to the use of *Stegomyia* indices to predict the risk of dengue outbreaks several articles in the literature questioned their efficiency (19, 28, 45, 46). A systematic review on the application of the *Stegomyia* indices to predict dengue outbreaks was conducted (2). Out of all the articles reviewed 15 were ranked as "weak studies" and no clear conclusion could be reached (2). Out of 13 articles directly dealing with the relationship between *Stegomyia* indices and dengue infection, 4 concluded on a correlation, 4 concluded on a lack of correlation, and 5 reported inconclusive discussions (2). More recent articles published on the subject also provided various conclusions. One article concluded on the lack of correlation (45), the second concluded on a correlation (46), and the last two were inconclusive, depending on the type of analysis performed (19, 29).

The work reported here brings explanations on the diverging conclusions reached by the previous studies. The first point to consider is that all the works previously reported on this topic were focused on a single place or a limited area. No studies were performed over a very large geographic area encompassing different local climates and environmental conditions. Therefore, each study was strongly influenced by local geographic and climatic conditions but also specific urbanization and socio-economic conditions, which could have biased the data. Furthermore, these previous studies were all independent investigations with variations in sampling schemes

and methodologies, making difficult a comparative analysis. Our study is based on a very large cross-section of locations of various sizes, with different urban environments throughout all of Indonesia. The geographic coverage of this work and the integration of a large set of data into a single analysis made data smoothing possible as well as elimination of variations due to specific environments or socio-economic conditions.

Data analysis in all previous studies utilized parametric statistics. However, as reported in this work, the data considered do not follow a normal, Gaussian distribution. Since parametric statistics are not well-suited for non-normal datasets, this could well-explain the contradictory conclusions previously reported. Consequently, we applied non-parametric methods to correct for bias. The dengue vectors are anthropophilic mosquitoes (52) and therefore the distribution of breeding sites is influenced by human societal aspects (53). The real drivers behind the distribution of *Aedes* breeding sites are demography, urbanization, and socio-economic level. This is supported by the correlation observed between the density of human populations and the incidence of dengue. These societal, sociological, and economical aspects do not follow a normal distribution and therefore the distribution of mosquitoes, thus the entomological indices, as well as the incidence of dengue do not either. Consequently, our application of non-parametric statistical analysis of the data, which to our knowledge was not done in any previous studies (2, 19, 25, 28, 30, 32–46), provides a very robust statistical conclusion strengthened by the size of the study and the multiplicity of sites and conditions.

We conclude that there is no correlation between the incidence of dengue and any of the *Stegomyia* indices. The very high level of explanation provided by the PCAs is a consequence of both the nature of the data studied and the absence of correlation between incidence and indices. Indeed, the first axis (abscissa on the graphs) explains the dispersion of the indices, which are necessarily correlated since they represent different elements of the mosquito population density in a study area. The second axis (ordered on the graphs) explains the dispersion of the incidence data. The lack of correlation between the two types of data is clearly represented by the orthogonality of the vectors of the various indices with respect to dengue incidence. None of the datasets influences the position of the other. Therefore, the data dispersion occurs in each set only, which considerably increases the explanation of the axes. This total lack of correlation is observed for both *Ae. aegypti* and *Ae. albopictus*, which eliminates any possibility of species-related interaction. This is also expected since the main drivers are linked to societal aspects and both species are anthropophilic (53).

The *Stegomyia* indices are not relevant descriptors for assessing the risk of dengue outbreak. They are not related to the vector competence. These indices are simply demographic descriptors. The higher the population, the higher the value of the descriptor. However, the main reason for this discrepancy is that they are targeting the wrong level of biological significance. The *Stegomyia* indices are targeting the species level, which is a good compromise between a reasonable work investment for collecting data and a systematic level accurate enough to avoid dispersion of data. Furthermore, the species is the widely recognized level of

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classification for the identification of living organisms. However, a species is an intellectual construction and is not biologically relevant. The relevant level of discrimination with respect to biological functions, and therefore vector competence, is the population or subspecies (54–56). A species should be regarded as a metapopulation or the combination of crossfertile genetically distinct populations displaying differing phenotypic traits (57). The vector competence of *Aedes* and other mosquitoes was shown to be related to specific populations (16, 56, 58–60) and not to the species *per se*. Targeting the species level with demographic descriptors can thus be misleading, hence the contradictory results obtained when assessing the efficiency of *Stegomyia* indices for predicting dengue outbreaks. A very high demography of a poorly vectoring population will lead to actions of prevention in the absence of risk of outbreak, whereas a low demography of a very good vectoring population would lead to a lack of action in the presence of a high risk of outbreak.

If not related to the *Stegomyia* indices, the dengue incidence is instead statistically related to the human population density. This is not really surprising since *Aedes* mosquitoes fly an average of 250 meters around their breeding site. Considering this short distance of flight, there is more chance for an infected mosquito to find a blood meal within flying distance in densely populated area than in a dispersed habitat. Other approaches than the *Stegomyia* indices, based on societal and urbanistic parameters should then be considered. The "One house/One inspector" approach recently implemented in Indonesia by the Ministry of Health is an interesting and sound alternative to the *Stegomyia* indices based on the monitoring and elimination of breeding sites at the household level (61). The philosophy of intervention developed in Indonesia is the prevention of dengue transmission through community participation. The approach implemented is the 3M approach, i.e., covering water containers (Menutup), cleaning water containers (Menguras), and burying discarded containers (Mengubur). The implementation is under the responsibility of families in each household. At least one person in each household is in charge of monitoring *Aedes* larvae in all water storage. However, to efficiently implement surveillance and risk analysis, people must be given reliable indices. It would therefore be important to communicate on the lack of reliability of the *Stegomyia* indices and to support the development of novel, more reliable, sociology-related markers, and actions taking into account the correlation between human population density and dengue incidence such as urbanism, type of housing, or socioeconomic level.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article.

AUTHOR CONTRIBUTIONS

TG participated in all part of the work. MH, R, RS, YA, and TS contributed to the conception and design of the study, and participated to the field work. J organized the field work. R, M, and WT did the field work and built the database. LG did the statistical analyses. SM did the supervision and corrections. RF did the analyses, supervision, and writing. All authors contributed to the article and approved the submitted version.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Conclusions

The study of the genetic characteristics among *Ae. aegypti* and *Ae. albopictus* populations was carried out in 43 study sites corresponding to 43 districts/municipalities in 25 dengue-endemic provinces in Indonesia. This study revealed that the *Ae. aegypti* population was homogeneous all over Indonesia. According to the COI sequences, all *Ae. aegypti* samples belong to the same maternal lineage. Variations were observed and clusters were described but they simply represent a limited polymorphism. All clusters identified correspond to co-circulating variants. The main difference is that one cluster, Cluster Aae2, comprised samples displaying a larger polymorphism. Cluster Aae1 and in particular the haplotypes H1 and H4 seemed to be populations with a very high colonizing and demographic potential. These two haplotypes represent each about 30% of the samples collected all over Indonesia. They represent indeed the very same population and the Cluster Aae1 makes up to 89% of all samples and is present everywhere in Indonesia. Other findings in this chapter are the report of what seems to be a dynamic of population replacement in Indonesia for both *Ae. aegypti* and *Ae. albopictus*. The population of Ae. *aegypti* found all over Indonesia is not only different from the populations characterized in 2013, but is also highly homogeneous with 89 % of samples showing very limited polymorphism or no polymorphism at all. The invasion of the Indonesian archipelago went very fast. The ITS2 marker, which is a nuclear DNA marker, showed a similar trend of occurrence of the same cluster all over Indonesia. The same phenomenon of population replacement in Indonesia is seen with *Ae. albopictus*. In this case, there are two populations, which do not correspond to those previously described from 2012 to 2015 (547,616). The domestication of *Ae. aegypti* and *Ae. albopictus* is a process closely linked to the development of the human society and in particular to long distance mobility, transportation of goods and international trade. To our knowledge, it is the first report of such massive and fast intraspecies replacement of existing populations in *Ae. aegypti* and *Ae. albopictus*.

The assessment of mosquito collection methods for dengue surveillance was conducted as part of the thesis to estimate the relative effectiveness of several methods for dengue vector surveillance, i.e. morning adult collection using an aspirator, pupal collection, animal baited trap, whole night collection using human landing methods, and larval collection. The implementation of the human landing method at night is

introducing a bias since the targeted *Aedes* mosquitoes are essentially diurnal. However, the Indonesian law forbids the implementation of human landing at day and the study ought to comply with the law. Furthermore, surveillance programs follow the same law and, therefore, this analysis reflects the reality. The study was conducted in 39 locations corresponding to 39 districts/municipalities in 15 dengue endemic provinces in Indonesia, from Aceh to North Maluku. A total of 44,675 mosquitoes were collected, of which, 32,525 specimens (72.8%) were *Ae. aegypti,* and 10,300 (23.1%) were *Ae. albopictus,* while 1,850 (4.1%) were undetermined mosquitoes. The highest number of captured individuals was obtained when targeting larvae. Larval collection by the single larva method was the most efficient in terms of number of individuals collected compared with rearing method, animal baited trap, human landing collection during the night and morning resting. A total of 89 pools of 25 *Aedes* specimens of the same species were positive for dengue virus. The results have also revealed that mosquito larvae were the almost exclusive source of dengue virus (93.3%), with 70.8% found the single larva method and 22.5% for the rearing method. Only 7.6% of total samples of adult collection were positive for dengue virus. Among the adult collections, 2.3% were found positive with human landing collection at the night, and 4.4% were found positive in the morning resting method. In addition, at least 76% of the dengue positive pools corresponding to *Ae. aegypti* (76.4%) comparing to *Ae. albopictus* (23.6%). However, there was no consistency in the efficiency of a given method for detecting dengue virus from on sampling site to another. In addition to the lack of correlation of the *Stegomyia* indices with the risk of dengue outbreak, there is evidence that targeting insects for assessing the risk of dengue or other arbovirus diseases is not a good approach. Therefore, there is a need for the development of a novel set of indices that can be used for efficiently managing the risk of dengue outbreaks.

Considering that natural vertical transmission may represent an important strategy to maintain the circulation of several arboviruses in the mosquito vector population, the confirmation of this phenomenon is needed and necessary to better understand the dynamics of the transmission of DENV. Although the evidence of transovarial transmission is not explained explicitly and in detail in this study, the result of analysis showed that DENV was detected in immature DENV vector mosquitoes. DENV was detected in 69 of 31,800 (0.22%) *Ae. aegypti* larvae that collected from the field. Meanwhile, DENV was also identified in 15 of 9325 (0.16%) *Ae. albopictus*

larvae. This finding of transovarial transmission has became a great concern as it demonstrated the autonomous circulation of the virus in populations of both DENV vector species, *Ae. aegypti* and *Ae. albopictus*. This evidences also the possibility of both mosquito species to play a role in the transmission dynamics of DENV.

A study related to the effectiveness of *Stegomyia* indices to predict the risk of dengue transmission was also conducted as part of the thesis based on the fact that Indonesia has been using *Stegomyia* indices for routine dengue transmission risk analysis for more than 3 decades. This study was conducted using a large sample size, consisting of 78 sampling sites of differing environmental and socio-economic conditions, climate, and population density across Indonesia, from Sumatra to Papua. To our knowledge this the largest study of that kind. A total of 65,876 mosquito larvae and pupae were collected for the study. The findings of this study have revealed that a correlation was identified between incidence and human population density, however absolutely no correlation was not found between the dengue incidence and any of the *Stegomyia* indices.

Chapter 5. The dynamic of Chikungunya virus in Indonesia

Introduction

The Chikungunya virus (CHIKV) is a mosquito-borne alphavirus that belongs to the *Togaviridae* family (639–641). In sub-saharan Africa, CHIKV is maintained in a sylvatic cycle that invoving forest-dwelling mosquitoes as disease vectors and non-human primates as reservoir hosts (484,642). In Asia, the spread of this virus is closely related to human settlement, both in urban and rural areas. CHIKV is transmitted among human through urban cycles (643–645).

