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METHODS

New Multilocus Variable-Number Tandem-Repeat Analysis (MLVA) Scheme for Fine-Scale Monitoring and Microevolution-Related Study of *Ralstonia pseudosolanacearum* Phylotype I Populations

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ABSTRACT Bacterial wilt caused by the *Ralstonia solanacearum* species complex (RSSC) is considered one of the most harmful plant diseases in the world. Special attention should be paid to *R. pseudosolanacearum* phylotype I due to its large host range, its worldwide distribution, and its high evolutionary potential. So far, the molecular epidemiology and population genetics of this bacterium are poorly understood. Until now, the genetic structure of the RSSC has been analyzed on the worldwide and regional scales. Emerging questions regarding evolutionary forces in RSSC adaptation to hosts now require genetic markers that are able to monitor RSSC field populations. In this study, we aimed to evaluate the multilocus variable-number tandem-repeat analysis (MLVA) approach for its ability to discriminate genetically close phylotype I strains and for population genetics studies. We developed a new MLVA scheme (MLVA-7) allowing us to genotype 580 *R. pseudosolanacearum* phylotype I strains extracted from susceptible and resistant hosts and from different habitats (stem, soil, and rhizosphere). Based on specificity, polymorphism, and the amplification success rate, we selected seven fast-evolving variable-number tandem-repeat (VNTR) markers. The newly developed MLVA-7 scheme showed higher discriminatory power than the previously published MLVA-13 scheme when applied to collections sampled from the same location on different dates and to collections from different locations on very small scales. Our study provides a valuable tool for fine-scale monitoring and microevolution-related study of *R. pseudosolanacearum* phylotype I populations.

IMPORTANCE Understanding the evolutionary dynamics of adaptation of plant pathogens to new hosts or ecological niches has become a key point for the development of innovative disease management strategies, including durable resistance. Whereas the molecular mechanisms underlying virulence or pathogenicity changes have been studied thoroughly, the population genetics of plant pathogen adaptation remains an open, unexplored field, especially for plant-pathogenic bacteria. MLVA has become increasingly popular for epidemiosurveillance and molecular epidemiology studies of plant pathogens. However, this method has been used mostly for genotyping and identification on a regional or global scale. In this study, we developed a new MLVA scheme, targeting phylotype I of the soilborne *Ralstonia solanacearum* species complex (RSSC), specifically to address the bacterial population genetics on the field scale. Such a MLVA scheme, based on fast-

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evolving loci, may be a tool of choice for field experimental evolution and spatial genetics studies.

KEYWORDS VNTR, microevolution, epidemiosurveillance, molecular epidemiology, population biology, bacterial wilt

Intensification of agriculture during the 20th century led to global changes in cropping practices and to intensification of rapid and large-scale exchanges. Thus, it increased risks for food security by contributing to significant changes in plant pathogen populations, such as emergence and/or reemergence of pathogens and selection of virulent pathogen populations (1). Among the different strategies to control plant pathogens, deploying resistant plant varieties appears to be the most promising solution (2). The implementation of efficient and durable disease management strategies requires deciphering of the ecological and genetic mechanisms that lead to the adaptation of a pathogen population to a new host or a new resistant cultivar (3). Thorough investigation of these mechanisms on the field scale requires the use of efficient *ad hoc* molecular markers.

To date, several markers have been developed through the use of DNA fingerprinting methods (restriction fragment length polymorphism [RFLP], amplified fragment length polymorphism [AFLP], and random amplified polymorphic DNA [RAPD] analyses) and housekeeping gene sequencing (multilocus sequence typing [MLST] and multilocus sequence analysis [MLSA]). Nevertheless, variable-number tandem-repeat (VNTR) markers, with their fast molecular clock (4), remain the most suitable tool for molecular epidemiology studies (5). VNTR are tandem repeats of sequences ranging from 5 to 100 bp; they have been used more and more for genotyping due to their portability between different laboratories, their reproducibility, and their high level of polymorphism on monomorphic bacteria (6). Multilocus VNTR analysis (MLVA) has been used widely in the medical field, especially to monitor epidemics of monomorphic bacteria, such as *Bacillus anthracis* (7), *Yersinia pestis* (8, 9), *Mycobacterium tuberculosis* (10, 11), *Burkholderia pseudomallei* (12, 13), and methicillin-resistant *Staphylococcus aureus* (14), and to assess their evolutionary history on different time and space scales (15, 16). Although there is a growing interest in the use of MLVA on plant-associated bacteria, only a few studies have been published, focusing on their population biology. These studies aimed at tracing sources of water contamination (*Ralstonia solanacearum* [17]), assessing the host-driven pathogen population structure (*Pseudomonas syringae* pv. maculicola and *P. syringae* pv. tomato [18], *Xanthomonas axonopodis* pv. manihotis [19], and *R. solanacearum* [20]), reconstructing the emergence history (*Xanthomonas citri* pv. citri [21–23]), evaluating the structure and evolution of pathogen populations (*X. citri* pv. citri [22] and *Erwinia amylovora* [24]), and global surveillance (*Xanthomonas oryzae* pv. oryzae and *X. oryzae* pv. oryzicola [25]). Studies investigating population structure on a small geographical scale have seldom been realized for plant-associated bacteria or for plant-associated oomycetes and fungi (22, 26–28).

Bacterial wilt caused by the *R. solanacearum* species complex (RSSC) (29, 30), a soilborne proteobacterium, is one of the most economically important bacterial plant diseases worldwide (31), mainly due to its wide geographical distribution and its large host range (250 host species in 54 botanical families [32]). The RSSC is considered a rather primitive vascular plant pathogen (33) and is composed of four phylotypes that correlate with their geographical origins (29). These phylotypes display specific evolutionary dynamics as estimated by their recombination rates and demographic histories (34). A splitting of the RSSC was proposed recently, reclassifying (i) phylotypes I and III into *Ralstonia pseudosolanacearum*, (ii) phylotype II into *R. solanacearum*, and (iii) phylotype IV into *Ralstonia syzygii* (35). The *R. pseudosolanacearum* phylotype I population structure presents molecular signatures of a recent and rapid demographic expansion (34), suggesting a high dissemination capacity. Moreover, phylotype I is highly recombinogenic (34, 36) and distributed worldwide (32), affects the largest number of hosts (37, 38), and contains all six pathoprofiles as described by Lebeau et

TABLE 1 Comparison of genetic diversity revealed by the MLVA-13 and MLVA-7 schemes for descending scales, i.e., worldwide, regional (Ivory Coast), and field (Vallon 2012) scales[b]

Phylotype I collection (reference)	MLVA-13 scheme[a]				MLVA-7 scheme			
	n	H_E	No. of haplotypes	G/N ratio	*n*	H_E	No. of haplotypes	G/N ratio
Worldwide (41)	34	0.374	31	0.91	34	0.666	31	0.91
Ivory Coast (41)	18	0.307	15	0.83	18	0.578	17	0.94
Vallon 2012 (this study)	319	0.013	3	0.007	319	0.298	43	0.135

[a]Described in reference 20.

