Larval Development of the Molecular Forms of *Anopheles gambiae* (Diptera: Culicidae) in Different Habitats: A Transplantation Experiment

ABDOULAYE DIABATÉ,[1] ROCH K. DABIRE,[1] EUN H. KIM,[2, 3] RYAN DALTON,[2, 3] NIAMA MILLOGO,[1] THIERRY BALDET,[4] FREDERIC SIMARD,[5] JOHN E. GIMNIG,[2, 6] WILLIAM A. HAWLEY,[2, 6] AND TOVI LEHMANN[2, 3, 7]

J. Med. Entomol. 42(4): 548–553 (2005)

ABSTRACT We compared the development of the molecular forms of *Anopheles gambiae* s.s. in different larval habitats. First stage larvae (L1s) of wild-caught females were placed into cages in natural habitats of the M form (rice fields) or the S form (puddles/quarries). Each cage was covered with cloth, allowing exchange of water, solutes, and small particles, including microorganisms, and was seeded with 100 L1s of a single form (M or S) or by a mixture of 50:50 of M and S forms. Emergence success of both forms in puddles and quarries was three-fold higher than in the rice fields. The emergence rate of the S form was higher than that of the M form in both habitats, but the form × habitat interaction was not significant. In temporary larval sites such as puddles, emergence success of the M form was lower in mixed cages than in single form cages, whereas the reverse was true for the S form, suggesting competition between the forms. The median developmental time was not significantly different between forms. Although these findings demonstrate differences between forms, they do not suggest that their spatial segregation is determined by differences in their exploitation of the physical and chemical conditions in these environments. These results should be regarded with caution because small numbers of first stage larvae could pass through the cloth of the cages.

KEY WORDS *Anopheles gambiae*, molecular forms, development, temporary habitats, rice fields

Anopheles gambiae s.s., the major malaria vector in Africa, is well adapted to exploit various domestic and peridomestic environments (Coluzzi et al. 1979). Originally considered as a single species, cytogenetic evidence for heterogeneity within this taxon has accumulated (Coluzzi et al. 1985; Touré et al. 1994, 1998). Analysis of paracentric inversions revealed deviations from Hardy–Weinberg equilibrium in many samples that led to the proposed division of the species into five chromosomal forms, named Mopti, Savanna, Bamako, Forest, and Bissau (Coluzzi et al. 1985). Molecular analysis, however, did not support the subdivision of *An. gambiae* into five incipient species but suggested the existence of two different entities referred to as molecular forms M and S (della Torre et al. 2001, Favia et al. 2001, Gentile et al. 2001, Mukabayire et al. 2001, Wondji et al. 2002). In Burkina Faso and Mali, there is a correspondence between the M molecular form and the Mopti chromosomal form. Similarly, the S form corresponds to Savanna and to Bamako chromosomal forms. However, the correspondence between molecular and chromosomal forms breaks down outside this geographical region (della Torre et al. 2001). The taxonomic and phylogenetic interpretation of the chromosomal and the molecular forms remains unresolved (Lanzaro et al. 1998, Black and Lanzaro 2001, Tripet et al. 2001, della Torre et al. 2002, Lehmann et al. 2003), but it is widely agreed that a comparison of the ecology and epidemiological roles of the different forms is greatly needed regardless of their taxonomic significance.

Studies in West Africa have shown clinal variation in the frequencies of inversions (and in chromosomal forms) along ecological transects (Touré et al. 1994, 1998). The Savanna form is often found farthest from main rivers and from flooded/irrigated areas. Its immatures occur mainly during the rainy season and in rain-dependent habitats. The Mopti form is often found in drier conditions and in flooded/irrigated areas, such as rice fields (Touré et al. 1994). In contrast,

[1] IRSS/Centre Muraz, Laboratoire de Parasitologie/Entomologie BP 390 Bobo Dioulasso, Burkina Faso.

[2] Entomology Branch, Division of Parasitic Diseases, Centers for Disease Control and Prevention, 4770 Buford Highway, Chamblee, GA 30041.

[3] Department of Biology, Emory University, Atlanta, GA 30322.

[4] Centre International de Recherches Agronomiques pour le Développement Programme Econap, campus international de Baillarguet 34398 Montpellier Cedex, France.

[5] IRD/OCEAC Yaoundé BP 288 Yaoundé, Cameroun.