Historically, the disease was first isolated from the serum of a febrile patient during an outbreak in Tanzania in the 1950s (646). Since then, CHIKV outbreaks have been reported in Africa, Asia, and Latin America between the 1960s and 2000s (647). CHIKV has become an important global public health problem worldwide after outbreaks that occured in 2004 in Kenya, Comores, and Indian ocean islands. Subsequent large CHIK outbreak was also reported in the Indian ocean basin occured in La Reunion Island in 2005 (647). At least 300,000 infected cases with an attack rate about 35% were reported in this area. Significant outbreaks also occured in Asia from 2005 to 2008, and in Italy in 2007. Subsequently, CHIKV spread to Southeast Asia, including Indonesia (482,496,499,503). The current spread of CHIKV in Europe and parts of Asia is associated with the spread of the anthropophilic *Ae. albopictus* mosquito outside Asia which is thought to have spread via global transportation and human migration (648).

In Indonesia, chikungunya was first reported in Samarinda (East Kalimantan) in 1973 (16). The first virologically confirmed chikungunya outbreak was reported in June 1982 in Jambi, Sumatra. Multiple outbreaks were reported between 1983 and 1984. However, chikungunya cases were not recorded in Indonesia for approximately 20 years until the early 2000s (35,36). The re-emerging Chikungunya was then reported in South Sumatra, Aceh and West Java in early 2001 (35,36). Two series of studies were conducted to assess the incidence of Chikungunya in Indonesia. One study took place in Bandung, West Java between 2000 and 2004 and between 2006 and 2008. These studies

have revealed the the CHIKV incidence was 10.1/1000 persons per year. The second study was conducted in three locations 2010-2011. The result showed that CHIKV incidence was 8.8/1000 person per year (649). In 2009, the highest CHIKV incidence rate recorded in Indonesia was 36.2 cases per 100,000 person per year (36). More than 83,000 cases were reported in 17 of the 34 provinces in Indonesia. Recently, in 2019, a total of 5,042 cases of CHIKV infection were reported in Indonesia (650). Despite the high number of chikungunya cases in Indonesia during a decade, comprehensive information about CHIK epidemiology, particularly the dynamic of the CHIKV genotypes and the diversity of CHIK vector is very limited. A better understanding of this dynamic is needed to identify the disease transmission pattern and to support appropriate prevention and control measures. To address this need, we conducted a study of the dynamic of CHIKV obtained from field-collected *Aedes* spp. in Indonesia, and from human samples collected during the chikungunya outbreak in Magelang, Central Java in 2014. These viruses were compared to previous human samples sequences from Indonesia present in Genbank.

Article displayed in Chapter 5

Article 7. Anggraeni YM, Garjito TA, Prihatin MT, Handayani SW, Kusumaningtyas SN, Yanti AO, Hidajat MC, Prastowo D, Satoto TBT, Manguin S, Gavotte L, Powers A, Frutos R. Fast expansion of the Asian-Pasific genotype of the Chikungunya virus in Indonesia. **(In preparation for submission to Frontiers in Microbiology)**

Article 7.

Fast expansion of the Asian-Pacific genotype of the Chikungunya virus in Indonesia

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Author contribution statement

Keywords

Abstract

Word count: 192

Chikungunya is repeatedly affecting Indonesia through successive outbreaks. The Asian genotype has been present in Asia since the late 1950s while the ECSA (East/Central/South Africa) genotype invaded Asia in 2005. In order to determine which genotype is circulating today in Indonesia, mosquitoes were collected in 28 different sites from 12 Indonesian provinces. The E1 subunit of the chikungunya virus (CHIKV) envelop gene (E1) was sequenced and mosquitoes were genotyped using the mitochondrial cox1 gene. Chikungunya viruses were found in Aedes aegypti, Aedes albopictus and Aedes butleri. These viruses, like all Indonesian CHIKV since 2000, belonged to an Asian-Pacific genotype, which also comprises the Yap isolates and viruses having emerged in Polynesia and the Caribbean. They differ from the CHIKV of the Asian genotype found earlier in Indonesia indicating a replacement. The collected Aedes aegypti mosquitoes all belonged to the same population, whereas the CHIKV-positive and CHIKV-negative Ae. albopictus mosquitoes belonged to separate clusters. However, the Ae. albopictus sample size is too small to reach a definitive conclusion. The Asian-Pacific genotype of the CHIKV populations in Indonesia found in this work raises the question of the mechanisms behind this fast and massive replacement.

Contribution to the field

This work is addressing the current chikungunya situation in Indonesia, a mosquito-borne disease repeatedly affecting this country. While two genotypes of chikungunya virus (CHIKV) are known to circulate in Asia, including the Asian genotype present since the late 1950s and the ECSA IOL genotype that invaded Asia in 2005, it was not clear which one was present in Indonesia. In order to determine which genotype is circulating today in Indonesia, mosquitoes were collected in 28 different sites from 12 Indonesian provinces to search for CHIKV. The E1 subunit of the CHIKV envelop gene (E1) was sequenced and mosquitoes were genotyped using the mitochondrial cox1 gene. CHIKVs were found in three vector species such as Aedes aegypti, Aedes albopictus and Aedes butleri, and compared to sequences in Genbank. Viruses up to 1985 belonged to the Asian genotype. CHIKVs collected after 2000 belong to a different genotype derived from the Asian one, which we propose to name the Asian-Pacific genotype. This Asian-Pacific genotype also comprises the Yap isolates and viruses having emerged in Polynesia and the Caribbean. This Asian-Pacific genotype has a strong invasive potential and its massive presence, replacing the previous Asian genotype, is an important information for better understanding the current worldwide dynamic of chikungunya, which was thought to be dominated by the ECSA genotype.

Ethics statements

Studies involving animal subjects
Generated Statement: No animal studies are presented in this manuscript.

Studies involving human subjects
Generated Statement: The studies involving human participants were reviewed and approved by Health research ethics committee, national institute of health research and development (HREC-NIHRD), Ministry of Health of Republic of Indonesia. The patients/participants provided their written informed consent to participate in this study.

Inclusion of identifiable human data
Generated Statement: No potentially identifiable human images or data is presented in this study.

Data availability statement

Fast expansion of the Asian-Pacific genotype of the Chikungunya virus in Indonesia

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24 **Running title:** Chikungunya replacement in Indonesia

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26 **Keywords:** Chikungunya, *Aedes aegypti, Aedes albopictus*, Genotyping, Indonesia

27

1

28 **Abstract**

29

30   Chikungunya is repeatedly affecting Indonesia through successive outbreaks. The
31 Asian genotype has been present in Asia since the late 1950s while the ECSA
32 (East/Central/South Africa) genotype invaded Asia in 2005. In order to determine
33 which genotype is circulating today in Indonesia, mosquitoes were collected in 28
34 different sites from 12 Indonesian provinces. The E1 subunit of the chikungunya virus
35 (CHIKV) envelop gene (E1) was sequenced and mosquitoes were genotyped using the
36 mitochondrial *cox*1 gene. Chikungunya viruses were found in *Aedes aegypti, Aedes*
37 *albopictus* and *Aedes butleri*. These viruses, like all Indonesian CHIKV since 2000,
38 belonged to an Asian-Pacific genotype, which also comprises the Yap isolates and
39 viruses having emerged in Polynesia and the Caribbean. They differ from the CHIKV
40 of the Asian genotype found earlier in Indonesia indicating a replacement. The collected
41 *Aedes aegypti* mosquitoes all belonged to the same population, whereas the CHIKV-
42 positive and CHIKV-negative *Ae. albopictus* mosquitoes belonged to separate clusters.
43 However, the *Ae. albopictus* sample size is too small to reach a definitive conclusion.
44 The Asian-Pacific genotype of the CHIKV populations in Indonesia found in this work
45 raises the question of the mechanisms behind this fast and massive replacement.

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265

47 **Introduction**

48

49   Chikungunya virus (CHIKV) is an arbovirus, member of the genus *Alphavirus*

50 in the family *Togaviridae*. CHIKV is a positive-strand RNA virus, 60-70 nm in

51 diameter, spherical, and enveloped. The genome length is ca.12 kb, capped in 5', with

52 a polyA tail in the 3' end. The genome has two open reading frames encoding two

53 polyproteins: non-structural polyproteins and structural polyproteins. The polyproteins

54 can be cleaved by proteases into four non-structural proteins: nsP1, nsP2, nsP3, nsP4;

55 and five structural proteins (C, E3, E2, 6K, E1) (Higashi et al., 1967; Simizu et al.,

56 1984; Powers et al., 2001; Khan et al., 2002). CHIKV was isolated for the first time in

57 Central Africa, in the Eastern part of the current Democratic Republic of Congo in 1958

58 (Osterrieth and Blanes-Ridaura, 1960, Osterrieth et al., 1961) and was introduced in

59 Asia through two separate waves. The first wave corresponded to the initial

60 diversification in three main genotypes: Asian, West African, and East/Central/South

61 African (ECSA) (Caglioti et al., 2013; Hammon et al., 1960; Powers et al., 2000; Presti

62 et al., 2014). Chikungunya was for the first time reported in Asia, specifically in

63 Thailand, in 1960 (Hammon et al., 1960). The second wave corresponded to a more

64 recent expansion of the re-emerging ECSA A226V mutant, also known as the Indian

65 Ocean Lineage or IOL, which evolved from the ECSA genotype in Kenya in 2004

66 (Tsetsarkin et al., 2011; Coffey et al.; 2014). This re-emerging CHIKV, more affine for

67 *Aedes albopictus* (Tsetsarkin et al., 2007; Tsetsarkin and Weaver, 2011), became even

68 more transmissible and virulent after additional mutations in Kerala, India (Tsetsarkin

69 and Weaver, 2011; Agarwal et al., 2016). It spread east to cover continental Southeast

70 Asia (Rianthavorn et al., 2010; Tsetsarkin et al., 2011; Duong et al., 2012; Sam et al.,

71 2009). Currently, these two genotypes, ECSA and IOL, circulate in many Asian

72 countries (Sam et al., 2012; Chen et al., 2016; Sam, 2018). We assessed in this work

73 the origin of the CHIKV and vectors present in several Indonesian islands.

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75 **Materials and methods**

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77 **Sampling sites.** During 2016-2017, adults and larvae of *Ae. aegypti* and *Ae. albopictus*
78 mosquitoes were collected from the field as previously described (IVRCRD, 2016) in
79 28 locations in 12 different Indonesian provinces: Riau, Riau Islands, Banten,
80 Yogyakarta, Central Kalimantan, East Kalimantan, South Sulawesi, Southeast
81 Sulawesi, Central Sulawesi, Maluku, North Maluku, and West Papua (Table 1,
82 Supplementary Table 1, Figure 1). Three additional virus samples were obtained from
83 the National Chikungunya Survey Program after a chikungunya outbreak in Magelang
84 (Central Java) in 2014 (Table 1).

85

86 **Mosquito collection.** Collected larvae were reared until emergence of adults and then
87 identified using standard taxonomic keys for *Stegomyia* (Rueda, 2004). Adult
88 mosquitoes were immediately identified following the same method. Samples were
89 segregated according to locality and date. Adult mosquitoes were transported on cold
90 chain and stored in pools of up to 25 individuals in 1.5-ml microtubes containing 250
91 µl of RNA later (Ambion-Thermo Fisher Scientific, Waltham, USA) and stored at -
92 80°C until further analysis.

93

94 **Chikungunya virus detection.** Chikungunya virus detection was carried out by one-
95 step RT-PCR, selective for the E1 gene as previously described (Gopal, 2016). Excised
96 head and thorax of each mosquito were homogenized in a sterile homogenizer and
97 RNAs were extracted by the silica-based methods (RNA-easy minikit, Qiagen, Hilden,
98 Germany). A single-step RT-PCR was performed using consensus primers (CHIK1: 5'-
99 ACC GGC GTC TAC CCA TTC ATG T-3'; CHIK2: 5'GGG CGG GTA GTC CAT
100 GTT GTA GA-3'). Master mix was prepared using Superscript III on step RT-PCR
101 with platinum *Taq* DNA polymerase (Invitrogen, Life Technologies, Carlsbad, USA).
102 The products were electrophoresed in alternate lanes in 2% agarose gels. Amplicons,
103 330-bp in size, were purified using Applied Biosystems ExoSAP-IT™ (Thermo Fisher
104 Scientific, Vilnius, Lithuania). Whole DNA from each mosquito was individually

4

105   extracted using a DNeasy® Blood & Tissue Kit (Qiagen, Hilden, Germany) according

106   to the manufacturer's standard protocol.