[b]*n*, number of strains; H_E (or H_{ub}), Nei's unbiased gene diversity index per locus; G/N ratio, ratio of the number of genotypes to the number of strains. All statistics were calculated with GenAlex v. 6.5.

al. (virulence patterns on a core collection of tomato, eggplant, and pepper plants [39–41]). Altogether, these features clearly indicate that phylotype I possesses a high evolutionary potential *sensu* McDonald and Linde (42). This phylotype is thus an interesting model for studying the evolutionary dynamics of plant-bacterium interactions. Although the molecular basis of its interactions with plants and the variability of its genome have been investigated widely (43–49), the population genetics and dynamics of this fascinating plant pathogen remain poorly understood and considered. Until now, only two studies have addressed the population genetic structure on a local scale. The first one was conducted in India by using RAPD markers (50), and the second was performed on Trinidad Island by using repetitive sequence-based PCR (REP-PCR) (51). Unfortunately, the low repeatability and reproducibility with RAPD markers make the results difficult to interpret, and REP-PCR portability between laboratories remains problematic (6). To date, three MLVA schemes have already been developed, to decipher RSSC population genetics on different scales (41), to investigate sources of water contamination by *Ralstonia solanacearum* (phylotype IIB) (17), and to characterize African *R. pseudosolanacearum* phylotype III strains on local and global scales (52). The MLVA phylotype I-specific scheme of N'Guessan et al. (20) (13 loci; MLVA-13) and the MLVA phylotype III-specific scheme of Ravelomanantsoa et al. (52) gave promising results in discriminating populations on scales ranging from cropping areas to continents (41).

In the present study, we first evaluated the MLVA-13 scheme for its discriminatory power on strains from the same field. Given that this scheme was poorly polymorphic on the field scale, we developed a new MLVA phylotype I-specific scheme and tested its use for conducting population genetics and microevolution studies on the field scale. More specifically, our objectives were (i) to identify new VNTR loci displaying potentially higher allelic diversity within phylotype I and III genomes, (ii) to evaluate their discriminatory power on collections sampled from the same location at different dates and collections sampled from different locations but on very small scales (single bulk soils and different plant locations), and (iii) to verify their suitability for assessing population differentiation and population evolution.

RESULTS

The MLVA-13 scheme is not adapted for analysis of field populations. We first used the previously developed MLVA-13 scheme (20) on the Vallon 2012 field collection, composed of 319 strains of *R. pseudosolanacearum* phylotype I. Within this collection, *egl-mutS* typing identified a single haplotype (egl-ST043, mutS-ST022, sequevar 31), identical to the sequevar 31 strain RUN0471, previously reported from Réunion Island (41). MLVA-13 identified only three haplotypes, which were most highly related to the reference haplotype H123, a singleton also corresponding to RUN0471. Only one locus (RS3L20) was polymorphic, with only three alleles detected. The genetic diversity detected was too low (Table 1) to properly perform population genetic analyses, leading us to develop a more discriminative MLVA scheme.

Tandem repeat screening leads to development of a new MLVA scheme composed of seven polymorphic loci (MLVA-7). TR searches using the Tandem Repeats

TABLE 2 Features of the VNTR loci composing the newly developed MLVA-7 scheme

Locus name[a]	Genome of origin[c]	Start position	Localization	Tandem repeat sequence	Motif size (bp)	VNTR locus size (bp) in the genome of origin	Primer sequences (5′-3′) for amplification of repeat region[d]
CMmp0131	CMR15	131842	Intergenic region	TGCGGA	6	69	FAM-CCCCGGTGGCTTTGTCATTCCCG and GATCGGGCAGCCGGATACCGC
CMmp0233	CMR15	233468	Putative secreted protein Popf1	GCCGGA	6	76	NED-GCCTGCAGTGCCCACTTCAGGTC and ACCTGCCGTCCGACCTGTTCTTCA
GMch0133	GMI1000	133827	Conserved hypothetical protein, putative 2-nitropropane dioxygenase	TCGCAA	6	41	PET-CATCGACCTGAAGCTGGCGCAGG and TGGACTACAGCGTGATGGTCGGCA
GMch0754	GMI1000	754254	Protein of unknown function or conserved protein of unknown function, proline-alanine-aspartic acid repeated motif	GGATCGGCA	9	142	VIC-GGCTCGTTGGCGGCTTCGATGTT and CGCTGTTCAACGACCTGCCCGAG
GMch3461	GMI1000	3461892	Putative pseudogene (dehydrogenase) protein	AATGGTTG	8	57	FAM-CGAGGTCGCTCTCCAGAAGGCGA and CGAGAAGGCCAGTCCCGAGCTGA
GMmp0266	GMI1000	266226	Conserved protein of unknown function	GAAGCGACC	9	108	NED-CCTGTATCGGCGCCGGAGATTGC and TGTCCTTGCCGAACACATCCGCG
CMmp0985 (RS3L20)[b]	CMR15	985	Putative cobalamin biosynthesis protein (CobN)	CGTGAT	6	128	VIC-GCCGCGCCCAGCTCGCACA and AAGGCGCACCGCCACCCGCA

[a]Loci were named according to their genome of origin (CM = CMR15; GM = GMI1000), replicon (mp = megaplasmid; ch = chromosome), and physical position on the replicon.

[b]The CMmp0985 locus (RS3L20) was described previously (20) and was used in this study because it was the only polymorphic locus in the Vallon 2012 collection when the first MLVA scheme (MLVA-13) was used.

[c]Genomic sequences of the reference strains CMR15 (phylotype III) (43) and GMI1000 (phylotype I) (46) were used.

[d]Forward primers were labeled with the following dyes giving the indicated colors: FAM, blue; NED, yellow/black; PET, red; and VIC, green. The annealing temperature was 63°C for all primers.

Database (TRDB) and the Microorganisms Tandem Repeats Database (MTRDB) led us to select 24 TR loci: 11 localized on the chromosome (four from strain GMI1000, three from strain Y45, and four from strain CMR15) and 13 localized on the megaplasmid (five from GMI1000, three from Y45, and five from CMR15). Four of the 11 chromosomal loci were intergenic, whereas all 13 megaplasmid loci were intergenic. No significant (NS) difference in polymorphism was observed either between chromosomal and megaplasmid loci ($P = 1.000$) or between intergenic and intragenic loci ($P = 0.625$). Prescreening of these 24 loci on a 19-strain collection subset by high-resolution MetaPhor gel electrophoresis revealed only six loci to be polymorphic. These were between 6 and 9 bp long and repeated 5 to 21 times in the genome of origin (Table 2). We thus combined these six loci with the RS3L20 locus (from the MLVA-13 scheme) to define the new MLVA scheme, called MLVA-7. The interexperimental (between plates) variation of fragment size calling by capillary electrophoresis reached 1 bp when the GeneScan-500 LIZ size standard was used and allowed unambiguous assignation of alleles. The number of expected TR (*in silico* prediction) and the number of observed TR for reference strains GMI1000 (phylotype I) and CMR15 (phylotype III) were statistically similar to each other (chi-square test P value = 0.976 [NS] and 0.988 [NS], respectively).

The MLVA-7 scheme is specific to the RSSC and to phylotype I strains. All MLVA-7 loci were amplified only from RSSC strains, not from the other *Ralstonia* species (*Ralstonia mannitolilytica*, *Ralstonia insidiosa*, and *Ralstonia pickettii*) strains, indicating that they were RSSC specific (Table 3). Amplification of the MLVA-7 loci showed clearly variable specificity for the different RSSC phylotypes of the worldwide collection. All seven loci were amplified from all *R. pseudosolanacearum* phylotype I strains. GMch3461 was the only phylotype I-specific locus, whereas all six other loci were amplified from the phylotype III strains, with rates ranging from 66.66% to 100%. The GMch0133 locus was also amplified from *R. solanacearum* (phylotype II) and *R. syzygii* (phylotype IV) strains (87.5% and 75%, respectively), whereas CMmp0233 was amplified from 25% of the phylotype II strains (Table 3).