[6] Malaria Epidemiology Branch, Division of Parasitic Diseases, Centers for Disease Control and Prevention, 4770 Buford Highway, Chamblee, GA 30041.

[7] Current address: Laboratory of Malaria and Vector Research, NIAID, National Institute of Health, 12735 Twinbrook Parkway, Room 2W13A, Rockville MD 20852.

0022-2585/05/0548–0553$04.00/0

surveys in Mali, where M and S forms were sympatric, revealed that both co-occurred in many larval sites (Edillo et al. 2002), as was the case for *An. gambiae* and *An. arabiensis* in Kenya (Gimnig et al. 2001). The co-occurrence of forms does not necessarily mean that they have the same ability to exploit these shared habitats. The current study was designed to compare the capacity of the molecular forms to develop in different habitats.

Materials and Methods

Study Areas. We compared larval developmental success in M-typical and S-typical habitats by selecting areas where each form is found nearly exclusively. Throughout this article, larval site represents a body of water where we found *An. gambiae* larvae, whereas habitat represents either rice fields in Bama or rain-dependent puddles and quarries in Kuinima. The village of Bama located 30 km from Bobo Dioulasso and surrounded by rice fields was selected as a typical M-form environment because this form predominated in collections of adult mosquitoes throughout the year (>95%; Robert 1989, Diabaté et al. 2002). The district of Kuinima on the periphery of Bobo-Dioulasso was selected as a typical S-form environment, where this form predominates (>90%; Diabaté et al. 2002) in collections made during the rainy season. Larval habitats in Kuinima consist mostly on rain-filled puddles and quarries. Based on indoor-resting adult (A) collections and larval (L) samples taken before experiments, the M form dominated in the village closest to the rice fields (94% of A, $n = 99$; 92%of L, $n = 76$) and the S form dominated in Kuinima (92% of A, $n = 24$; 94% of L, $n = 36$).

Transplantation Cages. One or more sets of three cages were placed in each habitat. A set consisted of a single-form M cage, a single-form S cage, and a mixed form (M:S) cage 50:50 placed ≈1 m apart (Fig. 1). Cylindrical cages (35 cm in diameter, 48 cm in height) were constructed from a metal frame covered from the bottom to the middle with cloth screen, 0.206 mm in width by 0.221 mm in mesh height, to contain the larvae but allow exchange of water, solutes, small particles, and microorganisms (Fig. 1). From the middle to the top, the cage was covered with a cloth of larger mesh size to prevent adult mosquitoes from entering or exiting the cage. The upper cloth was fitted with a sleeve through which adult mosquitoes were aspirated from the cage. The cages were free of predators and secured to the ground with stakes.

Larvae, Transplantations, and Emerged Adults. Gravid and blood-fed *An. gambiae* females were collected in Bama and Kuinima and provided with sugar water for 48–96 h in the laboratory. At that time, they were individually transferred into oviposition cups. After they laid eggs, they were killed and preserved in 85% ethanol, and their molecular form was determined by polymerase chain reaction performed on a single leg (Favia et al. 2001). Batches of 100 first instars, representing two to three families of each molecular form, were counted and quickly transferred into the field.

The cages were placed in sites where mixtures of *An. gambiae* developmental stages were observed (first or second stage larvae with either third or fourth stages or pupae). The cages were checked once a day, and emerged adults were collected until no pupae, larvae, or adults were observed for two consecutive days. Emerged adults were counted and preserved in 85% ethanol 24 h after emergence. All adults from mixed cages and 16 adults from each of the single-form cages were identified using the assay described by Fanello et al. (2002).

Data Analyses. Developmental success of the molecular forms in transplantation cages was measured by 1) the total number of male and female adults that emerged from each cage (adult emergence rate) and 2) the median developmental time of larvae in each cage. Because the number of larvae of each form (50) placed in the mixed form cages was different from that in the single-form cages (100), a separate analysis was performed for each type of cages. The total number of adults of each form in each cage and the median larval developmental time of each cage with seven or more emerging adults were subjected to an analysis of variance (ANOVA) or multiple analysis of variance (MANOVA) to test the effect of the molecular form, habitat (rice fields in Bama versus temporary pools/quarries in Kuinima), and their interactions. Statistical analyses were performed using SAS (SAS Institute 1999).