107

108   **Mosquito genotyping.** The amplification of the *cox*1 gene was conducted using the

109   primers CI-N-2087 (5'-AAT TTC GGT CAG TTA ATA ATA TAG-3') and TY-J-1460

110   (5'-TAC AAT TTA TCG CCT AAA CTT CAG CC-3') as previously described

111   (Rueanghiran et al., 2011). PCR reactions were carried out using the GoTaq® Green

112   Master Mix (Promega, Madison, WI, USA). The conditions for PCR amplification of

113   the *cox*1 gene were as follows: 1 cycle at 94 °C for 1 min for initial denaturation,

114   followed by five cycles of 94 °C for 30 s, 45 °C for 40 s and 72 °C for 1 min. This was

115   then followed by 35 cycles of 94 °C for 30 s, 44 °C for 40 s and 72 °C for 1 min, and

116   by a final extension step at 72 °C for 10 min (Rueanghiran et al., 2011).

117

118   **DNA Sequencing.** Cycle sequencing was performed using the primers listed above and

119   an Applied Biosystems BigDye™ Terminator v.3.1 Cycle Sequencing Kit (Life

120   Technologies Cooperation, Austin, TX, USA). To remove unincorporated BigDye®

121   terminators and salts, cycle sequencing products were purified using a BigDye®

122   Xterminator Purification Kit (Life technologies, Bedford, MA, USA). Sequence data

123   were obtained using a DNA sequencer (Applied Biosystems® 3500 Genetic Analyzer)

124   and analyzed using the Sequencing Analysis 6 program (Applied Biosystems). All

125   sequences have been deposited in Genbank under the accession numbers MW265951

126   to MW265970, MW270068 to MW270085 and MW270131 to MW270147.

127   **Phylogenetic analyses.** Sequences were aligned using the SeaView package (Gouy et

128   al., 2010). Multiple alignments and phylogenetic analyses were performed using the

129   SeaView package (Gouy et al., 2010). The *Aedes cox*1 gene tree was built using the

130   maximum likelihood method under the GTR+G model with 1,000 bootstrap repeats.

131   The CHIKV E1 gene tree was built using the maximum likelihood method under the

132   GTR model with 1,000 bootstrap repeats. Proteins trees were built using the maximum

133   likelihood method under the LG model with 500 bootstrap repeats.

134

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135

136 **Results**

137

138   **Mosquito sampling and chikungunya virus detection.** Mosquito collections were
139 conducted in 28 different locations in 12 provinces and a total of 15,197 mosquitoes,
140 11,776 *Ae. aegypti* and 3,421 *Ae. albopictus*, were collected (Supplementary Table 1).
141 Other *Aedes* species were found, i.e. *Aedes butleri, Aedes lineatopennis, Aedes*
142 *parasimilis, Aedes vexans, Aedes* sp. (*Verallina* sp.), *Aedes amesti, Aedes*
143 *andamanensis, Aedes aurantius, Aedes poicilius, Aedes flavipennis, Aedes*
144 *quadrifolium, Aedes longirostris* and *Aedes obsletus*. However, with the exception of
145 one *Ae. butleri* individual from Bengkalis in the province of Riau, all were negative for
146 CHIKV (Supplementary Table 1). *Aedes aegypti* was more frequent than *Ae. albopictus*
147 in 17 locations, whereas *Ae. albopictus* was more frequent in 6 sampling sites
148 (Supplementary Table 1). Only one place, Kulon Progo in Yogyakarta province, did
149 not yield any *Ae. aegypti* sample and only one place, Bombana in Southeast Sulawesi,
150 did not yield any *Ae. albopictus* sample (Supplementary Table 1). Mosquitoes were
151 pooled by species and location up to 25 individuals and CHIKV was found in 25 *Ae.*
152 *aegypti*, 8 *Ae. albopictus* and 1 *Ae. butleri* pools. A total of 37 mosquitoes from these
153 positive pools were found infected with CHIKV, including 28 *Ae. aegypti*, 8 *Ae.*
154 *albopictus*, and 1 *Ae. butleri* (Supplementary Table 1). The infection rate was 0.24%
155 and 0.23% for *Ae. aegypti* and *Ae. albopictus*, respectively.

156

157   ***Cox*1 phylogeny of *Aedes aegypti* and *Aedes albopictus* mosquitoes.** The *cox*1 genes
158 of 29 *Ae. aegypti* samples (14 CHIKV-positive and 15 CHIKV-negative) and of 6 *Ae.*
159 *albopictus* mosquitoes (3 CHIKV-positive and 3 CHIKV-negative) were sequenced
160 (Table 1). *The cox*1 phylogenetic tree of *Ae. aegypti* mosquitoes showed a monophyletic
161 topology with limited structuration (Figure 2). The CHIKV-positive sample
162 ktg_H05_Aae from Pulang Pisau in Central Kalimantan branched separately with a
163 bootstrap of 70. The samples mlk48_Aae and mlk654 from Maluku clustered separately
164 with a bootstrap of 57. Finally, a group comprising the CHIKV-negative samples
165 mlk73_Aae, 11_Aae and the CHIKV-positive mosquitoes 3_Aae, 6_Aae, 10_Aae,

6

166   15_Aae, 37_Aae, 9-18_Aae and 13-11_Aae separated with a bootstrap value of 63

167   (Figure 2). With the exception of 11_Aae from Banten (West Java), all other samples

168   from this last group were from Eastern Indonesia, i.e. Southeast Sulawesi, Maluku and

169   West Papua (Table 1). *Aedes albopictus* samples fell into two different clusters

170   depending on whether they were CHIKV-positive or -negative with a rather high

171   bootstrap value, i.e. 85 (Figure 2). However, the number of samples is too low to draw

172   any trend and reach any significant conclusion.

173

174   **Envelope gene (E1) phylogeny of the isolated chikungunya viruses.** The phylogeny

175   of the CHIKV based on the E gene indicated that all CHIKV samples from mosquitoes

176   collected in 2016-2017 for this work clustered into two related populations derived from

177   the Asian genotype (Figure 3). A first population, cluster 1, comprised the samples 9C,

178   a023, a027, a044, a070, a072, a078, a085, b084, a30 and a71. The second population,

179   cluster 2, comprised the samples 10, 12, 85, a20, a150, a024 and a050. These

180   populations were characterized by weak bootstraps (22 and 45 respectively) indicating

181   that they were not genetically distinct from each other (Figure 3). They were also

182   associated to different locations and mosquito species, including *Ae. butleri*, suggesting

183   that they did not correspond to specific geographical clusters or specific vector

184   populations. The three viruses isolated from human patients in 2014 did not correspond

185   to the same populations as the mosquito samples. The samples B14 and 8F from clinical

186   cases in Magelang, Central Java, in 2014 were identical to human isolates from totally

187   different provinces, such as Banten, Bali and Jambi from 2011, 2014 and 2015,

188   respectively (Figure 3, Table 1, Supplementary Table 2). The sample C7 from

189   Magelang, Central Java, in 2014 was related, but still different, to human samples from

190   the Eastern tip of Java (East Java) in 2011 and to the cluster 2 of mosquito samples

191   from this work (Figure 3, Supplementary Table 2). When compared to the Asian

192   genotype reference sequences, the E gene sequences from this work, and almost all

193   Indonesian CHIKV sequences from Genbank, grouped within a single Asian-Pacific

194   metapopulation comprising also CHIKV from Yap Island, French Polynesia and

195   Guadeloupe Islands in the Caribbean (Figure 3, Supplementary Table 2). The Asian

196   genotype reference sequences grouped with an isolate of 1963 from India (DQ520746),

197   an isolate of 1985 from The Philippines (HM045790) and three Indonesian isolates of

198   1983 and 1985 (HM045791, HM045797, AF192894). The ECSA IOL references

199   grouped with a CHIKV isolate of 2009 from Malaysia (HQ148971) and two 2011

200   Indonesian samples from West Kalimantan (KJ729851, KJ729852) identical to the

201   Malaysian sample suggesting the occurrence of imported cases (Figure 3,

202   Supplementary Table 2). The West African (HM045785) reference sequence of 1966

203   branched as expected in an ancestral position (Figure 3, Supplementary Table 2). The

204   protein tree did not show the same topology (Figure 4). All Asian-Pacific samples,

205   including the isolates from Guadeloupe and Yap Islands were strictly identical (Figure

206   4) and characterized by an alanine (A) in position 145 (Figure 5). From this Asian-

207   Pacific group, only the samples from French Polynesia (MF696160) and three

208   Indonesian samples (KC879569, EU192143, KX097982) from Bandung in 2002,

209   Indonesia-Taiwan introduction in 2007 and Jambi in 2015, respectively, differed by a

210   single amino acid replacement but outside the 145 position (Figure 4). Although the

211   West African reference branched in an ancestral position it was closer to the Asian-

212   Pacific genotype than to the Asian and ECSA genotypes. This isolate displayed the

213   alanine in position 145 as the Asian-Pacific isolates but differed by a single amino acid

214   change at position 162 (I>V) (Figure 5). The Asian genotype isolates, which grouped

215   separately, were characterized by a serine (S) in position 145 with the exception of the

216   1963 Indian human isolate AF192901, which displayed a tyrosine (Y) at this position.

217   The ECSA IOL isolates, which also made a separate group, all displayed a threonine

218   (T) at position 145 (Figure 4).

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271

219   **Discussion**

220

221   The CHIKV is a mosquito-borne arbovirus, which has been introduced in Asia
222 through two different waves. The first wave corresponded in the late 1950s to the
223 deployment of the ECSA genotype (East/Central/South Africa), which gave rise to the
224 Asian genotype (Hammon et al., 1960). The second wave was also associated to the
225 ECSA genotype, which in 2004 yielded the *Ae. albopictus*-affine A226V mutant, also
226 known as the Indian Ocean Lineage or IOL, which invaded the Indian Ocean in 2005
227 before spreading to India, Thailand, Cambodia, Singapore and Malaysia (Rianthavorn
228 et al.,2010; Tsetsarkin et al., 2011; Duong et al., 2012; Sam et al., 2009; Hapuarachchi
229 et al, 2010). However, the dynamic of CHIKV was not clearly established in Indonesia,
230 which has been exposed to several waves of chikungunya outbreaks over the past
231 decades (Harapan et al., 2019; MoH Indonesia, 2007). This work brings some
232 clarification on this dynamic.

233

234   The first conclusion is that a replacement of CHIKV strains occurred in
235 Indonesia between 1985 and 2000. Unfortunately, there is no sequence available from
236 this period to narrow down the time of the replacement and try to identify the causes.
237 No chikungunya cases were reported in Indonesia between 1985 and 2000. Until 1985,
238 CHIKV found in Indonesia belonged to the Asian genotype, which initially diverged
239 from the ECSA genotype in the 1950s-1960s. This Asian genotype was present in
240 several Asian countries. However, after 2000 another genotype was present in
241 Indonesia, which was found all over the country until now. This genotype expanded in
242 the Caribbean, South America, and Polynesia in 2013-2014, starting a real pandemic
243 (Leparc-Goffart et al., 2014; Lanciotti and Valadere, 2014, Freitas et al., 2018; Diaz-
244 Quinonez et al., 2016; Lanciotti and Lambert, 2016; Wimalasiri-Yapa et al., 2019,
245 Aubry et al., 2015, Nanh et al., 2014). The Caribbean genotype was found closer to the
246 Yap Island isolates (Lanciotti and Valadere, 2014). The contamination on French
247 Polynesia was traced back to the Caribbean (Nhan et al., 2014) indicating a worldwide
248 human-based mobility. These massive Caribbean, South American and Polynesian
249 epidemics were shown to have been caused by an Asian genotype (Lanciotti and

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250   Valadere, 2014; Nhan et al., 2014). However, it is not the Asian genotype present since
251   the 1960s, which was responsible, but instead a distinct genotype derived from the
252   Asian one that was already present in Indonesia, and perhaps other Asian-Pacific
253   countries, long before the 2013 Caribbean and Polynesian outbreak. It is not clear where
254   this genotype appeared at the first place, in Yap Island or Micronesia, in the Pacific area
255   or in Asia. We therefore propose to name it the Asian-Pacific genotype to distinguish it
256   from the Asian genotype.