MLVA-7 loci reveal genetic diversity from the field scale to the bulk soil scale. All MLVA-7 loci showed allelic diversity in field collections, ranging from 2 to 10 alleles per locus (see Table S2 in the supplemental material). The lowest diversity was found in the Pirogue 2007 soil sample (Martinique), as expected, with one or two alleles per

TABLE 3 Typeability of each MLVA-7 locus in the RSSC and other *Ralstonia* species, as estimated by the number (percentage) of strains from which the locus could be amplified within each RSSC phylotype and the *Ralstonia* outgroup[a]

Locus	No. (%) of strains				
	Phylotype I	Phylotype III	Phylotype II	Phylotype IV	*Ralstonia* spp.
CMmp0131	580 (100)	4 (66.6)	0 (0)	0 (0)	0 (0)
CMmp0233	580 (100)	4 (66.6)	2 (25)	0 (0)	0 (0)
CMmp0985	580 (100)	6 (100)	0 (0)	0 (0)	0 (0)
GMch0133	580 (100)	6 (100)	7 (87.5)	3 (75)	0 (0)
GMch0754	580 (100)	5 (83.3)	0 (0)	0 (0)	0 (0)
GMch3461	580 (100)	0 (0)	0 (0)	0 (0)	0 (0)
GMmp0266	580 (100)	5 (83.3)	0 (0)	0 (0)	0 (0)
Total	580	6	8	4	3

[a]The amplification success rates (in parentheses) were calculated based on the total number of strains per group (last row).

locus (Table S2) and five multilocus haplotypes (Table S3). The highest diversity was found in the Vallon 2012 field collection (Table 4), with two to nine alleles per locus (Table S2) and 32 haplotypes (Table S3). Genetic diversity was thus highest in the Vallon 2012 collection, with decreasing levels in the Vallon 2009, Vallon 1999, and Pirogue 2007 collections (Table 4; see also Table S3). However, it is striking that the ratios between numbers of haplotypes and numbers of strains (G/N ratios) were similar for the Vallon 2012 and Pirogue 2007 collections (Table 4), despite the huge difference in sampling efforts.

MLVA-7 is more discriminant than MLVA-13 on both global and field scales. To compare the discriminatory power between MLVA-13 and the newly developed MLVA-7 scheme on the global (worldwide), regional (countrywide), and local (field-wide) scales, we considered the following different subsets of strains: (i) the worldwide phylotype I collection ($n = 34$), among which was defined (ii) a subset composed of representative phylotype I strains from the Ivory Coast (referred to here as the "country-scale" collection; $n = 18$), and (iii) the Vallon 2012 field collection ($n = 319$) (Table 1).

Considering the worldwide collection, the unbiased diversity index for the MLVA-7 scheme was almost 2 times higher than that for MLVA-13, though the G/N ratios were the same (0.91) (Table 1). Considering the country-scale collection (Ivory Coast strains [41]), a similar trend was observed, with MLVA-7 revealing a genetic diversity 2 times higher than that with the MLVA-13 scheme, as well as a higher G/N ratio (0.94 versus 0.83). For the Vallon 2012 field collection, the MLVA-7 scheme revealed a genetic diversity 23 times higher than that with the MLVA-13 scheme. The G/N ratio followed the same trend (Table 1).

MLVA-7 is congruent with MLVA-13 on the global scale, giving geographically relevant clusterings. We evaluated the congruence of both MLVA schemes toward the worldwide collection of *R. pseudosolanacearum* phylotype I strains ($n = 34$) by using

TABLE 4 Summary statistics for the different collections of *R. pseudosolanacearum* phylotype I strains, based on MLVA-7 loci[a]

Collection	Host	*n*	No. of haplotypes	G/N ratio	Mean N_a	*I*	H_E	Allelic richness
Pirogue 2007 (Martinique)	Soil, *Musa* fallow	38	5	0.132	1.429	0.160	0.101	1.22
Vallon 1999	*Solanaceae*, *Geraniaceae*	96	8	0.083	1.860	0.198	0.111	1.28
Vallon 2009	*Solanaceae*	90	7	0.078	1.714	0.279	0.182	1.48
Vallon 2012	*Solanum lycopersicum*	319	32	0.100	4.714	0.511	0.298	1.64

[a]*n*, number of strains; G/N ratio, ratio of the number of genotypes to the number of strains; N_a, number of different alleles; *I*, Shannon's information index; H_E (or H_{ub}), Nei's unbiased diversity index. All statistics were calculated with GenAlex v. 6.5.

TABLE 5 Correspondence between clusters identified by DAPC within the MLVA-13 data set and within the MLVA-7 data set[a]

DAPC13 cluster	No. of strains in DAPC7 cluster													
	14	2	6	13	10	4	7	1	8	11	9	12	5	3
1	**5**													
2		**3**												
3			2	1	1									
4					1	1								
5							**1**							
6								**2**						
7									**1**					
8										**3**				
9											1			
10											1	**2**		
11				2										
12													**3**	
13														2
14														2

[a]For MLVA-13, the DAPC clusters are named DAPC13.01 to DAPC13.14; the same notation was used for MLVA-7 (DAPC7.01 to DAPC7.14). Clusters that match each other are indicated in bold.

two approaches: Mantel tests on distance matrices and comparison of clusterings obtained via discriminant analysis of principal components (DAPC).

Distance matrices (Euclidean and Manhattan distances) were highly correlated, with respective Mantel correlation coefficients of 0.666 and 0.640 ($P = 0.001$ for both Euclidean and Manhattan matrices), indicating that MLVA-13 and MLVA-7 data were significantly congruent.

DAPC was performed on the worldwide collection of phylotype I strains ($n = 34$) genotyped by both MLVA-13 and MLVA-7. DAPC clusters were named by giving the MLVA scheme ("DAPC13." for data from the MLVA-13 typing scheme and "DAPC7." for data from the MLVA-7 scheme) followed by the number of the cluster (cl01 to cl14). In both DAPC analyses, all strains were assigned to a single cluster (Fig. S1), with one exception (RUN1533 with MLVA-7). Among the 14 clusters identified in both data sets, 10 DAPC clusters obtained with the MLVA-7 scheme each perfectly matched a single MLVA-13 cluster (Table 5). DAPC7.03 was split between DAPC13.13 and DAPC13.14, DAPC7.09 was split between DAPC13.9 and DAPC13.10, and DAPC7.13 was split between DAPC13.03 and DAPC13.11 (Fig. 1). Strains RUN0044 (Australia), RUN0215 and RUN0119 (Cameroon), and RUN1533 and RUN1878 (Ivory Coast) were thus ranked differently by MLVA-13 and MLVA-7 (Fig. S1). We also checked for correspondence between the clonal complexes (CC) previously identified with MLVA-13 (20) and the DAPC clusters. Most of the DAPC13 clusters corresponded to a single CC, except for DAPC13.03, which grouped strains of CC02 and CC05. The correspondence between CC and DAPC7 clusters was a little lower, since two clusters corresponded to more than one CC: (i) DAPC7.09 (CC02 and CC10) and (ii) DAPC07.13 (CC02 and CC03). CC02 was split into three clusters with both MLVA-13 and MLVA-7 (Fig. 1); CC07 and CC10 were each split into two DAPC7 clusters, whereas CC11 was split into two DAPC13 clusters. DAPC clusters were globally correlated with geographical origins for both data sets, even though 4 of 14 clusters gathered strains from two or three countries (Fig. S3).