Results

In total, 2,445 adults were collected from 79 transplantation cages between day 5 and day 17 post-transplantation (pt). Molecular identification of adults (Fanello et al. 2002) was performed on 1,719 specimens comprising all adults that emerged in the mixed form cages and on 16 adults from each single-form cage. Unexpectedly, 30 *An. arabiensis* adults (1.7%) were found among the mosquitoes that emerged from 16 cages, suggesting that the screen used for cages allowed some larval immigration. Likewise, 61 adults collected in 53 single-form cages were of the "wrong" molecular form. Subsequent experiments revealed that first stage larvae could pass through the cloth in small numbers (≈3.5 per 100 during 24 h, and ≈7 in total because the larvae molt to the second instar stage by the time they are 3 d old). To minimize the effect of migration of L1 into the cages, we excluded from the analysis adults collected after the seventh day of emergence (day 11 pt), and all data from 11 cages with >6% of the adults belonging to the "wrong" molecular form. This procedure reduced the total number of adults to 1,657 (of which 1,112 were identified molecularly), the total number of *An. arabiensis* adults was reduced to seven (0.6%), and the total number of wrong molecular forms was reduced to seven, representing 2.6% of the adults collected in cages where this determi-

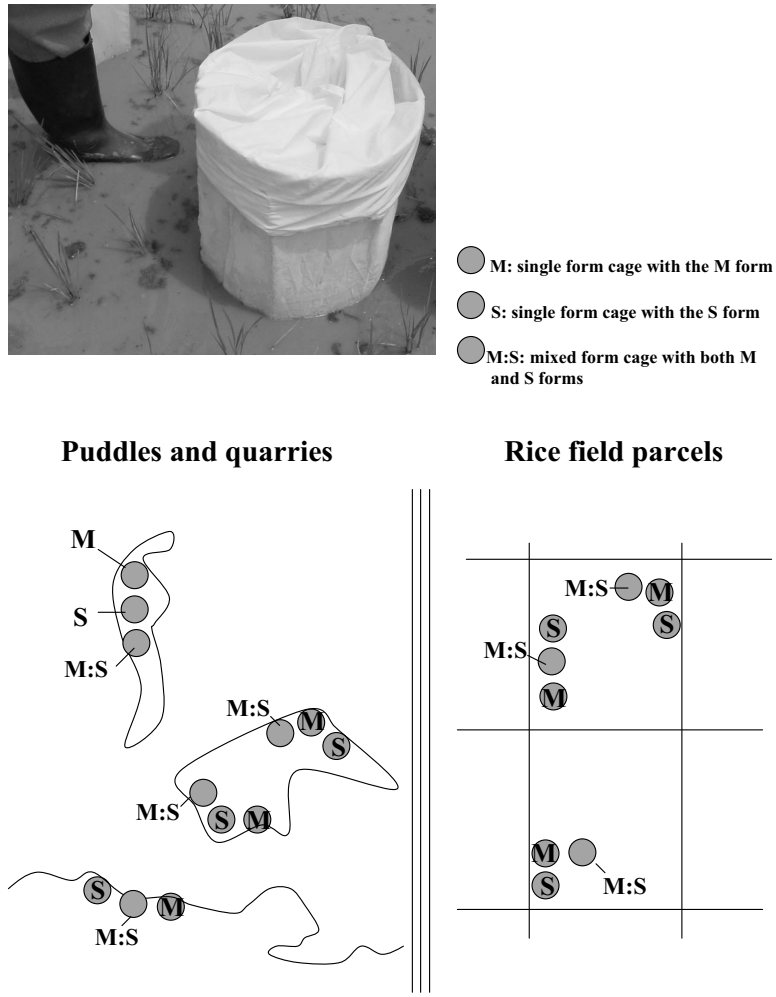

Fig. 1. Developmental time of larvae of the M and S molecular forms in different habitats by sex. Bar charts are based on all cages. Arrows represent the overall median developmental time after pooling males and females. Adults emerging after the day 11 post-transplantation were excluded (see text for details).

nation could be made (i.e., single-form cages of S larvae transplanted into rice fields dominated by M form and single-form cages of M larvae transplanted into puddles and quarries in Kuinima where S larvae dominated).