257

258   This Asian-Pacific genotype displays traits different than those of the Asian one.
259   Unlike the Asian genotype, which is polymorphic, the Asian-Pacific genotype is
260   homogeneous. This is particularly visible with the amino acid 145, which is a conserved
261   alanine (A) in all Asian-Pacific samples, whereas it can be either a serine (S) or a
262   tyrosine (Y) in the Asian genotype samples. This position 145 in the E1 envelop protein
263   is a signature position also differentiating the ECSA IOL genotype, which displays a
264   threonine (T). The significance of this variation of amino acids at position 145 suggests
265   the existence of a specific selective pressure, although its nature is unknown. It does
266   not seem to be vector-related like the A226V mutation conferring a higher affinity to
267   *Ae. albopictus* (Tsetsarkin et al., 2007; Tsetsarkin and Weaver, 2011) since it is found
268   both in *Ae. aegypti* and *Ae. albopictus,* while both mosquito species show the same rate
269   of infection. The only difference between both mosquito species is a demographic
270   difference reported in the Indonesian collection sites with *Ae. aegypti* being 3.5 times
271   more frequent than *Ae. albopictus*. The infection rate being the same, there is thus no
272   visible difference in vector competence. This selective pressure might be a higher
273   transmissibility of virulence in human populations. Interestingly, the West African
274   genotype, which is ancestral to all other genotypes and branching at an ancestral
275   position, is displaying the same alanine residue at position 145 as the Asian-Pacific
276   genotype. This could be seen as a reverse mutation from the Asian and the ECSA
277   genotypes, which historically are ancestral to the Asian-Pacific genotype. What does
278   this mutation mean, in terms of mode of action and selective pressure, is not known and
279   further analyses are needed to determine the role of this mutation. Another trait is the
280   capacity of the Asian-Pacific genotype to have established all over Indonesia and have
281   replaced the former Asian genotype. This suggests a higher transmissibility rate, a trait

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273

282   perhaps linked to the S>A mutation at position 145. This is also further supported by
283   the invasive capacity of the Asian-Pacific genotype demonstrated in the Caribbean,
284   South America and Polynesia (Leparc-Goffart et al., 2014; Lanciotti and Valadere,
285   2014, Freitas et al., 2018; Diaz-Quinonez et al., 2016; Lanciotti and Lambert, 2016;
286   Wimalasiri-Yapa et al., 2019, Aubry et al., 2015, Nhan et al., 2014). The invasiveness
287   of IOL was seemingly due to a higher affinity for *Ae. albopictus* and a wide
288   transportation by this mosquito species due to international trade (Benedict et al., 2007).
289   IOL showed invasiveness in the Indian Ocean and in Continental South and Southeast
290   Asia where it replaced the initial Asian genotype (Rianthavorn et al.,2010; Tsetsarkin
291   et al., 2011; Duong et al., 2012; Sam et al., 2009; Hapuarachchi et al, 2010). Conversely,
292   the invasiveness of the Asian-Pacific genotype does not seem to be related to the
293   mosquito species since it can be transmitted by *Ae. aegypti, Ae. albopictus, Ae. hensilli,*
294   *Ae. polynesiensis* and, as shown in this work, by *Ae. butleri*, making it perhaps at higher
295   risk of global dispersion.

296

297   The diversity of vectors was also found to be very low in Indonesia. *Aedes*
298   *aegypti* samples, whether CHIKV-positive or not, belonged to the same population.
299   This suggests that in parallel to the uniformity of CHIKV in Indonesia, there might also
300   be a uniformity of *Ae. aegypti* vectors with a single population occupying the country.
301   However, the number of samples is not high enough to bring a definitive conclusion
302   and a larger genotyping study must be conducted at the scale of the whole country to
303   determine the structure of the *Ae. aegypti* populations. This is even truer for *Ae.*
304   *albopictus.* This dynamic of *Aedes* vectors is not only of importance for chikungunya
305   but also for dengue, Zika fever and other arbovirus diseases transmitted by the same
306   mosquito species.

307

308   The Asian-Pacific genotype of the CHIKV populations in Indonesia found in
309   this work raises the question of the mechanisms behind this fast and massive
310   replacement. Further studies must be conducted on the impact of the observed mutation
311   on the rate of transmissibility but also on societal aspects, which could favor the
312   expansion of the virus. Indonesia being an archipelago with no permanent contact

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313 between populations from different islands, trade and people mobility must be
314 analyzed. Although IOL invaded largely Continental South and Southeast Asia owing
315 to intensive international maritime trade, it does not seem to have invaded Indonesia.
316 Further studies are therefore needed to understand why, unlike what happened on the
317 continent, it is the Asian-Pacific genotype that expanded and is now dominating in
318 Indonesia. Beyond chikungunya, these investigations will be useful to understand the
319 dynamic of other arboviral diseases, such as dengue, which are among the most widely
320 distributed and fast-spreading diseases worldwide.

321

322

Ethics approval

324 Formal approval to use human samples for virus sequence analyses was provided by
325 the Ethical Commission Board of the NIHRD, Ministry of Health, Indonesia No.
326 LB.02.01/2/KE.645/2020.

327

Consent for publication

329 Not applicable

330

Availability of data and materials

332 Data supporting the conclusions of this article are included within the article. All
333 sequences have been deposited in Genbank under the accession numbers MW265951
334 to MW265970, MW270068 to MW270085 and MW270131 to MW270147.

335

Conflict of interest

337 The authors declare that the research was conducted in the absence of any commercial
338 or financial relationships that could be constructed as a potential conflict of interest.

339

Authors Contributions

341 YMA, TAG, MCH, MTP, DP, SWH conceived and designed the field studies. YMA,
342 TAG, MCH, MTP, AOY, KSN, DP, SWH prepared samples. YMA, MTP, TAG, and
343 KSN ran molecular analyses and laboratory experiments. TAG, LG and RF analyzed
344 the data. MA prepared the map. TAG and RF wrote the manuscript. SM and LG
345 provided critics and significant revisions to the manuscript.

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509

20

510   **Figures legends**

511

512   **Figure 1. Map of the sampling locations in Indonesia with the presence of** *Aedes*
513   *aegypti***,** *Aedes albopictus* **and** *Aedes butleri* **being CHIKV positive or negative.**

514

515   **Figure 2.** *Cox*1 **gene phylogeny of** *Aedes aegypti* **and** *Aedes albopictus* **mosquitoes**

516   The tree was built using the maximum-likelihood (ML) method under the GTR+G
517   model with 1,000 bootstrap repeats. Red: CIHKV-positive *Ae. albopictus* samples;
518   Green: CHIKV-negative *Ae. albopictus* samples; Blue: CHIKV-positive *Ae. aegypti*
519   samples; Purple: CHIKV-negative *Ae. aegypti* samples. The tree was rooted on the *cox*1
520   gene of *Culex quinquefasciatus* used as outgroup (beige).

521

522   **Figure 3. Phylogenetic analysis of the partial E1 gene of chikungunya viruses**

523   The tree was built using the maximum-likelihood (ML) method under the GTR model
524   with 1,000 bootstrap repeats. The tree was rooted on the E1 gene of the O'nyong-nyong
525   virus (HM045785) used as outgroup.

526

527   **Figure 4. Distribution tree of the partial E1 protein sequence of chikungunya**
528   **viruses**

529   The tree was built using the maximum-likelihood (ML) method under the LG model
530   with 500 bootstrap repeats. The tree was rooted on the E1 protein of the O'nyong-nyong
531   virus (HM045785) used as outgroup.

532

533   **Figure 5. Alignment of the partial E1 protein sequences of chikungunya viruses**

534   The arrow indicates the amino acid at position 145.
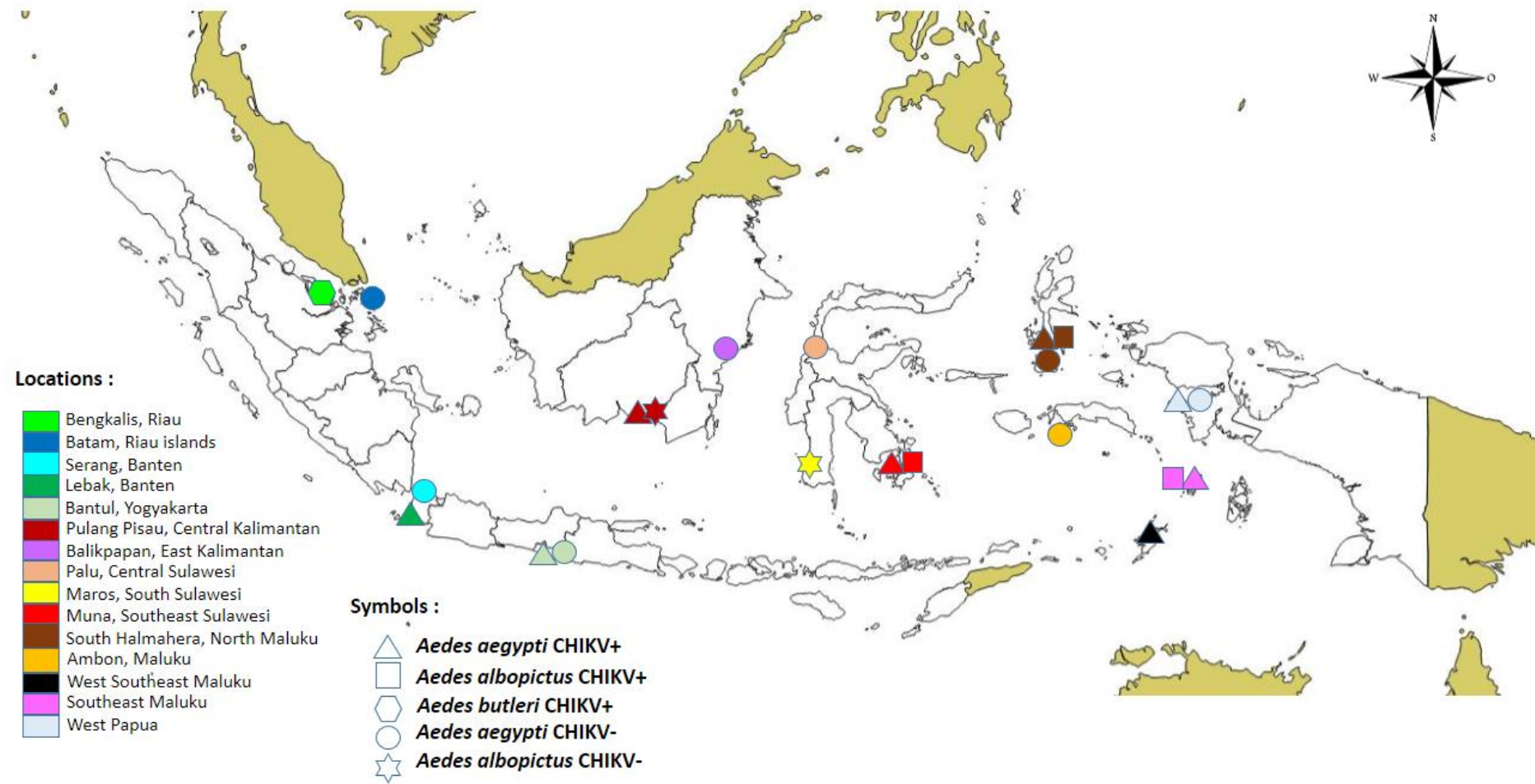
21

Figure_1

Figure_2

sls16_Aal
sls11_Aal
r15_Aal
14_Aal
5_Aal
22-30_Aal
85
100
H05_Aae
57
mlk48_Aae
mlk654_Aae
70
2_Aae
15-18_Aae
19_Aae
16_Aae
8_Aae
tb131_Aae
pl26_Aae
pl1_Aae
mlk73_Aae
11_Aae
37_Aae
15_Aae
13-11_Aae
10_Aae
9-18_Aae
3_Aae
6_Aae
17_Aae
JGJ5_Aae
mlk40_Aae
mlk38_Aae
blp3_Aae
JGJ6_Aae
MK265737_Cqui
0.02

