

New Multilocus Variable-Number Tandem-Repeat Analysis Tool for Surveillance and Local Epidemiology of Bacterial Leaf Blight and Bacterial Leaf Streak of Rice Caused by *Xanthomonas oryzae*

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Multilocus variable-number tandem-repeat analysis (MLVA) is efficient for routine typing and for investigating the genetic structures of natural microbial populations. Two distinct pathovars of *Xanthomonas oryzae* can cause significant crop losses in tropical and temperate rice-growing countries. Bacterial leaf streak is caused by *X. oryzae* pv. *oryzicola*, and bacterial leaf blight is caused by *X. oryzae* pv. *oryzae*. For the latter, two genetic lineages have been described in the literature. We developed a universal MLVA typing tool both for the identification of the three *X. oryzae* genetic lineages and for epidemiological analyses. Sixteen candidate variable-number tandem-repeat (VNTR) loci were selected according to their presence and polymorphism in 10 draft or complete genome sequences of the three *X. oryzae* lineages and by VNTR sequencing of a subset of loci of interest in 20 strains per lineage. The MLVA-16 scheme was then applied to 338 strains of *X. oryzae* representing different pathovars and geographical locations. Linkage disequilibrium between MLVA loci was calculated by index association on different scales, and the 16 loci showed linear Mantel correlation with MLSA data on 56 *X. oryzae* strains, suggesting that they provide a good phylogenetic signal. Furthermore, analyses of sets of strains for different lineages indicated the possibility of using the scheme for deeper epidemiological investigation on small spatial scales.

Molecular typing of pathogen populations is essential to gain insight into their genetic diversity and population dynamics in order to elaborate efficient strategies for disease control (1, 2). In agricultural systems, pests are ideally controlled by integrated approaches, including eradication or treatment of diseased organisms and planting of resistant varieties. However, the durability of resistance can be challenged if pathogen diversity is significant. Importantly, gene flow between pathogen populations can facilitate the breakdown of resistance in crop plants (3). Hence, efficient and precise molecular-typing tools for identifying strains and differentiating among related bacterial isolates are essential for microevolutionary reconstruction as a population genetics approach for integrated plant protection.

Rice, one of the major crops worldwide, is affected by two bacterial diseases that are caused by strains of *Xanthomonas oryzae*, bacterial leaf blight (BLB), caused by *X. oryzae* pv. *oryzae*, and bacterial leaf streak (BLS), caused by *X. oryzae* pv. *oryzicola*. Collectively, these two diseases cause significant yield losses in tropical and temperate rice-growing areas. *X. oryzae* pv. *oryzae* colonizes xylem vessels upon entry into the vascular system. *X. oryzae* pv. *oryzicola* infects the plant via natural openings and colonizes the mesophyll (4). Genomes of members of both pathovars have been sequenced; however, the determinants of tissue specificity are still largely unknown (5, 6). While *X. oryzae* pv. *oryzicola* has been shown to be seedborne and seed transmitted (7, 8), the evidence that *X. oryzae* pv. *oryzae* is seedborne is still controversial (7, 9).

Because both pathogens infect the same host species and cause symptoms that at later stages of infection may be difficult to distinguish, both BLB and BLS diseases are not easy to unambigu-

ously diagnose in the field. In the laboratory, strains of the two pathovars are identified by inoculation methods on susceptible host plants (10). Upon leaf inoculation, *X. oryzae* pv. *oryzicola* colonizes the mesophyll, resulting in water-soaked lesions, while *X. oryzae* pv. *oryzae* colonizes and spreads in the vascular system, resulting in long lesions along the leaf blade (4, 10). Besides the observation of visual symptoms, and for a more reliable diagnosis, a genomics-based multiplex PCR has been developed to differentiate the two pathovars (11). Recently, these diagnostic loci were converted into a loop-mediated isothermal amplification (LAMP) assay (12). In addition to pathovar discrimination, other assays

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were developed that can differentiate *X. oryzae* strains from Africa and Asia (13). Currently, phenotypic, multiplex PCR, and LAMP methods are routinely used by several laboratories to identify, to characterize, and to detect *X. oryzae* strains.

BLB was first described in Fukuoka Prefecture, Japan, in 1884 (14, 15) and has been reported in a number of rice-growing countries from Iran to Japan and Philippines (16). The disease was also reported in African countries, including Mali, Senegal, Niger, Gabon, Nigeria, Cameroon, Burkina Faso, Mauritania, and the Gambia (17). Reports for northern Australia (18) and South America (19) exist but are rather sporadic and less important (16).

BLS was first described in Philippines in 1918 and is prevalent in Asia (16), as well as in Africa in Mali (20) and Burkina Faso (21), and was recently reported in Madagascar (22), Burundi (23), and Uganda (24).