A DAPC analysis performed on the MLVA-7 data set obtained from the Vallon 2012 (Réunion Island) ($n = 319$), Pirogue 2007 (Martinique) ($n = 38$), and worldwide ($n = 37$) collections clustered Réunion Island strains into several clusters, together with strains from different African countries (Fig. 2).

Altogether, these results indicate that MLVA-7 gave clusterings similar to those obtained with MLVA-13 on the global scale, and the geographical relevancy of these clusters seemed equivalent between both MLVA schemes. Since several clusters had mixed origins (Fig. 2), the usefulness of such an MLVA scheme for biogeographic studies looks questionable.

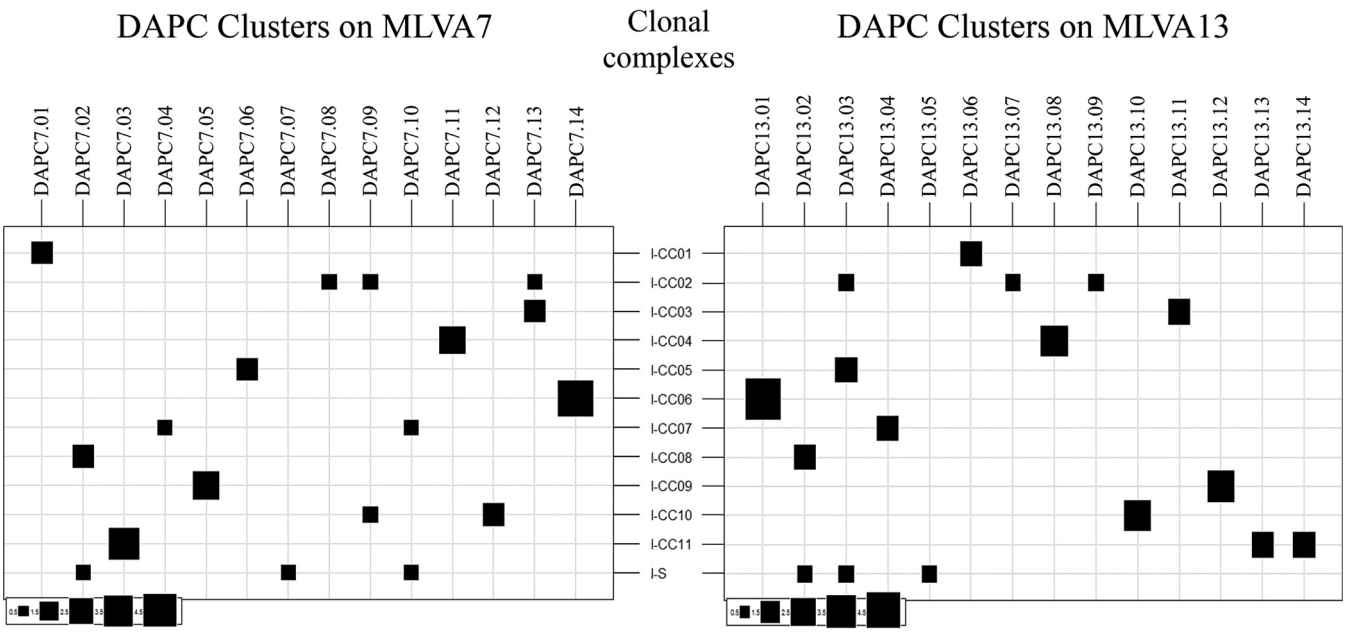

FIG 1 Correspondence between clonal complexes (defined in reference 41) and DAPC clusters inferred by MLVA-13 (right) and MLVA-7 (left). CC, clonal complex; S, singleton. The figure was created using the table.value function in the R adegenet package (87).

The MLVA-7 scheme allows for identification of epidemiological relationships within and between field collections. The epidemiological relationships between the 78 haplotypes identified within the entire phylotype I collection (Table S3) were visualized using a minimum spanning tree (Fig. 3). Two clonal complexes were identified. The main clonal complex (Fig. 2), whose founder (H018) was specific to the Vallon 2012 collection, consisted mostly of Vallon 2012 haplotypes, Vallon 2009 haplotypes, Vallon 1999 haplotypes, and three worldwide haplotypes: H58 (Ivory Coast), H20 (Ivory

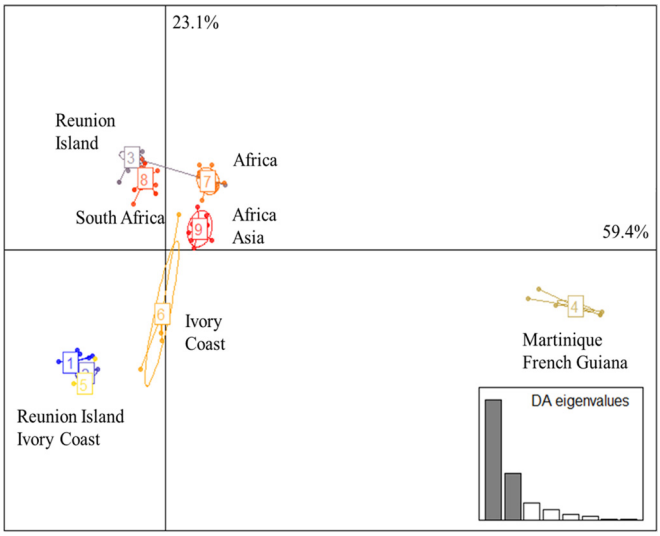

FIG 2 Scatterplot representing DAPC clusters for the Vallon 2012, Pirogue 2007 (Martinique), and worldwide collections, inferred by use of the MLVA-7 scheme. Réunion Island-specific clusters and the Martinique cluster (box 4) are associated with negative and positive values, respectively, on the *x* axis. Some clusters are shared across countries, i.e., Ivory Coast and Réunion Island (boxes 2 and 3); Martinique and French Guiana (box 4); Cameroon, Ivory Coast, Réunion Island, and Taiwan (box 7); Cameroon, Ivory Coast, South Africa, and Réunion Island (box 8); and Cameroon, French Guiana, Ivory Coast, Réunion Island, and South Africa (box 9). Numbers on axes indicate respective inertia rates. The figure was created using the find.cluster and scatterplot functions in the R package adegenet (87).