CHIKV-positive *Aedes aegypti*
CHIKV-negative *Aedes aegypti*
CHIKV-positive *Aedes albopictus*
CHIKV-negative *Aedes albopictus*

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Figure_3

Figure_4

Figure 5.TIF
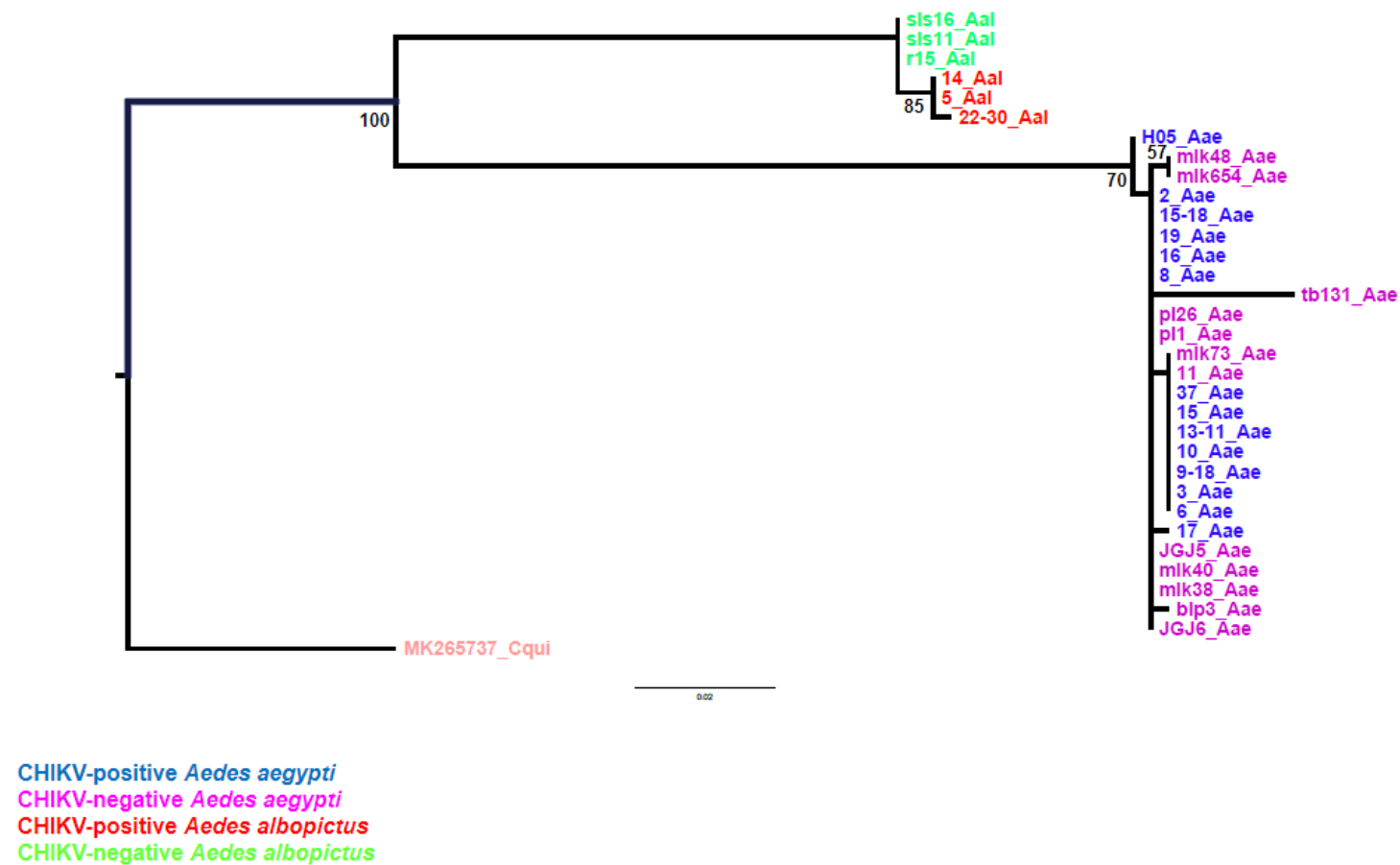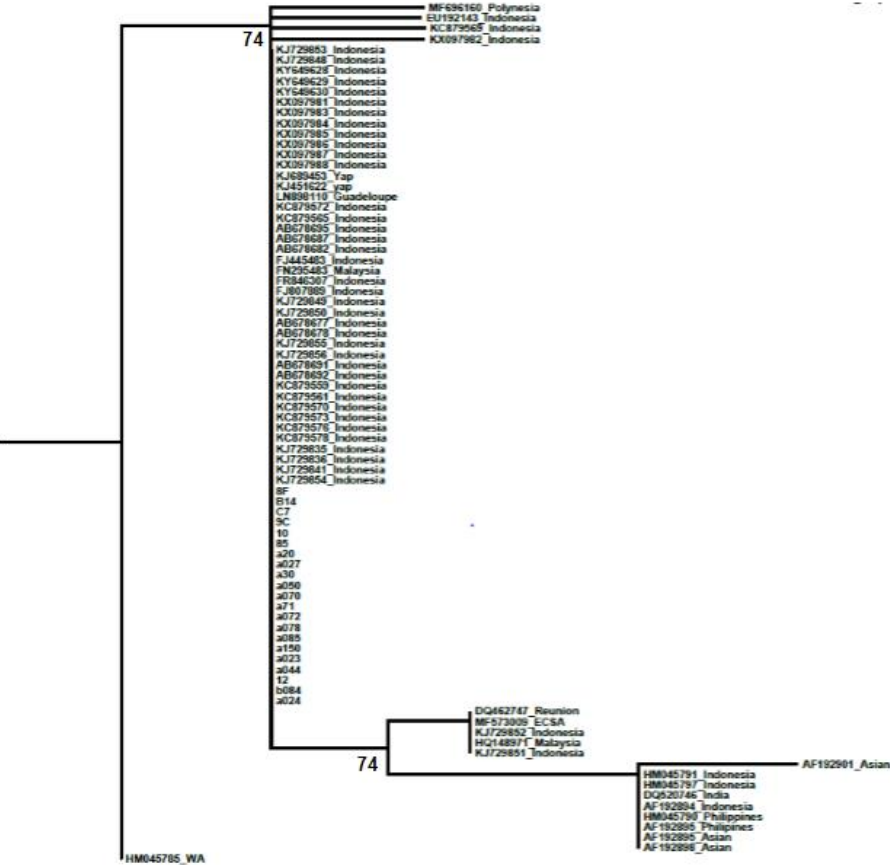
Supplementary table_1

Supplementary Table 1. Mosquito collection and chikungunya virus positivity

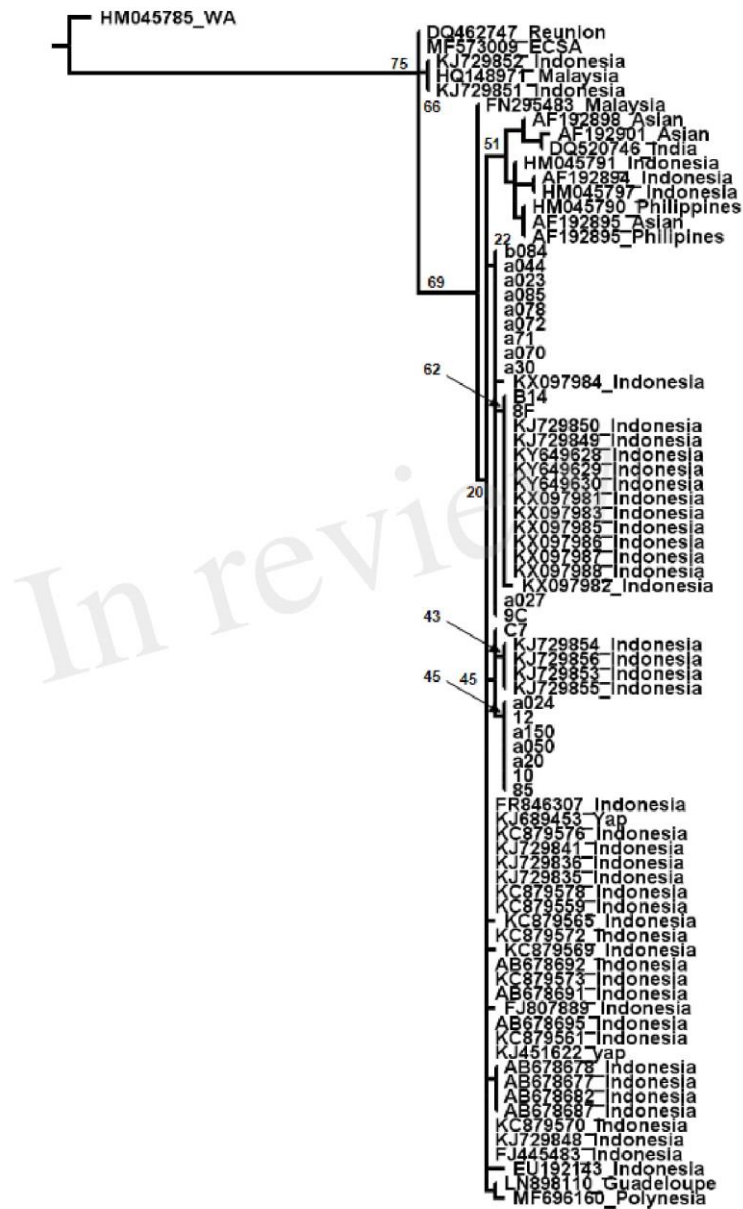| Province	Location	Species Number	Number of of pools	Number of mosquitoes	Number of positive pools	positive mosquitoes
Banten	Pandeglang	*Ae. aegypti*	11	33	0	0
		Ae. albopictus	2	7	0	0
Banten	Lebak	*Ae. aegypti*	9	22	1	1
		Ae. albopictus	51	353	0	0
Banten	Serang	*Ae. aegypti*	4	7	0	0
		Ae. albopictus	11	108	0	0
Riau	Bengkalis	*Ae. aegypti*	3	5	0	0
		Ae. albopictus	55	261	0	0
		Ae. butleri	36	165	1	1
Riau	Meranti	*Ae. aegypti*	24	132	0	0
		Ae. albopictus	14	141	0	0
Riau	Dumai	*Ae. aegypti*	23	115	0	0
		Ae. albopictus	13	82	0	0
Yogyakarta	Kulon Progo	*Ae. aegypti*	0	0	0	0
		Ae. albopictus	12	68	0	0

Yogyakarta	Bantul	*Ae. aegypti*	29	124	1	1
		Ae. albopictus	9	79	0	0
Yogyakarta	Gunung Kidul	*Ae. aegypti*	2	2	0	0
		Ae. albopictus	31	163	0	0
Central Kalimantan	Gunung Mas	Ae. aegypti	24	146	0	0
		Ae. albopictus	21	66	0	0
Central Kalimantan	Murung Raya	*Ae. aegypti*	2	8	0	0
		Ae. albopictus	7	54	0	0
Central Kalimantan	Pulang Pisau	*Ae. aegypti*	33	122	1	4
		Ae. albopictus	6	23	0	0
Southeast Sulawesi	Muna	*Ae. aegypti*	148	1042	5	5
		Ae. albopictus	10	56	0	0
Southeast Sulawesi	Konawe	*Ae. aegypti*	18	108	0	0
		Ae. albopictus	1	25	0	0
Southeast Sulawesi	Bombana	*Ae. aegypti*	33	632	0	0
		Ae. albopictus	0	0	0	0
Maluku	West Southwest	*Ae. aegypti*	19	274	0	0
Maluku	*Ae. albopictus*		2	4	1	1
Maluku	Southeast Maluku	*Ae. aegypti*	200	4709	6	6
		Ae. albopictus	38	913	2	2

Maluku	Aru Islands	*Ae. aegypti*	96	1732	0	0
		Ae. albopictus	4	55	0	0
North Maluku	Central Halmahera	*Ae. aegypti*	5	117	0	0
		Ae. albopictus	3	44	0	0
North Maluku	South Halmahera	*Ae. aegypti*	73	1800	10	10
		Ae. albopictus	32	716	1	1
North Maluku	Morotai Islands	*Ae. aegypti*	5	16	0	0
		Ae. albopictus	22	157	4	4
West Papua	Manokwari	*Ae. aegypti*	9	142	0	0
		Ae. albopictus	5	23	0	0
West Papua	Fak-Fak	*Ae. aegypti*	30	488	1	1
		Ae. albopictus	5	23	0	0
Total *Ae. aegypti*			800	11776	25	28
Total *Ae. albopictus*			354	3421	8	8
Total[a]			**1154**	**15197**	**33**	**36**