Adult Emergence Rate. Emergence success of males and females was similar (overall 857 females and 853 males, df = 1, $P > 0.8$, χ^2 test; sex ratio difference from 1:1 was not significant when tested by habitat or by day of emergence). Accordingly, analyses were done on the total number of adults that emerged from each cage. Emergence success in the puddles and quarries near Kuinima was more than three-fold higher than in the rice fields (Table 2) for both forms regardless of the cage type (single-form and mixed-form), resulting in a significant habitat effect in both analyses (Table 1). Likewise, emergence success of the S molecular form was higher than that of the M form in both habitats (Table 2), but this difference was highly significant in the mixed cages and not significant in the single-form cage type (Table 1). The interaction term form × habitat was not statistically significant (Table 1).

To evaluate the role of interform competition, we tested the following predictions. In temporary habitats where the S form predominates, 1) emergence success of the M molecular form in the mixed form cages (with competitor) will be lower than that in the single-form cages (without competitor), whereas 2) the emergence success of the S form in the mixed form cages will be higher than that in the single-form cage because being a superior competitor in this habitat, intraform competition will affect the S form more negatively than interform competition with an inferior competitor. Corresponding predictions also were made for the rice fields where the M form predominates, with the reverse form relations. The results were tested using one-tailed Fisher's exact tests of four

Table 1. Emergence success (number of adults per cage) of the molecular forms in different habitats and transplantation cages

Source	Single form ($r^2 = 0.46$)			Mixed form[a]		
	df	*F*	*P*	df	Wilks' λ[c]	*P*
Model	3	13.2	<0.0001	—	—	—
Habitat[b]	1	31.9	<0.0001	2/20	0.52	0.002
Form	1	3.2	0.08	1/21	0.68	0.005
Form × habitat[d]	1	0.2	0.67	1/21	0.31[e]	0.58
Error	38					

[a] Number of adults (per cage) produced by both forms in the mixed form cages were subjected to MANOVA to test the effect of habitat and form simultaneously.

[b] Habitat distinguishes between rice fields versus puddles and quarries.

[c] Wilks' λ is a likelihood ratio test statistic commonly used in MANOVA.

[d] In the mixed form cages, the test of form × habitat interaction was performed using a single ANOVA on the ratio M:S per cage with habitat as the independent factor.

[e] Mean square of the source and error terms are given in this column instead of the multivariate Wilks' λ.

contingency tables, contrasting the number of L1 that developed into adults and the number that failed to do so (columns) in each cage type (rows). The results are consistent with these predictions, but they are statistically significant only in temporary larval sites (Table 2). In the rice fields, there were minimal (and nonsignificant) differences between cage types of both forms possibly because of the lower overall density of developing larvae. Overall, these results provide some support for the hypothesis that competition plays a role in segregating the molecular forms, at least in temporary larval sites dominated by the S molecular form.

Larval Developmental Time. The median developmental time of males and females was not significantly different when tested over the whole data and separately for each form and habitat (paired *t*-test and paired Wilcoxon test performed on the difference between the median developmental time of the males and females in each cage, $P > 0.3$; Fig. 2). Thus, subsequent analyses were performed on the total number of adults.

The ANOVA model testing the effect of form, habitat, and their interaction on the median larval developmental time was applied to the cages with a minimum of six emerging adults during days 5–11 pt. Developmental time of both forms was longer in rice fields than in temporary sites near Kuinima (the average median developmental times ± standard deviation were 9.6 ± 1.2 and 7.3 ± 1.7 days for the M form and 8.7 ± 1.4 and 7.9 ± 1.4 days for the S form; Fig. 2). The effect of form and the interaction between form and habitat were not statistically significant (Table 3). There was no evidence of competition between forms affecting larval developmental times that were not longer in the mixed cages (Table 3).

Discussion

In arid environments of West Africa, where the Savanna and Mopti chromosomal forms correspond to the S and M molecular forms, respectively, these taxa differ in the way they exploit the environment because their spatial and temporal distributions are different (Coluzzi et al. 1979; Coluzzi 1999; Touré et al. 1994, 1998). Our study area reflects these differences as the M form predominated near the rice fields of Bama, whereas the S form predominanted in Kunima, only 30 km away. If the M form is better adapted to rice fields and the S form is better adapted to temporary habitats (Sagnon et al. 2000), then we would expect the results of the transplantation experiment to reflect this difference. In contrast our results revealed that the developmental success of the S-form larvae was higher than that of the M form in both habitats. Although this finding demonstrates a difference between forms, it does not suggest that the spatial segregation between forms is determined by larval adaptations to different habitats. Possibly, the spatial segregation between forms is based on adaptive differences in the adult stage. Preferences for different oviposition sites remain to be studied, although it is probably related to the prospects of the larvae to successfully develop in the larval site. Alternatively, our transplantation cages did not represent all the conditions in the habitat, and in particular they excluded predators. Accordingly, predator avoidance in predator-rich permanent habitat such as a rice field may be the key adaptation of the M from, whereas the S form specializes in exploiting temporary habitats such as puddles, with low predation pressure. Finally, we cannot entirely rule out that movement of L1s in and/or out of the cages confounded our results (but see below).