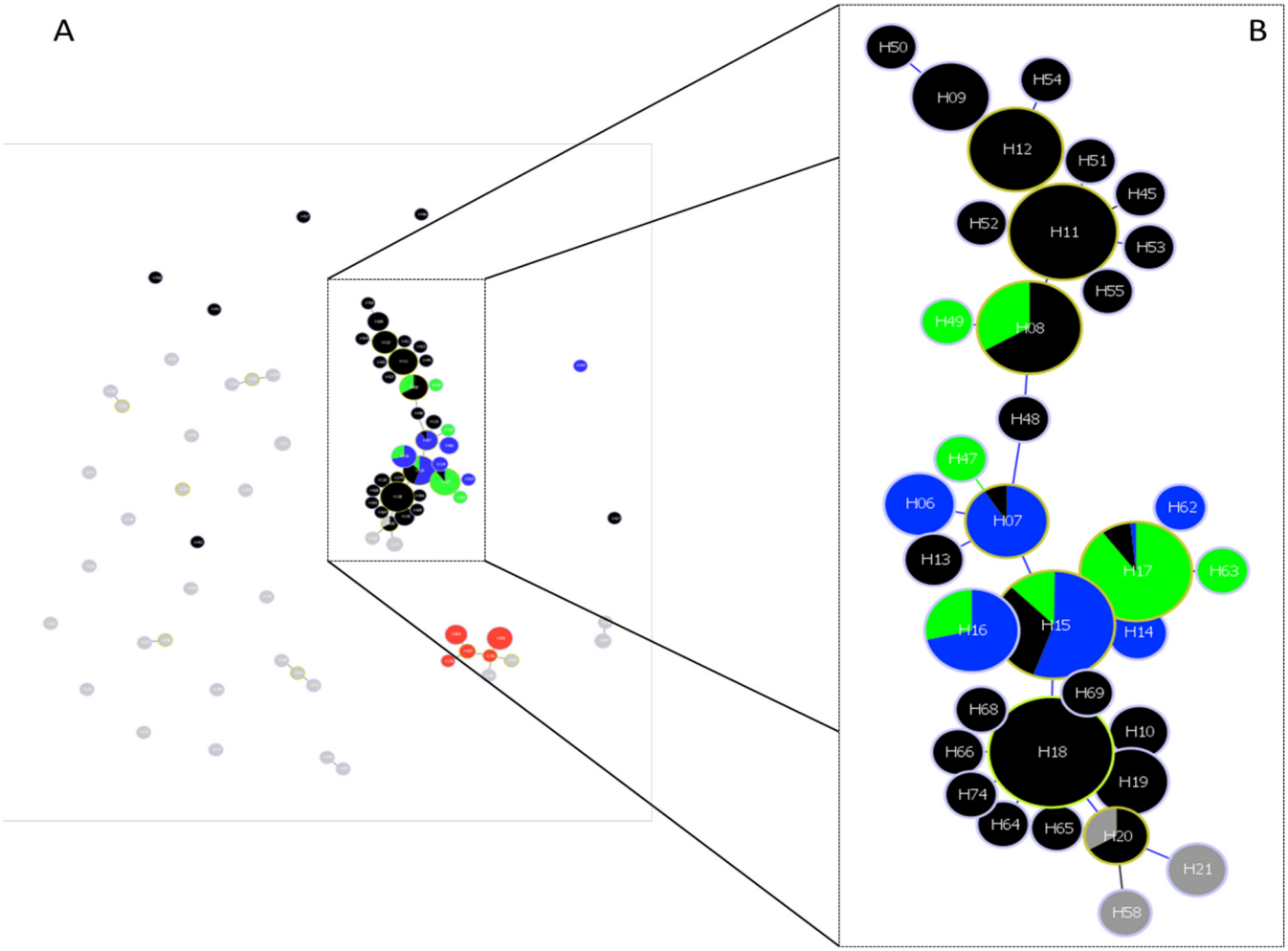

FIG 3 Haplotype network built from the MLVA-7 data sets for different collections. The Vallon 1999 collection is represented in blue, the Vallon 2009 collection is represented in green, the Vallon 2012 collection is represented in black, the Pirogue collection (Martinique) is represented in red, and the worldwide collection is represented in gray. Networks link single-locus variants (SLVs) in blue, double-locus variants (DLVs) in green, and triple-locus variants (TLVs) in red. Labels within circles indicate haplotype numbers. (A) Global view. (B) Detailed view of clonal complex 01 (CC01). The founder of CC01 is circled in yellow (H18), whereas the founders of subgroups within CC are circled in light green. This figure was built using the goeBURST algorithm implemented in PHYLOViZ 1.1.

Coast and Vallon 2012), and H21 (Kenya/Ivory Coast). The other small clonal complex gathered all five Pirogue collection-specific haplotypes (Martinique) (shown in red in Fig. 3) and two haplotypes (H035 and H039) that were specific to French Guiana. Double- and triple-haplotype clonal complexes and singletons were mostly composed of worldwide haplotypes, as well as six Vallon 2012 haplotypes and one Vallon 2009 haplotype.

The MLVA-7 scheme allows monitoring of the temporal evolution and genetic structure of a field population ("Vallon" collections). To investigate the temporal evolution of the genetic diversity and structure of a field population, we considered the three "Vallon" collections only. Ten DAPC clusters were inferred, among which two (cl07 and cl08) were common to all three collections ("universal" clusters) and six (cl01, cl02, cl03, cl04, cl09, and cl010) were specific to the 2012 sampling (Fig. 4). DAPC strongly differentiated the universal clusters from four of the Vallon 2012-specific clusters (cl01, cl02, cl04, and cl10) and from cluster 05, composed of Vallon 2009 and Vallon 2012 strains. Interestingly, cluster 05 was first detected within the Vallon 2009 collection and then increased in size within the Vallon 2012 collection. Since the Vallon 1999 and Vallon 2009 collections were sampled from plant stems only, we hypothesized that the Vallon 2012-specific clusters may have been isolated from a specific habitat (rhizo-

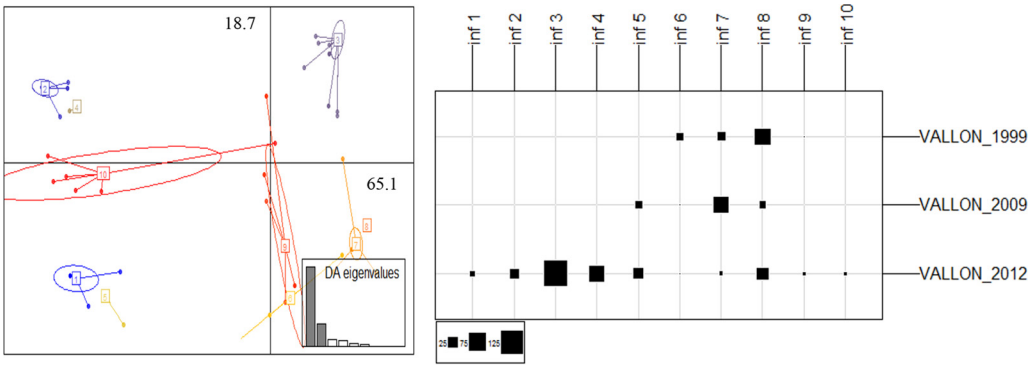

FIG 4 DAPC clusters and plot inferred for the "Vallon" collections. Strains in the Vallon 1999 ($n = 96$), Vallon 2009 ($n = 90$), and Vallon 2012 ($n = 319$) collections were genotyped using the MLVA-7 scheme (left), and their distribution within the three collections was determined using the table.value tool (right). Among the 10 clusters, five (cl01, cl02, cl07, cl08, and cl09) are specific to the Vallon 2012 collection, whereas three (cl03, cl04, and cl10) are common to all three collections. Numbers on axes indicate respective inertia rates. The figure was obtained using the find.cluster, scatterplot, and table.value functions in the R package adegenet (89). Inf, DAPC clusters inferred from the data set mentioned.

sphere or soil). However, there was no correlation between DAPC clusters and the habitat of isolation (data not shown).

MLVA-7 allows inference of the reproductive mode of *R. pseudosolanacearum* phylotype I within field collections as estimated by multilocus LD. The Pirogue 2007 (Martinique) collection, though collected from a very small amount of soil, displayed a significant linkage disequilibrium (LD), suggesting a clonal reproductive mode (Table 6). For the Réunion Island "Vallon" collections, the results contrasted: the Vallon 1999 collection appeared to be nearly recombining, while the Vallon 2009 and Vallon 2012 collections appeared to be highly clonal.