Supplementary Table 2. Chikungunya virus sequences used for phylogenetic analysis

Isolate	Location	Year	Host	Genotype	Accession #
CHK-Banten.INA:2011*066	Banten	2011	Human	Asian	KJ729849
CHK-Banten.INA:2011*068	Banten	2011	Human	Asian	KJ729850
CHK-Bali.INA:2011*016	Bali	2011	Human	Asian	KJ729836
CHK-Bali.INA:2011*014	Bali	2011	Human	Asian	KJ729835
2001918633-BDG	Bandung	2001	Human	Asian	KC879561
2001908323-BDG	Bandung	2001	Human	Asian	KC879559
2007904923-BDG	Bandung	2007	Human	Asian	KC879578
2003902452-BDG	Bandung	2003	Human	Asian	KC879570
2008900345-BDG	Bandung	2008	Human	Asian	KC879573
2008900207-BDG	Bandung	2008	Human	Asian	KC879576
2002918314-BDG	Bandung	2002	Human	Asian	KC879569
2004904879-BDG	Bandung	2003	Human	Asian	KC879565
CHK-Jatim.INA:2011*108	East Java	2011	Human	Asian	KJ729856
CHK-Jatim.INA:2011*107	East Java	2011	Human	Asian	KJ729855
CHK-Jatim.INA:2011*105	East Java	2011	Human	Asian	KJ729854
CHK-Jatim.INA:2011*096	East Java	2011	Human	Asian	KJ729853
JKT23574	Jakarta	1983	Human	Asian	HM045791
CHIK/SBY10/10	Surabaya	2010	Human	Asian	AB678678
CHIK/SBY8/10	Surabaya	2010	Human	Asian	AB678677
CHIK/SBY6/10	Surabaya	2010	Human	Asian	AB678691
CHK-NTB.INA:2011*064	West Nusa Tenggara	2011	Human	Asian	KJ729848
CHK-NTB.INA:2011*048	West Nusa Tenggara	2011	Human	Asian	KJ729841
CHK-Kalbar.INA:2011*075	West Kalimantan	2011	Human	ECSA	KJ729851
CHK-Kalbar.INA:2011*077	West Kalimantan	2011	Human	ECSA	KJ729852

JMB-230	Jambi	2015	Human	Asian	KX097988.
JMB-192	Jambi	2015	Human	Asian	KX097986
JMB-154	Jambi	2015	Human	Asian	KX097982
JMB-205	Jambi	2015	Human	Asian	KX097987
JMB-187	Jambi	2015	Human	Asian	KX097985
JMB-172	Jambi	2015	Human	Asian	KX097984
JMB-167	Jambi	2015	Human	Asian	KX097983
JMB-015	Jambi	2015	Human	Asian	KX097981
GianyarBali-4-E1	Bali	2014	Human	Asian	KY649630
GianyarBali-3-E1	Bali	2014	Human	Asian	KY649629
GianyarBali-1-E1	Bali	2014	Human	Asian	KY649628
RSU1	Indonesia	1985	Human	Asian	HM045797
RSU1	Ambon	1985	Human	Asian	AF192894
SGEHICHS412308	Indonesia/Singapore	2008	Human	Asian	FJ445483
Pt11352	Indonesia/France	2009	Human	Asian	FR846307
0804aTw	Indonesia/Taiwan	2008	Human	Asian	FJ807889
0706aTw	Indonesia/Taiwan	2007	Human	Asian	EU192143
PH H15483	Philippines	1985	Human	Asian	AF192895
1455/75	Thailand	1975	Human	Asian	AF192898
Gibbs 63-263	India	1963	Human	Asian	AF192901
IND63WB1	India	1963	Human	Asian	DQ520746
Ph15483	Philippines	1985	Human	Asian	HM045790
MY/06/37348	Malaysia	2006	Human	Asian	FN295483
3807	Yap, Micronesia	2013	Human	Asian	KJ451622
Yap 13-2148	Yap, Micronesia	2013	*Aedes hensilli*	Asian	KJ689453
G106	Guadeloupe	2014	Human	Asian	LN898110
NA	French Polynesia	2014	NA	Asian	MF696160
IMT/6470	Reunion Island	2006	Human	ECSA	DQ462747
IND-2015-MH_Pune1517011	India	2015	Human	ECSA	MF573009
216-NSembilan-2009	Malaysia	2009	Human	ECSA	HQ148971

PM2951	Senegal	1966	*Ae. aegypti*	WA	HM045785
A023	Muna	2016	*Ae. albopictus*	Asian	This work
a027	Muna	2016	*Ae. aegypti*	Asian	This work
a30	Muna	2016	*Ae. aegypti*	Asian	This work
a044	Muna	2016	*Ae. aegypti*	Asian	This work
10	Fak Fak	2016	*Ae. aegypti*	Asian	This work
12	Bengkalis	2016	*Ae. butleri*	Asian	This work
85	Southeast Maluku	2016	*Ae. aegypti*	Asian	This work
a70	Southeast Maluku	2016	*Ae. aegypti*	Asian	This work
a150	Southeast Maluku	2016	*Ae. albopictus*	Asian	This work
b084	Southeast Maluku	2016	*Ae. aegypti*	Asian	This work
a20	Southeast Maluku	2016	*Ae. aegypti*	Asian	This work
a024	South Halmahera	2016	*Ae. aegypti*	Asian	This work
a050	South Halmahera	2016	Ae. aegypti	Asian	This work
a072	South Halmahera	2016	*Ae. aegypti*	Asian	This work
a085	South Halmahera	2016	*Ae. albopictus*	Asian	This work
a71	Lebak	2016	*Ae. aegypti*	Asian	This work
a078	Pulang Pisau	2016	*Ae. aegypti*	Asian	This work
8F	Magelang	2014	Human	Asian	This work
B14	Magelang	2014	Human	Asian	This work
C7	Magelang	2014	Human	Asian	This work

Table 1.

Virus	ChikV code	sample type	CHIKV-positive *Aedes* Species	Sample	District	Province
ChikV	c7	human sample			Magelang	Central Java
ChikV	B14	human sample			Magelang	Central Java
ChikV	8F	human sample			Magelang	Central Java
ChikV	a023	mosquito sample	*Aedes albopictus*	14_Aal	Muna	Southeast Sulawesi
ChikV	a027	mosquito sample	*Aedes aegypti*	15_Aae	Muna	SoutheastSulawesi
ChikV	a30	mosquito sample	*Aedes aegypti*	16_Aae	Muna	Southeast Sulawesi
ChikV	a044	mosquito sample	*Aedes aegypti*	17_Aae	Muna	Southeast Sulawesi
ChikV	10	mosquito sample	*Aedes aegypti*	9-18_Aae	Fak-Fak	West Papua
ChikV	12	mosquito sample	*Aedes butleri*	012_Abt	Bengkalis	Riau
ChikV	85	mosquito sample	*Aedes aegypti*	8_Aae	Southeast Maluku	Maluku
ChikV	a070	mosquito sample	*Aedes aegypti*	6_Aae	Southeast Maluku	Maluku
ChikV	a150	mosquito sample	*Aedes albopictus*	5_Aal	Southeast Maluku	Maluku
ChikV	b084	mosquito sample	*Aedes aegypti*	3_Aae	Southeast Maluku	Maluku
ChikV	a20	mosquito sample	*Aedes aegypti*	37_Aae	Southeast Maluku	Maluku
ChikV	a024	mosquito sample	*Aedes aegypti*	15-18_Aae	South Halmahera	North Maluku
ChikV	a050	mosquito sample	*Aedes aegypti*	13-11_Aae	South Halmahera	North Maluku
ChikV	a072	mosquito sample	*Aedes aegypti*	19_Aae	South Halmahera	North Maluku
ChikV	a085	mosquito sample	*Aedes albopictus*	22-30_Aal	South Halmahera	North Maluku
ChikV	a71	mosquito sample	*Aedes aegypti*	2_Aae	Lebak	Banten
ChikV	a078	mosquito sample	*Aedes aegypti*	ktg_H05_Aae	Pulang Pisau	Central Kalimantan
NA	NA		*Aedes aegypti*	10_Aae	Bantul	Yogyakarta

| CHIKV-negative *Aedes* | | Location | |
Species	Sample	District	Province
Ae.aegypti	**pl1_Aae**	Palu	Central Sulawesi
Ae.aegypti	**pl26_Aae**	Palu	Central Sulawesi
Ae.albopictus	**sls11_Aal**	Maros	South Sulawesi
Ae.albopictus	**sls16_Aal**	Maros	South Sulawesi
Ae.aegypti	**19_18_1_Aae**	Fak-Fak	West Papua
Ae.aegypti	**tb131_Aae**	Batam	Riau islands
Ae.aegypti	**mlk38_Aae**	Ambon	Maluku
Ae.aegypti	**mlk40_Aae**	Ambon	Maluku
Ae.aegypti	**mlk73_Aae**	Ambon	Maluku
Ae.aegypti	**mlk654_Aae**	Ambon	Maluku
Ae.aegypti	**mlk48_Aae**	Ambon	Maluku
Ae. aegypti	**15-18_1_Aae**	South Halmahera	North Maluku
Ae. aegypti	**13_11_1_Aae**	South Halmahera	North Maluku
Ae. aegypti	**19_1_Aae**	South Halmahera	North Maluku
Ae. albopictus	**ktg28_Aal**	Pulang Pisau	Central Kalimantan
Ae. aegypti	**11_Aae**	Serang	Banten
Ae.aegypti	**blp3_Aae**	Balikpapan	East Kalimantan
Ae.aegypti	**jgj5_Aae**	Bantul	Yogyakarta

Conclusions

This study has revealed that a replacement of CHIKV strains occured in Indonesia between 1985 and 2000. However, the exact time of the replacement is not known due to the lack of CHIKV sequences during that time. CHIKV isolates characterized up to 1985 belonged to the Asian genotype which is also found in several Asian countries. However, the isolates found in Indonesia after 2000 throughout the country correspond to a different genotype derived from the Asian genotype. This genotype we propose to call Asian-Pacific corresponds to the samples found on Yap Island in Micronesia but also in the Carribean, South America and Polynesia in 2013-2014. The potential of invasiveness of the Asian-Pacific genotype seems to be higher than that of the ECSA/IOL genotype. Considering its presence all over Indonesia and the lack of polymorphism, this invasion and replacement event occurred rapidly. This genotype does not seem to be species dependent like the ECSA/IOL genotype since it is found in all mosquito species with the same infection rate. Furthermore, this genotype is under strong selective pressure to conserve an alanine in position 145, a trait differentiating it from the other genotypes. All together, these suggest that the selective advantage might be a higher transmissibility in humans. Another interesting feature is that the Asian-Pacific genotype was circulating in Indonesia, and perhaps other Asia-Pacific countries, long before the 2013 pandemic outbreak in the Carribean and Polynesia. However, the exact origin of this genotype is not known.

The *cox1* genotyping of the CHIK vectors in Indonesia also showed that all the field-collected *Ae. aegypti* mosquitoes belonged to the same population. CHIKV-positive and –negative *Ae. albopictus* seemed to belong to different populations. However, the sample size is not large enough to conclude.

Chapter 6. Discussion and conclusions

Discussion

Studies delivered in the previous chapters summarized the dynamic of the main mosquito-borne diseases in Indonesia, with focus on the epidemiology of Japanese encephalitis and its vector distribution, *Anopheles* species diversity and implications for malaria control and intervention, genetic diversity of dengue vector, vector surveillance methods and entomological indices to assess the risk of transmission. A last study addressed the dynamic of chikungunya in Indonesia.

The study of Japanese encephalitis (JE) is discussed in Chapter 2. JE has been reported as an important mosquito-borne disease since it was first identified in Indonesia in 1960. Although JE has posed a major public health threat for quite a while in Indonesia, surprisingly, studies to understand factors that play a role in Japanese encephalitis virus (JEV) transmission and its risk factors are still very limited (4,5,7,30,31,33,342,344,349,359,560,563,651–660).