The average developmental time of both forms was shorter in temporary habitats than in rice fields, and adult emergence rate was considerably higher in the former sites. Possibly, the average temperature in rice fields is lower than in puddles, explaining the difference in the habitat productivity. Our results suggest that the lower productivity of rice field sites is "com-

Table 2. Emergence success of the molecular forms in mixed and single-form cages

Cage type	Temporary larval sites (Kuinima)		Rice fields	
	M form	S form	M form	S form
Mixed form	34.4% (155/450)	58.7% (264/450)	9.7% (68/700)	15.6% (109/700)
Single form	39.8% (171/600)	53.6% (589/1100)	7.8% (70/900)	16.2% (259/1,600)
Fisher's one-tailed exact test	$P = 0.042$	$P = 0.037$	$P > 0.17$	$P > 0.6$

The proportion of adults produced per first stage larvae was derived by pooling across all cages corresponding to each group. Number of adults produced over the number of first stage larvae are in parentheses (see text for details).

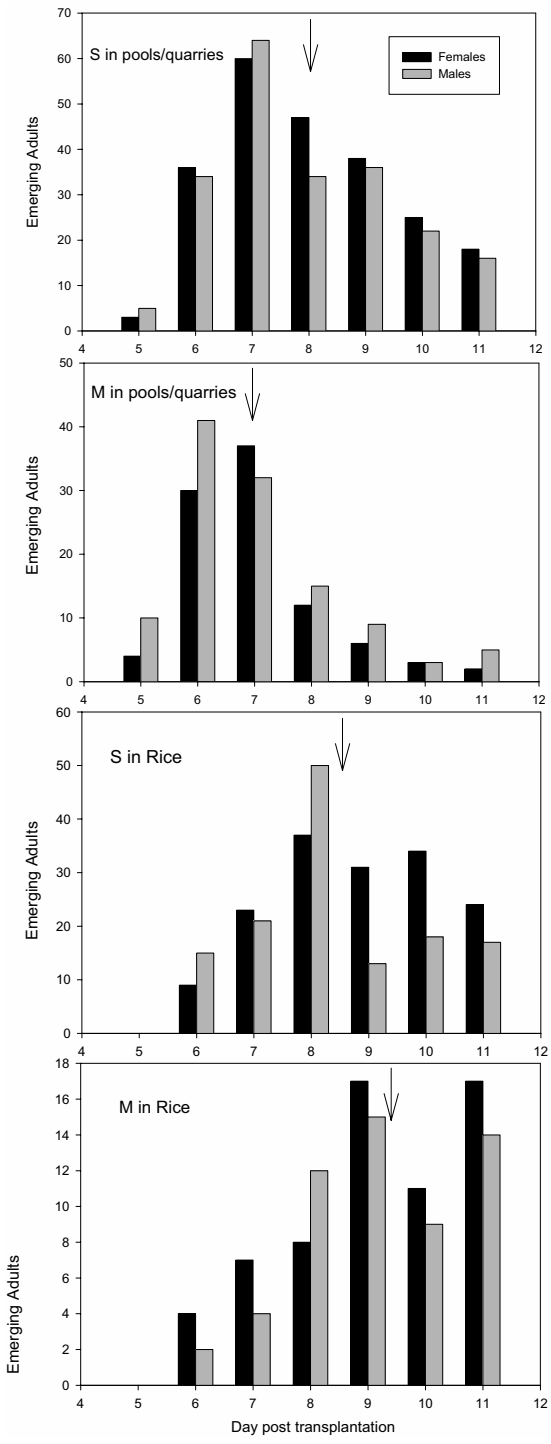

Fig. 2. A picture of a transplantation cage and a schematic showing the experimental design.

pensated" by the abundance of larval sites in rice cultivation areas (year-round).