Estimation of a TR locus mutation model. In order to estimate the mutation model followed by our molecular markers, we looked at the locus variation along the haplotype network of the minimum spanning tree, specifically into the TR number variation across single-locus variants (SLV) (Fig. 3). Three loci (GMch0133, GMch3461, and RS3L20) clearly followed the stepwise mutation model (more than 80% of −1/+1 TR variation), while CMmp0131 gave a majority (55.6%) of single-TR variants (STRV) but also 44.44% 2-TR variants. Two other loci gave a majority of STRV, but at much lower rates. GMch0754 indeed gave 40% STRV, but it also gave 2-, 5-, and 6-TR variants, at a rate of 20% each. GMmp0266 gave 26.09% STRV, as well as 2-TR (8.70%), 4-TR (13.04%), 5-TR (13.04%), and 7-, 8-, 9-, 10-, and 15-TR variants (at rates of 4.35, 8.70, 4.35, 17.39, and 4.35%). Finally, the CMmp0233 locus gave only 3- and 4-TR variants (50% for both).

DISCUSSION

The proposed new MLVA scheme is the fourth one developed for the RSSC, after the initial work of N'Guessan et al. (including the phylotype I-specific MLVA-13 scheme) (20), the phylotype IIB-1-specific scheme of Parkinson et al. (17), and the phylotype III-specific scheme of Ravelomanantsoa et al. (52). These three schemes, used on regional scales, allowed the inference of possible dissemination routes of phylotype I

TABLE 6 Estimates of multilocus linkage disequilibrium[b]

Collection	I_A value	*P* value[a]	r_d value	*P* value[a]
Pirogue 2007 (Martinique)	0.301	0.014*	0.172	0.014*
Vallon 1999	0.193	0.024*	0.061	0.033*
Vallon 2009	1.179	0.001***	0.437	0.001***
Vallon 2012	1.252	0.005***	0.283	0.005***

[a]*P* values were estimated from 999 randomized permutations by using the parametric bootstrap method. Randomly permuted data sets were obtained using the "permutation over alleles" method. *, significant; ***, very highly significant.

[b]Estimation was realized on field populations within the "Vallon" collections and the Martinique collection (Fig. 3).

within the Ivory Coast and Africa (41), addressed the global surveillance of phylotype III (52), and identified sources of contamination of the Thames by phylotype IIB-1 (17). These studies improved the comprehension of RSSC epidemiology, its spread, its structuration on different scales, and its colonization pattern. However, all these studies focused only on large scales and did not pay particular attention to how microevolution acted on the field scale. Until now, field-scale studies focusing on RSSC genetic diversity were achieved only with markers with poor interlaboratory portability (RAPD [50], REP-PCR [51, 53], and AFLP [54, 55]).

In this study, we decided to develop a new MLVA scheme, as VNTR loci have already proved their use in field-scale microevolution studies of plant-pathogenic bacterial epidemics, e.g., for local populations of *Xanthomonas citri* pv. citri from different citrus orchards in Vietnam (22, 27) and in Mali and Burkina Faso (23) as well as local populations of *X. oryzae* pv. oryzae and *X. oryzae* pv. oryzicola (25).

Our primary goal in developing this new MLVA scheme was to differentiate genetically closely related strains of *R. pseudosolanacearum* phylotype I from a unique field, and even from a unique bulk soil. We indeed demonstrated that the first phylotype I-specific MLVA-13 scheme (20) was not powerful enough on the field scale, with only one polymorphic locus (CMmp0985; also named RS3L20). We thus identified six new, polymorphic, fast-evolving loci of 6 to 9 nucleotides that were repeated 7 to 22 times in the genomes of origin, thus matching the rules previously enounced for fast-evolving loci (20). The search for loci that were both potentially fast evolving and polymorphic had a relatively low success rate, since only six loci were retained among 24 selected. Our VNTR loci were specifically present in RSSC genomes and were even specific to phylotype I as well as phylotype III, to a lesser extent. These findings, which confirmed earlier reports (17, 20), tend to support the proposed concept of three genomic species within the RSSC: the first (*R. pseudosolanacearum*) gathering phylotypes I and III, the second (*R. solanacearum*) corresponding to phylotype II, and the third (*R. syzygii*) corresponding to phylotype IV (35, 43, 56, 57).

Our second goal was to assess the discriminatory power of the newly developed MLVA scheme on the field scale. The new MLVA-7 scheme is clearly adapted to monitoring the evolution of strains sampled from the same location at different dates and from different fields on very small scales (either from single bulk soils or from different plant locations). On the field scale, the discriminatory power of MLVA-7 is much higher than that of MLVA-13 (20). Genetic diversity could be revealed on a scale as small as a single soil sample (5 g) collected in a banana fallow in Martinique; this point may open research prospects for study of bacterial microevolution in soil habitats. The MLVA-7 scheme is also discriminant enough to reveal evolutionary patterns of a bacterial population sampled from the same field at different dates ("Vallon" collections). Interestingly, our markers could distinguish between haplotypes prevalent over 14-year and 4-year periods (from 1999 to 2012 and from 2009 to 2012, respectively) and haplotypes specific to one or another sampling date. This point also paves the way for future experimental evolution assays performed on the field scale.

The third goal of this study was to evaluate the suitability of our markers for assessing population genetics and evolution studies. We demonstrated that clustering inferred by use of MLVA-7 on a worldwide collection of isolates was globally congruent with clustering inferred from the previously published MLVA-13 scheme (20). MLVA-7 loci also identified the specificity of African phylotype I populations toward Asian ones and the close relatedness between some Réunion Island strains and some Ivorian strains, previously established by multilocus sequence analysis (C. A. N'Guessan and E. Wicker, unpublished data). The geographical coherence of our markers was good, as we did not identify common allelic profiles between field collections and the worldwide collection (with one exception), though we realize that these markers are of limited value for biogeographic studies. This geographical coherence looked better than that of phylotype IIB-1 markers, which identified common allelic profiles in English and Swedish strains (17). Finally, we demonstrated that these markers could be used to decipher the evolution of field populations over time, even with different sampling

efforts. We were able to distinguish different clusters in a single field and their evolution, as previously evoked. We could also address the reproductive mode of *R. pseudosolanacearum* phylotype I field populations, distinguishing some nearly recombining populations from more clonal populations. Such evolutionary patterns need to be investigated further in collections generated by similar sampling efforts and with complementary approaches, including spatial genetics.

The genetic diversity estimated for our *R. pseudosolanacearum* phylotype I field collections ($0.101 < H_E < 0.298$) appears to be low compared to values reported for plant-pathogenic bacteria, such as *E. amylovora* ($H_E = 0.50$) (24), *X. citri* pv. citri ($H_E = 0.77$) (22, 27) and *R. solanacearum* ($H_E = 0.595$) (41). It is important that all these studies analyzed the genetic diversity in areas that were larger than ours (continent, country, and regional scales, respectively). Interestingly, our values were higher than that reported for an *R. pseudosolanacearum* phylotype III field population sampled in Cameroon ($H_E = 0.03$; G/N = 0.235) (52). An explanation for the low genetic diversity detected, apart from a possible sampling bias effect, might be that the RSSC is capable of surviving in plants and in soil in a viable but not cultivable (VBNC) state (58). Therefore, this means that the haplotypes identified might not reflect the total variability in the field and that we detected only haplotypes specifically able to grow on an agar medium.