Since JE was designated as a national priority, the Indonesian government has shown a commitment to improve clinical management and treatment of the disease (562). The Indonesian Ministry of Health (MoH), in collaboration with WHO, has then implemented hospital-based sentinel site surveillance for JE in 11 selected provinces in 2016. At sentinel sites, all clinical cases of acute encephalitis syndrome (AES) were identified based on a case definition according to the WHO JE surveillance standards. Patients (cerebrospinal fluid (CSF) or serum) were then tested regularly by JE IgM-capture ELISA at the National reference laboratory for JE, National Institute of Health Research and Development (NIHRD)-MoH Indonesia. In addition, the JE mass vaccination program was also set as a priority by the Indonesian MoH for implementation in areas of high JE transmission. The JE vaccination campaign started in 2018 with Bali as the first target province. Currently, the Chengdu SA14-14-2 live attenuated JE vaccine is applied in a single dose through 2 phases in Bali (350,661).

Case management of JE surveillance in the sentinel areas has been carried out routinely over the last few years, however, routine epidemiological surveillance data for JE, which can provide an overview of the latest situation and patterns of JEV transmission, is still very limited and sporadic in certain areas.

The review and original research studies reported in this thesis had several aims, all targeting to provide an updated situation on the epidemiology, the circulation of virus genotypes, and vector distribution in Indonesia. This information is needed to provide baseline data on JE in Indonesia. It is essential to optimize the national planning and the targeting of appropriate JE prevention and control strategies other than vaccination campaigns. Several issues have been identified as challenges to JE prevention and control in Indonesia, including: 1) The JE burden needs to be clearly assessed and appropriate control measures must be implemented; 2) Long term and systematic JE surveillance across the country being a priority, they must be strengthened and pursued. Our study showed for the first time the presence of Genotype-1 (GI) in Indonesia. This is a very important information and further studies are needed to assess the distribution of GI JEV in Indonesia. National and local health authorities must be alerted in order to address potential risks to public health. The GI JE, which is currently replacing GIII in all over Asia, is not detected in cerebrospinal fluid by JEV-specific IgM antibodies raised against GIII JEV. There is thus a risk of misdiagnosis in the presence of GI. Furthermore, all vaccines currently available against JEV are derived from GIII JEV and several studies have reported human confirmed cases with GI JEV infection in areas where effective JEV vaccination programs are implemented.

Chapter 3 investigated the importance of understanding *Anopheles* species diversity and its implications for malaria control and intervention. In Indonesia, the analysis of the distribution of *Anopheles* species, in particular those recognized as important malaria vectors, is of great importance in the objective of malaria elimination by 2030 (284). However, *Anopheles* species in Indonesia are quite complex with numerous taxa and various epidemiological contexts. At least 90 *Anopheles* taxa have been identified with 25 confirmed malaria vector species (3,192). Furthermore, a comprehensive understanding of transmission dynamics and appropriate malaria vector control efforts is quite complicated due to several factors, including intraspecific variation and vector status across species distribution (271). The complexity and

diversity of *Anopheles* species could be attributed to natural selection, historical processes, ecological changes and gene flow. This has led to divergence and homogenization of variations within or between species which could be the key to understanding the dynamic of malaria transmission and the basis for appropriate vector control (271).

We reported the genetic homogeneity of *Anopheles maculatus* as a case study of malaria vector in Indonesia. *Anopheles maculatus* (*s.s.*) is one of the major malaria vector in Indonesia. This species has been reported to transmit both *Plasmodium falciparum* and *P. vivax*, particularly in the Menoreh Hills of Central Java and Tenang in Southern Sumatra (197,209,238,239). Previously, this species was considered the only member of the Maculatus group present in Indonesia (1,3,187,188). A specific population in the Kulon Progo district, in Central Java turned out to be a different species. In this study, we analyzed the diversity and phylogeny of *An. maculatus* samples collected in 6 different locations and islands in Indonesia, including the proposed novel species present in Kulon Progo district to derive its putative origin. The findings showed that two species of the Maculatus group are present in Indonesia. The novel sibling species is confirmed as present in Kulon Progo, the most important malaria endemic area in Java. This novel species is considered a major vector in the Menoreh Hills, Central Java. The results of this study also indicated that the genetic structure of the *An. maculatus* population in Indonesia is likely greatly influenced by geographic barriers. The divergence of this species is explained by the stable refugia during the quaternary period of intense volcanic activity throughout most of Java. This had an impact on gene flow patterns and dispersal of these species, which causes the novel species in Kulon Progo to become increasingly divergent from the *An. maculatus* (*s.s*) population due to a process of neutral genetic drift and differential natural selection.

The occurrence of geographic barriers for a long period of time is likely to have driven differential local adaptation leading to a divergence between populations leading to speciation (271,662). These conditions will have consequences for the emergence of interspecific and intraspecific variations, which will affect the bionomics, blood-seeking preferences and habitat of malaria vector species (271). These differences in bionomics have been reported when comparing *An. maculatus* (s.s) collected from West Sumatra with the novel sibling species previously identified as *An. maculatus* (s.s). in Kulon

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Progo, in Central Java. *An. maculatus* (s.s.) from West Sumatra has been found to bite humans both indoors and outdoors, while the novel sibling species from Kulon Progo is more a human outdoor biting mosquito (3,202,211,222,238,663). *An. maculatus* (s.s.) from West Sumatra tends to bite during the first half of the night, while the novel sibling species from Kulon Progo bites during both the first half of night and early morning (dawn) (3,202,211,213,222,663). This characterization is an important step in determining the appropriate vector control strategy to implement.

This study highlights the importance of molecular identification of the *Anopheles* fauna of Indonesia to determine more exactly the *Anopheles* species composition and to help undertanding the real role of the various *Anopheles* mosquitoes as malaria vectors in Indonesia. The current *Anopheles* identification key used in Indonesia is a compilation of entomological/*Anopheles* taxonomy studies that from the early 1900s to several decades ago (early 1980s) (1,3,185,187). The identification key used is entirely based on morphological traits. In practice, the use of morphology-based keys may be complicated by outdated references, contradictory parameters and difficulty of interpretation (667). In addition, the identification of *Anopheles* species using only the variability of the morphological characteristics often faces obstacles, especially in areas with the presence of cryptic species within *Anopheles* taxa which are very difficult to distinguish based on morphological identification alone. St Laurent et al. reported a low level of accuracy when the *Anopheles* specimens were identified morphologically in Jayapura, Papua, compared with molecular identification, with only 51% accuracy using morphology alone (234). A comprehensive survey of *Anopheles* species diversity in Karama, West Sulawesi, has also revealed that no species were correctly identified using morphological features with 100% accuracy when compared with molecular identification. High rate of morphological identification accuracy was found for the most dominant *Anopheles* in the area, i.e. *An. barbirostris* with 92.1% and *An. vagus* with 87.6%. However, the rate of correct identification for *An. aconitus, An. karwari, An. peditaeniatus* and *An. tesselatus* was very variable, ranging from 0% to 83% (667). Differences in biological traits, including bionomics and characteristic habitats, of members in the *Anopheles* complex also have important influences on the dynamics of malaria transmission. Therefore, it is important to determine exactly the siblings species, their bionomics and their respective role in the transmission of malaria. Misidentification of vector species can have a negative effect in determining the species bionomic,

vectorial capacity, entomological inoculation rate, and impact on disease control, especially malaria (668). Advanced molecular entomology tools can provide a better understanding of the identification of *Anopheles* species and of the genetic structure of the populations (669).

Chapter 4 concerns dengue transmission and its vectors. This disease is an environmental issue with several factors, such as population growth, population movement, transportation, household water supplies, sanitation services and community behavior that contribute to create optimal conditions for the reproduction of *Aedes* mosquitoes and dengue virus (DENV) circulation. Critical strategies need to be implemented for efficient dengue prevention, vector surveillance and control and disease management. Strengthening community participation for dengue control and developing cross-sectorial network in local and central government is a key issue (9). In order to find solutions for appropriate vector surveillance, studies on the genetic diversity of dengue vectors, vector surveillance methods and assessment of entomological indices to assess risk of dengue transmission have been carried out.

Considering the role of the main *Aedes* mosquito vectors in the dissemination of dengue in hyperendemic dengue areas of Indonesia, it is essential to study the genetic characteristics of *Ae. aegypti* and *Ae. albopictus* populations to better understand their genetic variability and relationship (664). However, the information about genetic diversity of *Ae. aegypti* is still insufficient in Indonesia. This information is important to determine any correlation between *Ae aegypti* populations and vector competence, ecological adaptation and resistance to insecticides (26,27,547). The study of the genetic characteristics of *Ae. aegypti* and *Ae. albopictus* has revealed the fast replacement of *Ae. aegypti* and *Ae. albopictus* populations in Indonesia. This dynamic of replacement represents a threat for inundative strategies of dengue vector control, such as sterile male release and *Wolbachia* establishment in existing populations of *Ae. aegypti*. These strategies are population dependent and any massive and fast replacement of population will impair all efforts to establish the inundative population. A consequence is that vector control should not be population-based. Established or invasive *Ae. aegypti* and *Ae. albopictus* mosquitoes will have to breed in the human environment and the best way to prevent any vector population from thriving is certainly to implement vector control as a very local level, at maximum at the community level, essentially by eliminating

breeding sites using very simple and affordable means of control such as containers and garbage removal. The strategy of prevention of dengue transmission, through community participation currently recommended in Indonesia, is likely to be the most effective way.

Study to assess the relative effectiveness of several methods, i.e. morning adult collection using an aspirator, pupal collection, animal baited trap, whole night collection using human landing methods, and larval collection for dengue surveillance is also discussed in this chapter. However, the results have revealed no consistency in the efficiency of a given method for detecting dengue. Therefore, more effective and appropriate vector surveillance methods are needed to determine vector distribution, density, larval habitats, and risk factors related to dengue transmission and evaluation of vector control efforts. In addition, the development of a novel set of indices is needed for implementing more efficient tools to manage and anticipating the risk of dengue outbreaks.

While an effective vaccine is still under study, vector control is the only effective way to prevent and control dengue. Although a variety of vector control methods have been employed, only a few of them have been successfully carried out (634). Several factors led to the failure of these control programs including lack of public health commitment, rapid unplanned population growth in many tropical countries, large, rapid and chaotic urbanization that has created crowded human populations living in urban areas with poor sanitation facilities, inadequate water supplies and numberless *Ae. aegypti* and *Ae. albopictus* breeding sites. Furthermore, globalization with high and fast connectivity transporting different dengue virus strains from endemic areas to other areas has provided ideal conditions for increased dengue transmission (9,530,634,665). In addition, dengue vector surveillance methods has remained mostly unchanged in Indonesia for more than three decades (9). Larval surveys are the most adopted dengue vector surveillance methods to locate larval habitats and to measure the abundance of *Ae. aegypti* and *Ae. albopictus* (407). As an indicator of the main implementation of vector surveillance in Indonesia, the traditional *Stegomyia* indices [e.g. the house index (HI), container index (CI) and Breteau index (BI)] and free larval index have been widely used as standards for calculating abundance and predicting the risk of dengue transmission (9). However, previous studies in several countries have yielded

contradictory results about the reliability of these traditional entomological indices to assess the risk of dengue transmission (407,634).

As part of this thesis, a study aiming at assessing the effectiveness of the *Stegomyia* indices has been carried out to determine in definitive way the correlation between *Stegomyia* indices and the risk of dengue transmission over a very large zone covering 78 sampling sites throughout Indonesia from Sumatra to Papua. This study is the first one of this magnitude. No such comprehensive study has been cnduted before. The *Stegomyia* indices have been developed as quantitative indicators of the risk of dengue transmission and outbreak and Indonesia has been using *Stegomyia* indices for dengue transmission risk analysis for more than 3 decades. The findings of this study have revealed that no correlation was found between the incidence of dengue and the *Stegomyia* indices. This study brings a definitive conclusion regarding the effectiveness of using *Stegomyia* indices for assesing the risk of dengue transmission. There is absolutely no correlation. It is important to note that the ability to infer associations between one or more of these *Stegomyia* indices and transmission risk analyzes have been empirically developed over the years and only for specific regions (407). Other more accurate and sensitive indices need to be developed to monitor and predict efficiently and precisely the risk of dengue transmission in Indonesia.