Observing that the M form in tropical savannah areas of West Africa is found almost exclusively and at very high densities near rice fields, even where they are surrounded by areas dominated by the S form, Robert (1989) suggested that competitive exclusion of the S form by the M form in rice fields is involved. Our results provide some evidence in support of this hypothesis, albeit in temporary habitats, based on the lower emergence success of the M form in mixed cages compared with that in single-form cages, and the higher emergence success of the S form in mixed cages compared with that in single-form cages. Nevertheless, the observed differences seem modest and therefore suggest that competition contributes to the sharp spatial segregation between forms rather than fully explains it. The lower density of larvae in the rice fields might have been below the level in which competition (inter- and intraform) would be manifested. Importantly, these results reflect predator-free settings; therefore, they are compatible with the hypothesis that predation is important for the segregation of larval sites. Currently, we are conducting studies to evaluate this hypothesis.

Our results suggest that both forms successfully exploit different habitats with regard to the chemical, physical, and microbiological conditions and that larval competition between forms is a process of some importance in the spatial segregation between forms. However, our results should be considered preliminary because movement of first stage instars through the cloth of the transplantation cages may have confounded our results. The estimate of total immigration into the cage was low (<3%). Assuming that emigration out of the cage did not differ between molecular forms and between habitats, our results are expected to hold true. Importantly, our main conclusion is conservative with respect to immigration into the cages. Because in the rice fields dominated by the M form, migration into the cages would involve M larvae, and in the temporary larval sites near Kuinima, migration would involve S larvae, the stronger this migration is, the more pronounced should be the form × habitat interaction. Contrary to this expectation, the interaction was nonsignificant, lending support to the perception that the effect of the movement of first instars through the mesh was negligible.

Table 3. ANOVA for developmental time of the molecular forms in different habitats and transplantation cages

Source	ANOVA model (r^2 = 0.28)		
	df	*F*	*P*
Model	7	3.4	0.004
Habitat	1	11.9	0.001
Form	1	0.2	0.65
Form × habitat	1	2.2	0.14
Cage type	1	0.1	0.80
Habitat × cage type	1	0.5	0.47
Form × cage type	1	0.4	0.42
Habitat × form × cage type	1	2.3	0.13
Error	61		

Acknowledgments

We are grateful to T. Kaberou, A. Ouari, S. Mamourou, T. Souleymane, and T. Ibrahim for help in mosquito collection. The cooperation of many villagers facilitating our work in rice fields and gardens is deeply appreciated. Special thanks for the editor and two anonymous referees for providing helpful comments on earlier versions of this article.

References Cited

Black, W. C., and G. C. Lanzaro. 2001. Distribution of genetic variation among chromosomal forms of *Anopheles gambiae* s.s: introgressive hybridization, adaptive inversions, or recent reproductive isolation? Insect Mol. Biol. 10: 3–7.

Coluzzi, M. 1999. The clay feet of the malaria giant and its African roots: hypotheses and inferences about origin, spread and control of *Plasmodium falciparum.* Parassitologia 41: 277–283.

Coluzzi, M., A. Sabatini, V. Petrarca, and M. A. Di Deco. 1979. Chormosomal differentiation and adaptation to human environments in the *Anopheles gambiae* complex. Trans. R. Soc. Trop. Med. Hyg. 73: 483–497.

Coluzzi, M., V. Petrarca, and M. A. Di Deco. 1985. Chromosomal inversion intergradation and incipient speciation in *Anopheles gambiae.* Boll. Zool. 52: 45–63.

della Torre, A., C. Fanello, M. Akogbeto, J. Dossou-Yovo, G. Favia, V. Petrarca, and M. Coluzzi. 2001. Molecular evidence of incipient speciation within *Anopheles gambiae* s.s. in west Africa. Insect Mol. Biol. 10: 9–18.

della Torre, A., C. Costantini, N. Besansky, A. Caccone, V. Petrarca, J. R. Powell, and M. Coluzzi. 2002. Speciation within *Anopheles gambiae*-the glass is half full. Science (Wash. DC) 298: 115–117.

Diabaté, A., T. Baldet, F. Chandre, M. Akogbeto, T. R. Guiguemdé, F. Darriet, C. Brengues, P. Guillet, J. Hemingway, G. Small, et al. 2002. The role of agricultural insecticides in *Anopheles gambiae* s.l. resistance to pyrethroids in Burkina Faso, West Africa. Am. J. Trop. Med. Hyg. 67: 617–622.