The mutation model for our MLVA-7 loci has been determined. It has been reported that VNTR loci evolve following a stepwise mutation model (SMM) or another model, e.g., an infinite allele model (IAM) (59), a two-phase model (TPM) (60), or a generalized stepwise mutation model (GSM), or k-allele model (KAM) (61). We showed that within the MLVA-7 scheme, six loci strictly or predominantly follow SMM, whereas one locus does not follow this model. This finding is important information for future population genetics analyses, notably the assessment of population differentiation. It also illustrates that mutation dynamics of VNTR loci can be different depending on the loci and on the species studied (62, 63). SMM is the predominant mutational model for some plant-pathogenic bacteria (24, 27, 64), but data remain scarce on the actual mutation model at play and the possible variations around this model within the *Proteobacteria* phylum.

In conclusion, this study provides an adapted tool for characterizing the genetic diversity and structure of *R. pseudosolanacearum* phylotype I on fine scales, from fields to bulk soil. This MLVA-7 scheme opens research prospects toward an understanding of the evolutionary mechanisms underlying population structure and adaptation as well as plant selective pressure on the field scale. This will thus allow characterization of the impacts of resistant or susceptible plant accession and of crop successions on the spatial genetic structure of soilborne bacterial plant pathogens. Such tools will allow unraveling of the bacterial population dynamics on the plant and field scales, help us to understand plant resistance bypass processes, and pave the way to optimization of innovative disease control strategies, such as the use of resistant/susceptible varietal mixtures.

MATERIALS AND METHODS

Bacterial strains. A total of 601 strains—including 598 RSSC strains—were used in this study (Table 7; see Table S1 in the supplemental material). The species specificity of the MLVA markers was checked with DNAs from *Ralstonia pickettii* (LMG5942^T), *R. mannitolilytica* (LMG6866^T), and *R. insidiosa* (LMG21421^T) (Table S1). The phylotype specificity and phylogenetic coherence of our markers were tested on 55 RSSC strains of different phylotypes representative of the worldwide diversity (Table S1). This collection is referred to here as the "worldwide collection." The suitability of our markers for discriminating populations on the field and soil scales was assessed on (i) three collections sampled from the same *R. pseudosolanacearum* phylotype I-infected field on Réunion Island at different dates ("Vallon" collections) and (ii) a collection extracted from 5 g of soil sampled at one single location point ("Pirogue" collection) in another *R. pseudosolanacearum* phylotype I-infected field in Martinique (Table 1; Table S1).

Strains of the Vallon 1999 collection were isolated as previously described by Poussier (54). For the other collections, bacterial extractions were performed on 5-cm stem segments following a previously described protocol (40). For bacterial extraction from rhizospheres, the complete root systems were soaked in 200 to 500 ml of Van Elsas and Smalla buffer (VES buffer) (65) and shaken overnight at 120 rpm and 28°C. For bacterial extraction from soil, 20 g of fresh soil (0- to 20-cm top layer) was soaked in 100

TABLE 7 Summary of the collections of RSSC strains

Collection	Phylotype[a]	Sampling location	Country	GPS location (latitude, longitude)[b]	Sampling date	Sampled material	Host	Total no. of strains	No. of strains genotyped by:	
									MLVA-13	MLVA-7
Worldwide	I	Table S1	Table S1		Table S1	Stem	Table S1	37	34[c]	37
	II	Table S1	Table S1		Table S1	Stem and tuber	Table S1	8	8	8
	III	Table S1	Table S1		Table S1	Stem	Table S1	6	6	6
	IV	Table S1	Table S1		Table S1	Stem	Table S1	4	4	4
Field collections										
Vallon 1999	I	Le Vallon field, CIRAD exptl station of Ligne Paradis, St. Pierre	Réunion Island	−21.3208°, 55.4845°	1999	Stem and rhizosphere	*Solanaceae* and *Geraniaceae*	96		96
Vallon 2009	I	Le Vallon field, CIRAD exptl station of Ligne Paradis, St. Pierre	Réunion Island	−21.3208°, 55.4845°	2009	Stem	*Solanaceae*	90		90
Vallon 2012	I	Le Vallon field, CIRAD exptl station of Ligne Paradis, St. Pierre	Réunion Island	−21.3208°, 55.4845°	January to March 2012	Soil and stem	*Solanum lycopersicum*	319	319	319
Soil collection										
Pirogue	I	Habitation Pirogue, Le Lorrain	Martinique	+14.8030°, −61.1044°	2007	Soil	*Musa* fallow	38		38
Total phylotype I field and soil strains								543	319	543
Total phylotype I strains (field, soil, and worldwide)								580	353	580

[a]Phylotypes I and III now belong to *R. pseudosolanacearum*, phylotype II to *R. solanacearum*, and phylotype IV to *R. syzygii*.
[b]Positive latitude coordinates correspond to the North, whereas negative coordinates correspond to the South; positive longitude coordinates correspond to the East, whereas negative coordinates correspond to the West.
[c]Strains RUN1955, RUN1985, and RUN0157 could not be genotyped by MLVA-13.

ml of VES buffer, rotated for 30 min at 100 rpm, and then left to settle for 5 min. Series of 1-ml aliquots were sampled and diluted down to 10^{-4}. Afterwards, macerates (50 μl) were streaked either on Imazaki medium (rhizosphere and soil) (66) or on modified Sequeira medium (67). Plates were then incubated at 28°C for 2 to 3 days. Isolates presenting the typical RSSC morphology—mucoid, "bird's eye"-shaped beige colonies with formazan coloration—were finally plated on Kelman's triphenyltetrazolium chloride (TZC) medium (68). Finally, bacteria were stored on microbeads (Microbank ProLab Diagnostic, Neston, Wirral, United Kingdom) or in 20% glycerol at −80°C.

Molecular typing and DNA extraction. Molecular identification of the RSSC and determination of the phylotype were performed using multiplex PCR (mxPCR) on bacterial suspensions ($\sim 10^8$ cells ml^{-1} in high-pressure liquid chromatography [HPLC]-grade water) as previously described (69, 70). PCR products were then subjected to 2% agarose electrophoresis and visualized under UV light after ethidium bromide staining (5 $\mu g\ ml^{-1}$). For isolates identified as belonging to the RSSC, bacterial suspensions ($\sim 5 \times 10^8$ to 1×10^9 CFU ml^{-1}) were washed with 1 ml of 0.5 M NaCl and centrifuged at 12,000 × *g*. The pellets were subjected to Wizard genomic DNA purification lysis buffer (Promega, Fitchburg, WI), and DNA was extracted following the protocol provided by the manufacturer. The DNA concentration and quality were checked using a NanoDrop ND-8000 device (NanoDrop Technologies Inc.). For genotyping, DNA was diluted to 2 ng μl^{-1} in HPLC-grade water and stored at −30°C.

Sequence-based molecular typing (*egl* and *mutS*). Multilocus sequence typing based on partial sequences of DNA mismatch repair (*mutS*) and virulence-associated endoglucanase (*egl*) genes has been a reference method for RSSC molecular diagnostics (40, 69, 71, 72). *egl* sequevars and *mutS* sequence types were determined using the Endo-F/Endo-R and mutS-RsF.1570/mutSRsR1926 primer pairs, respectively. The PCR conditions and the sequencing procedure were described previously (40). Sequences were assembled with Geneious v. 6.0.3 (Biomatters Ltd., Auckland, New Zealand), aligned with the algorithm ClustalW (73) implemented in MEGA5 (74), and then trimmed as explained previously (34). *egl*-based sequevars and STs (eSTs) and *mutS*-based STs (mSTs) were assigned using *egl* and *mutS* reference sequences (34) within DnaSP, using the "generate haplotype list" command (75).