So far, studies have focused on *X. oryzae* pv. *oryzae*, describing the genetic and pathotypic diversity of different populations. Most of these studies analyzed regional populations of *X. oryzae* pv. *oryzae*, mainly using pathogenicity assays and/or restriction fragment length polymorphism (RFLP) profiling (25–30). For instance, Mishra and coworkers (2013) analyzed more than 1,000 isolates of *X. oryzae* pv. *oryzae* based on their reactions to 10 resistance genes. They also differentiated a subset of strains by an RFLP analysis using *IS1112* as a probe. However, the study included only strains from India (30).

Much less is known about *X. oryzae* pv. *oryzicola* diversity. In Philippines, a study evaluated the diversity of 123 *X. oryzae* pv. *oryzicola* strains by RFLP analysis, concluding that the pathovar is endemic due to the large diversity of strains (31). A repetitive-element palindromic (rep)-PCR analysis of 141 Chinese *X. oryzae* pv. *oryzicola* strains revealed significant genetic diversity among *X. oryzae* pv. *oryzicola* strains in southwest China (32). One of the most exhaustive phylogenetic studies of *X. oryzae* was performed by Gonzalez and coworkers, who analyzed a set of 26 Asian *X. oryzae* pv. *oryzae* strains, 21 African *X. oryzae* pv. *oryzae* strains, and 14 *X. oryzae* pv. *oryzicola* strains from different origins (20). Using a polyphasic approach, including RFLP, rep-PCR, and fluorescent amplified fragment length polymorphism (AFLP), three lineages were defined within the species as Asian *X. oryzae* pv. *oryzae*, African *X. oryzae* pv. *oryzae*, and *X. oryzae* pv. *oryzicola* (from Asia and Africa). More recently, multilocus sequence typing (MLST) targeting nine housekeeping genes of a few strains of *X. oryzae* confirmed the designation of the three lineages (33). Other studies combining multilocus sequence analysis (MLSA) with the analysis of the type III effector repertoires of 40 *X. oryzae* strains belonging to different pathovars and from different geographical origins clearly confirmed that both *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola* pathogens belong to closely related but distinct phylogenetic groups formerly defined as lineages (20, 34). Finally, MLST and RFLP analyses focusing on a large collection of *X. oryzae* pv. *oryzicola* strains revealed a high level of genetic diversity among African strains (35).

Molecular methods previously used to evaluate the genetic diversity of *X. oryzae* were either cumbersome, poorly reproducible, insufficiently discriminative, or difficult to interpret evolutionarily. Over the past several years, MLST based on a set of housekeeping genes has become popular to investigate microbial populations of bacterial pathogens (36). However, this technique does not have enough resolution for an in-depth study of *X. oryzae* populations or epidemic outbreaks. Now, with easy access to

nearly complete genome sequences, single-nucleotide polymorphism (SNP) analyses of genomes receive more attention due to the unprecedented increase in resolution they provide and the consequent possibility of investigating epidemics more accurately (37, 38). Although the costs of genome sequencing have decreased tremendously in the last few years, such typing methods are unlikely to become widely adopted as a standard due to bioinformatic and infrastructural constraints. Most developing countries that are concerned with epidemiological surveillance of plant diseases are not yet equipped for such analyses.

First reported in eukaryotic species, DNA motifs that are repeated in multiple copies were identified in bacterial genomes (39). Variation between strains is reflected by a change in the size of the repeat array, called variable-number tandem repeats (VNTRs) or mini- or microsatellites. Among the different models of mutations of microsatellites, the stepwise mutation model (SMM) was widely adopted for microsatellites, even if large jumps in repeat numbers may occasionally occur (40, 41). The SMM postulates that the size of a VNTR locus evolves through the addition or deletion of one repeat unit per mutation event. Consequently, VNTR loci provide us with connectible data reflecting patterns of evolutionary descent that can be used for epidemiological tracing of bacterial strains. The flanking regions next to the repeats are generally well conserved, sometimes even among different species. Consequently, PCR primers could be designed allowing the analysis of VNTR polymorphisms at different levels, e.g., the species or subspecies level (42). Since 2001, shortly after the first genome sequence became available, VNTR studies of bacterial plant pathogens became more and more popular, as exemplified by *Xylella fastidiosa* (43). In 2009, the first VNTR typing scheme was developed for *Xanthomonas* species (44). Later, a 25-locus-based VNTR scheme (named MLVA-25) was developed for *X. oryzae* pv. *oryzicola* (45) and evaluated on a limited collection of *X. oryzae* pv. *oryzicola* strains. Preliminary *in silico* analyses indicated that a few loci would be useful to characterize the *X. oryzae* pv. *oryzae* strains, as well, but the discriminatory power would have been rather low for the two *X. oryzae* pv. *oryzae* lineages in comparison to the *X. oryzae* pv. *oryzicola* lineage. Hence, a highly discriminatory multilocus variable-number tandem-repeat (MLVA) scheme that could at the same time identify the different *X. oryzae* pathovars and distinguish strains within pathovars would be very useful.