Edillo, F. E., Y. T. Touré, G. C. Lanzaro, G. Dolo, and E. T. Taylor. 2002. Spatial distribution of *Anopheles gambiae* and *Anopheles arabiensis* (Diptera: Culicidae) in Banambani village, Mali. J. Med. Entomol. 39: 70–77.

Fanello, C., F. Santolamazza, and A. della Torre. 2002. Simultaneous identification of species and molecular forms of the *Anopheles gambiae* complex by PCR-RFLP. Med. Vet. Entomol. 16: 461–464.

Favia, G., A. Lanfrancotti, L. Spanos, I. Sidén-Kiamos, and C. Louis. 2001. Molecular characterization of ribosomal DNA polymorphisms discriminating among chromosomal forms of *Anopheles gambiae* ss. Insect Mol. Biol. 10: 19–23.

Gentile, G., M. Slotman, V. Ketmaier, J. R. Powell, and A. Caccone. 2001. Attempts to molecularly distinguish cryptic taxa in *Anopheles gambiae* s.s. Insect Mol. Biol. 10: 25–32.

Gimnig, J. E., M. Ombok, L. Kamau, and W. Halley. 2001. Characteristics of larval anopheline (Diptera: Culicidae) habitats in western Kenya. J. Med. Entomol. 38: 282–288.

Lanzaro, G. C., Y. T. Touré, J. Carnahan, L. Zheng, G. Dolo, S. Traore, V. Petrarca, K. D. Vernick, and C. E. Taylor. 1998. Complexities in the genetic structure of *Anopheles gambiae* populations in west Africa as revealed by microsatellite DNA analysis. Proc. Natl. Acad. Sci. U.S.A. 95: 14260–14265.

Lehmann, T., M. Licht, N. Elissa, B. T. Maega, J. M. Chimumbwa, F. T. Watsenga, C. S. Wondji, F. Simard, and W. A. Hawley. 2003. Population Structure of *Anopheles gambiae* in Africa. J. Hered. 94: 133–147.

Mukabayire, O., J. Caridi, X. Wang, Y. T. Touré, M. Coluzzi, and N. J. Besansky. 2001. Patterns of DNA sequence variation in chromosomally recognized taxa of *Anopheles gambiae:* evidence from rDNA and single-copy loci. Insect Mol. Biol. 10: 33–46.

Robert, V. 1989. La transmission du paludisme humain: la zone des savane d'Afrique de l'Ouest. Mémoire de thèse Université Paris 6, Paris, France

Sagnon, N. F., C. Costantini, A. della Torre, and M. Coluzzi. 2000. Random distribution of larval populations of sympatric members of the *Anopheles gambiae* complex in Burkina Faso. SOIPA XXI (abstr.) Parassitologia 42: 157.

SAS Institute. 1999. SAS version, 8th ed. SAS Institute, Cary, NC.

Touré, Y. T., V. Petrarca, S. F. Traoré, A. Coulibaly, H. M. Maïga, O. Sankaré, M. Sow, M. A. Di Deco, and M. Coluzzi. 1994. Ecological genetic studies in the chromosomal form Mopti of *Anopheles gambiae* s.s. in Mali, west Africa. Genetica 94: 213–223.

Touré, Y. T., V. Petrarca, S. F. Traoré, A. Coulibaly, H. M. Maïga, O. Sankaré, M. Sow, M. A. Di Deco, and M. Coluzzi. 1998. The distribution and inversion polymorphism of chromosomally recognised taxa of the *Anopheles gambiae* complex in Mali, West Africa. Parasitologia 40: 477–511.

Tripet, F., Y. T. Toure, C. E. Taylor, D. E. Norris, G. Dolo, and G. C. Lanzaro. 2001. DNA analysis of transferred sperm reveals significant levels of gene flow between molecular forms of *Anopheles gambiae.* Mol. Ecol. 10: 1725–1732.

Wondji, C., F. Simard, and D. Fontenille. 2002. Evidence for genetic differentiation between the molecular forms M and S within the Forest chromosomal form of *Anopheles gambiae* in an area of sympatry. Insect Mol. Biol. 11: 11–19.

Received 29 June 2004; accepted 31 December 2004.