Genotyping with the MLVA-13 scheme (20). To assess whether MLVA-13, the first phylotype I-specific MLVA scheme, developed by N'Guessan et al. (20), was suitable for analysis on the field scale, 319 phylotype I strains from one field (Vallon 2012 collection) were genotyped using this scheme. Multiplexed primer mixes and the PCR conditions were described previously (20). Capillary electrophoreses were performed in an ABI Prism 3130XL sequencer, and the electropherograms generated were analyzed with GeneMapper v. 4.0 (Applied Biosystems, Life Technologies, Carlsbad, CA).

Development of a new MLVA scheme (MLVA-7). Three fully sequenced and annotated genomes (phylotype I strains GMI1000 [46] and Y45 [49] and phylotype III strain CMR15 [43]) that are publicly available via the MaGe interface (RalstoniaScope [76]) were screened for detection of tandem repeats using the Microorganisms Tandem Repeats Database (MTRDB) (77, 78) and the Tandem Repeats Database (TRDB) (79). It has been reported that the number of alleles correlates with the number of TR in the genome and conversely correlates with the pattern size (80), but so far, no general rules have been published. From the literature, expert personal communications (O. Pruvost, personal communication), and the development of the first RSSC MLVA scheme (20), we observed that the highest level of polymorphism was observed for loci with more than 10 repeats and pattern sizes ranging from 5 to 9 bp (20). As a consequence, after a preliminary search, we set the following criteria for TR detection in the MTRDB: total length in the range of 50 to 600 bp, TR length in the range of 5 to 9 bp, number of copies in the range of 9 to 50, matching percentage above 80%, and the default setting for other parameters. TR detection in the TRDB was performed using default parameters (alignment weights at 2 [match], 7 [mismatch], and 7 [indels]) (see the detailed explanation at the TRDB website [79]). PCR primers (18 to 27 bp) were designed within the 500-bp flanking sequences of the TR by using Geneious software v. 6.0.3 (Biomatters Ltd.). Primer sequences were then subjected to BLAST searches with the BLASTN program (81), available on the MaGe website (76), to determine the locations of loci and to check the specificity of each pattern to a single locus per genome. This first step led to the identification of 24 loci (3 detected by both platforms, 5 detected only by the MTRDB, and 16 detected only by the TRDB). These loci were further screened for amplification ability, repeatability, and polymorphism on a 19-strain collection subset (Table S1) including (i) 11 Vallon 2012 strains, selected on the basis of their allelic profiles according to the MLVA-13 scheme, their locations in the field, their hosts (tomato or susceptible or resistant eggplant), and their sampling habitats (stem, rhizosphere, or soil), and (ii) 8 worldwide strains (41), selected on the basis of the clonal complex to which they belong and their geographic origin. Simplex PCRs were performed as previously detailed (20), followed by electrophoresis migration in high-resolution Metaphor agarose (3% Metaphor and 1% SeaKem agarose; Lonza Ltd., Basel, Switzerland). Based on these results, six loci and primer pairs were selected to develop the new MLVA scheme (Table 2). As we found that CMmp0985 (RS3L20) from the MLVA-13 scheme (20) was the only polymorphic locus in the Vallon 2012 collection, this locus was added to the six other loci to compose the new MLVA-7 scheme. Primers were multiplexed into two pools of four and three pairs (Table 2), labeled on the forward primers with different fluorescent dyes, i.e., 6-carboxyfluorescein (FAM) (blue), VIC (green), NED (black/yellow), and PET (red) (Applied Biosystems). Primer concentrations and annealing temperatures were optimized for amplification of loci on four reference strains according to the manufacturer's instructions (Applied Biosystems). Capillary electrophoresis was performed as mentioned above.

VNTR analysis and performance measures. Peak data were analyzed using GeneMapper v. 4.0 (Applied Biosystems) and automatically converted to fragment sizes (in base pairs). The analyses described below were realized by using the amplification sizes. When needed, amplicon sizes were converted to repeat numbers by use of the following formula: observed size = extra TR sequence length + (TR size × repeat

number). The number of TR was rounded up to the nearest integer, as previously recommended (82). The reproducibility of the amplification of our markers was assessed using GMI1000, the reference phylotype I strain (46), as a positive control for each genotyping run, and also by twice amplifying the phylotype I worldwide collection from independent DNA extractions. Allelic profiles were identical across the different replicates.

Analysis of genetic data. Haplotypes (defined as allelic profiles), Nei's unbiased diversity index (H_E or H_{ub}) (83), the total number of alleles (N_a), and their frequencies were determined using GenAlex software v. 6.5 (84) (Table 7). To assess the correlation between locus location (chromosome versus megaplasmid and intergenic versus intragenic) and locus polymorphism, allele numbers of loci were compared statistically by the Fisher exact test under the null hypothesis (H0) that a locus has equal probabilities of being polymorphic for any position on the gene.

Haplotype networks were achieved with the globally optimized eBURST analysis (goeBURST) implemented in PHYLOViZ v. 1.0 (85). These analyses allowed us to highlight different clonal complexes, defined as groups of SLVs. It also allowed us to visualize allelic profiles diverging by one locus (SLVs) or several loci (double-locus variants [DLVs], three-locus variants [TLVs], etc.). Using the eBURST algorithm, STs are clustered based on their numbers of SLVs, DLVs, and TLVs and occurrence frequencies. The group founder is first connected to all its SLVs; then, each of those, following the ST ordering, is connected to its SLVs not yet present in the tree. This process is repeated until each element of the disjoint graph is present in the tree. The second step consists of a local optimization of the tree. The goeBURST algorithm provides a globally optimal solution and corrects links that do not strictly follow the eBURST rules.

To assess the congruence of MLVA-13 and MLVA-7 clusterings, distance matrices were calculated and compared by the Mantel test, using the CADM.post function of the R package ape 3.1-4, with 9,999 permutations (86). The Mantel correlation coefficients were computed on rank-transformed distances among the distance matrices.

The population structure of *R. pseudosolanacearum* phylotype I (the worldwide strain collection and the three "Vallon" collections) was assessed using DAPC with the adegenet 1.4.2. package (87) implemented in R software v. 3.3.1. This clustering method, free of any assumption related to a population genetics model (e.g., Hardy-Weinberg equilibrium or an absence of linkage disequilibrium) (6), provides an efficient description of genetic clusters by using a few synthetic variables (called the discriminant functions). DAPC yields linear combinations of the original variables (alleles) that maximize differences between groups while minimizing variation within clusters. Clusters were defined by independent k-means, using the Bayesian internal criterion (BIC). Twenty DAPC runs were performed in order to assess the stability of clusters. The xvalDAPC function was used to choose the optimal number of PCs for DAPC.

To detect signatures of recombination and population structure within field collections, multilocus linkage disequilibrium (LD) was estimated using the index of association calculated by the R package poppr (88). The poppr tool calculates both Brown's index (I_A), which was found to increase with the number of loci, and r_{d}, which corrects for this scaling and forces the index to lie between 0 (linkage equilibrium) and 1 (full disequilibrium). To test the significance of both indices, the package generated 999 resamplings of the data under the null hypothesis (H0) of free recombination, using the "permutation over alleles" and "parametric bootstrap" methods, and then calculated the *P* value.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.03095-16.

TEXT S1, PDF file, 0.04 MB.
DATA SET S1, XLSX file, 0.08 MB.
DATA SET S2, XLSX file, 0.01 MB.
DATA SET S3, XLSX file, 0.01 MB.

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