Based on these results, although several attempts have been made to identify risk factors for dengue transmission and more effective dengue vector control efforts, the strategy of prevention and control of dengue transmission through vector control efforts by community participation is still the best way to achieve efficient control and should be still recommended in Indonesia. In 1992, The Indonesian Ministry of Health initiated a national program for the community known as 3M, i.e. covering water containers (Menutup), cleaning water containers (Menguras), burying discarded containers (Mengubur) (8,9). Later, this program was upgraded to 3M plus, with additional spesific activities aiming at eliminating moquito breeding places and implementing education on protective behaviour. This program was designed to be implemented at the household level under the responsibility of the head of family with at least one person in each family in charge of monitoring and controlling *Aedes* larvae in all water storage. This program has been implemented comprehensively throughout Indonesia under the coordination of local health authorities (670). Although community empowerment efforts in controlling

dengue have been successful in several places, most of the efforts to control DHF did not reach the expectations (671). To reaffirm this program, the prevention and control programs need to be undertaken with specific commitments from stakeholders from the top to bottom. Coordination and collaboration by all sectors within the government, communities, private sector, media and civil societies need to be strengthened (672). Results from this PhD work, in particular this on the entomological indices, collection methods and replacement of mosquito populations are very valuable in this framework.

Chapter 5 discusses the dynamic of CHIKV isolated from field-caught *Ae. aegypti, Ae. albopictus* and *Ae. butleri* mosquitoes. The study has revealed that all the CHIKV identified all over Indonesia in this study were similar to those isolated in Indonesia since 2000. This CHIKV all belonged to the Asian-Pacific genotype, the name of the new CHIKV genotype proposed in this study, which is different from the Asian genotype. Although, all collected *Ae. aegypti* mosquitoes belonged to the same population, it was not the case for *Ae. albopictus* samples. CHIKV-positive and CHIKV-negaive *Ae. albopictus* belonged to separate clusters. However, the sample size was too small and a large study is needed to correctly analyze the structure of the population of *Ae. albopictus* in relation with chikungunya vector competence. The evidence of the replacement of the CHIKV population and the low diversity of vectors in Indonesia may facilitate the management and prevention of potential outbreaks by properly implemented local mosquito control actions at the community level.

Conclusions and perspectives

This thesis provides an overview of the current dynamics and risk of transmission of the main mosquito-borne diseases in Indonesia. The main objective of this work was to provide basis and knowledge on some critical points which are priorities for the Ministry of Health. With the exeption of Japanese encephalitis, there is no vaccine available for the mosquito-borne diseases present in Indonesia. The main means of control is therefore the control of the vectors. Understanding the dynamic of these diseases and of their vectors is a key element to successfully control those diseases. This work is expected to help the public and authorities for implementing more efficient national programs for the control of the main vector-borne diseases addressed during

this PhD work: Japanese encephalitis, malaria, dengue and chikungunya. Several perspectives are coming out of this work.

Japanese encephalitis

With respect to Japanese encephalitis, the main efforts must devoted to the prevention of transmission.

Development of an appropriate JE vector control system. The mosquitoes that transmit JE are breeding in a variety of habitats, from clean water to highly polluted water, with more exophagic biting behavior. However, the breeding habitat of the main JE vector is rice fields. Therefore, rice fields are a potential habitat as a target for vector control, especially during the harvest season and starting the rice growing season. Implementation of a cattle barrier in combination with mina rice, a method developed in agricultural technology in Indonesia by releasing freshwater fish in rice fields to improve the farmer's economy as well as a mosquito larva predator. This effort can be integrated with the malaria and lymphatic filariasis vector control so that it will be more cost-effective and more efficient.

Strengthening JE surveillance in hospitals. With the report of the presence of the GI genotype in Indonesia, the survey and vaccination programs must adapted. A long term and systematic JE surveillance must be implemented across the country. Currently, surveillance is only carried out in 12 sentinel hospitals in JE endemic areas. Strengthening JE surveillance across the country is currently constrained by the high running costs of providing detection equipment (IgM ELISA) in all hospitals. Strengthening the capacity of JE surveillance in hospitals by supporting pro-active efforts of the hospital itself to send samples of suspected JE to reference laboratories in Indonesia with financial support from the respective local governments is needed to determine the magnitude of the threat.

Operational research to support the monitoring of GI JE. The GI genotype of JE is definitely the main risk in Indonesia due to screening and vaccine avoidment all based on the GIII genotype. It is essential to survey and map the human cases of GI JE to assess the distribution and dynamic of this genotype in Indonesia. International

cooperation will be needed to develop the proper tools and appropriate methods for an efficient detection of this genotype.

Malaria

Malaria is still an important issue in terms of efforts to control vector-borne diseases in Indonesia. The study we conducted is an effort towards eliminating malaria in Indonesia, especially in relation to the problems faced by entomologists in the field when conducting surveillance of malaria vector entomology. Our study has revealed that an update of the identification key of *Anopheles* is needed. A complete information about cryptic species is nedeed to help field entomologists identifying the vectors and better analyze the results of entomological surveys. In addition, the preparation of guidelines for molecular identification of *Anopheles* species that can be used in all regions of Indonesia. This comes along with the needed strengthening the capacity of molecular laboratories for acurately identifying mosquito species, especially those having an important role in the transmission of malaria. Currently, several areas are recorded as low malaria endemic areas but the elimination is difficult due to the presence of cryptic species of Anopheles in these areas (3,209). This effort is of course highly expected and will greatly support the malaria elimination program in Indonesia which is expected to be achieved by 2030 (283).

***Aedes*-borne diseases**

A major part this PhD work was devoted to *Aedes*-borne diseases. A national priority is to develop an appropriate and effective dengue vector control method that can be applied at the household level. This is still to be done. The implementation must be done thoroughly, comprehensively, be sustained, and can be integrated with the national dengue control program based on household that launched in 2016, namely 1 house 1 inspector program (673). A key issue for the success of such an approach is to have reliable predictors. Since entomological survey and indices do not work as shown in this PhD work. Novel types of predictors, societal predictors, based on urbanism and socio-economic criteria will have to be developed. This is a priority project to be developed as

quickly as possible. This effort will be integrated with the community empowerment that has been taking place and has become an important part in controlling dengue in Indonesia (8,9). Appropriate predictors that can efficiently assess the risk of dengue transmission are needed as dengue remains the main vector-borne disease priority in Indonesia. Another issue to consider as a priority is the risk of mosquito population replacement. This PhD work showed that populations of the two main dengue and chikungunya vectors, *Ae. aegypti* and *Ae. albopictus*, have been replaced in a short time by invasive populations. It is therefore essential to extend the genotyping study conducted in this work to a larger area, i.e. Asia-Pacific coverage, through a collaborative regional project with regular updates. Specifc tools will have to be developed such as a dedicated database, standardized sampling methods and detectin procedures. This is particularly important considering the risk this replacement is representing to the mass vector control strategies. An integration into a community survey strategy will be needed. There is here a community-based operational project to fully develop. The replacement of the CHIKV population is the last issue raised by this PhD work. A broad study of the haplotype of *Ae. albopictus* throughout Indonesia needs to be carried out to correctly analyze the structure of the population of *Ae. albopictus* and *Ae. aegypti* and their respective role as a vector for Chikungunya and other arboviruses. This effort requires collaborative support from various parties, both from Indonesia and the international community.

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Résumé

Cette thèse porte sur l'étude de la dynamique des principales maladies transmissibles par les moustiques en Indonésie, à savoir l'encéphalite japonaise, le paludisme, la dengue et le chikungunya. Plusieurs résultats utiles en santé publique ont été obtenus durant ce travail de thèse. Sur l'encéphalite japonaise, la présence du génotype I a été démontrée en Indonésie. C'est essentiel car ce génotype n'est pas détecté par les tests actuels et contourne la vaccination. En ce qui concerne le paludisme, les travaux ont permis la mise en évidence d'une nouvelle espèce d'*Anopheles* vecteur sur l'île de Java. Les travaux sur les méthodes de surveillance des moustiques *Aedes* vecteurs d'arbovirus apportent des données importantes pour la lutte contre des maladies comme la dengue ou le chikungunya. Les travaux de cette thèse ont montré pour la première fois au travers d'une étude statistique très large, que les indices *Stegomyia*, recommandés par l'OMS et utilisés en Indonésie et dans d'autres pays, n'ont aucune utilité pour gérer le risque d'épidémie de dengue. Ces travaux ont également montré un remplacement massif et rapide des populations d'*Aedes* en Indonésie ce qui a un impact majeur sur les stratégies de lutte. Il y a eu également un replacement des lignées de virus chikungunya qui correspond à un génotype très invasif que nous proposons de nommer Asie-Pacifique. En conclusion, il faut changer les outils de gestion et de surveillance, et développer de nouveaux prédicteurs.

Mots-clés : Dynamique, Maladies à transmission vectorielle, Indonésie

Summary

This PhD work focuses on the study of the dynamics of the main mosquito-borne diseases in Indonesia, namely Japanese encephalitis, malaria, dengue and chikungunya. Several useful results in public health were obtained during this PhD work. With respect to Japanese encephalitis, the presence of genotype I has been demonstrated in Indonesia. This is essential because this genotype is not detected by current tests and bypasses vaccination. With regard to malaria, the work has led to the identification of a new species of *Anopheles* vector on the island of Java. The work on surveillance methods for arbovirus *Aedes* mosquito vectors provides important information for the control of diseases such as dengue or chikungunya. This PhD work has shown for the first time through a very large statistical study, that the *Stegomyia* indices, recommended by the WHO, and used in Indonesia and other countries, are of no use in managing the risk of epidemics of dengue fever. This work has also shown a massive and rapid replacement of *Aedes* populations in Indonesia, which has a major impact on control strategies. There was also a replacement of the chikungunya virus lineages, which corresponds to a very invasive genotype that we propose to name Asia-Pacific. In conclusion, management and monitoring tools must be changed and new predictors must be developed.

Keywords: Dynamic, Vector-borne diseases, Indonesia

Ringkasan

Penelitian yang merupakan bagian dari studi PhD ini fokus pada studi tentang Dinamika penyakit tular nyamuk yang utama di Indonesia, yaitu Japanese encephalitis, malaria, dengue dan chikungunya. Beberapa hasil yang bermanfaat bagi kesehatan masyarakat berhasil diperoleh selama penelitian berlangsung. Terkait dengan studi terhadap Japanese encephalitis, keberadaan genotipe I telah dibuktikan bersirkulasi di Indonesia. Hal ini penting karena genotipe ini tidak terdeteksi dengan menggunakan uji yang selama ini digunakan dan genotipe ini dapat lolos dari vaksinasi. Terkait dengan studi malaria, penelitian ini lebih mengarah pada identifikasi spesies baru *Anopheles* yang berperan sebagai vektor di pulau Jawa. Evaluasi terhadap metode surveilans yang digunakan untuk *Aedes*, nyamuk penular arbovirus, juga telah memberikan informasi yang penting dalam pengendalian penyakit, seperti demam berdarah dengue dan chikungunya. Dalam studi PhD ini menunjukkan bahwa indeks stegomyia yang direkomendasikan WHO dan digunakan di Indonesia dan negara endemik DBD lainnya tidak efektif digunakan dalam memperkirakan risiko epidemi DBD. Untuk pertama kalinya, analisis terhadap efektifitas indeks stegomyia sebagai prediktor dilakukan dengan menggunakan analisis statistik dengan jumlah sampel yang sangat besar. Bagian lain dari studi ini adalah pembuktian secara masif dan cepat adanya penggantian populasi *Aedes* di Indonesia yang dikawatirkan dapat berdampak besar dalam strategi pengendalian vektor DBD. Studi bagian dari thesis lainnya menunjukkan adanya penggantian garis keturunan virus chikungunya oleh genotipe yang sangat invasif yang dalam studi ini kita usulkan sebagai genotipe Asia-pasifik. Metode monitoring dan manajemen pengendalian vektor harus diubah dan prediktor baru harus dikembangkan

Kata kunci : Dinamika, penyakit tular vektor, Indonesia