Here, we report on a new multilocus VNTR analysis that allows the production of robust and reproducible genetic data to efficiently characterize *X. oryzae* strains and to study epidemics of BLB and BLS on rice. For this purpose, a collection of 338 strains of the *X. oryzae* pathovars *oryzae* and *oryzicola*, originating from 20 countries, was analyzed. Both global and small-scale MLVAs of the three *X. oryzae* genetic lineages are discussed in the context of the geographical origins of the strains, the sampling dates, and the host plants. With this work, we wish to promote the worldwide use of MLVA-16 (a 16-locus-based VNTR scheme) for monitoring of BLB and BLS on various temporal and geographical scales.

MATERIALS AND METHODS

Bacterial strains and DNA isolation. *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola* strains were obtained from different sources (see Table S1 in the supplemental material). The strains were representative of the different lineages (127 *X. oryzae* pv. *oryzae* strains from Asia, 59 *X. oryzae* pv. *oryzae* strains from Africa, and 152 *X. oryzae* pv. *oryzicola* strains from

TABLE 1 Multiplexing scheme for primer pairs in pools

Pool	Locus	Discriminative for (polymorphic):	Diagnostic for (monomorphic) ^a :	Primer 1		Primer 2	
				Label ^b	Sequence	Label	Sequence
1	Xo_G06	<i>X. oryzae</i> pv. oryzae Asia, <i>X. oryzae</i> pv. oryzae Africa, and <i>X. oryzae</i> pv. oryzicola		VIC	GCAGACGGATGGGCGTTG	6-FAM	GCTCGCCGGCACTCTCCT
	Xo_G07			NED	CAGGGCGAACGCGATGAG		GCTCCATGGTGCCGGAGA
	Xo_G58			PET	CAACGAGGTGCCCGGCAA		GGAGCGCGCACATCATCG
	Xo_G81				GTGCCGTCTCCCACGCT		GCCATCGTACGCTGCCG
2	Xo_G2553	<i>X. oryzae</i> pv. oryzae Asia and <i>X. oryzae</i> pv. oryzae-Africa	<i>X. oryzae</i> pv. oryzicola (372)	VIC	GCTGGCGGTGACCACCAC	6-FAM	GTCCAGCAGGTGCTCGCG
	Xo_G88				CTCACTGCGGCGGTGTTG		TAAGCTGCGCAAGGCGCG
	Xo_G44				GCCGTGCTCCGTCTGCA		CCGCATCCATCGCGACAG
	Xo_G62				GCTTCGCGCACACGTGA		ACGACAAGCAGCGCCTGC
3	Xo_G55	<i>X. oryzae</i> pv. oryzae Africa and <i>X. oryzae</i> pv. oryzicola	<i>X. oryzae</i> pv. oryzae-As (294)	VIC	ACCCGGCAACTCGCAACC	6-FAM	GGCAGCAGCAAGCGGCAT
	Xo_G59				TCATTGCGTCACCCATCGG		GGCGGGCGCTCAACTGTCC
	Xo_G60				GTTGCCGCCCGCGGTAC		CGGCGTCTTGCCAACCCT
	Xo_G67				GCTTGCGGGTACATCG		TGGATCGACGCCGGACTG
4	Xo_G09	<i>X. oryzae</i> pv. oryzae Asia and <i>X. oryzae</i> pv. oryzicola	<i>X. oryzae</i> pv. oryzae-Af (133)	VIC	CCGCGATAGGCCGAGGTC	6-FAM	TGGCCTATCTGGCAGCGC
	Xo_G15				CGCAACGATGTGCTGGCG		CGCAATCGCTCGAACGC
	Xo_G80				ACGGATGGCGTTGGCCAG		CGGCATGATCTGGGCG
	Xo_G83				CACCCGGCCGGCAATATC		TGCACCACCAGTTGGCG

^a Numbers in parentheses indicate predicted sizes of PCR amplicons (in base pairs) based on the analysis of 10 genome sequences.

^b The following fluorescent labels of PCR primers were used (excitation/emission wavelengths in parentheses): VIC (535/555 nm), NED (550/570 nm), PET (570/590 nm), and 6-FAM (495/515 nm).

Africa and Asia). The isolates were maintained in 15% glycerol at -80°C . For DNA extraction, the bacteria were streaked on PSA plates (10 g/liter peptone, 10 g/liter sucrose, 1 g/liter glutamic acid, 15 g/liter Bacto Agar) and incubated for 24 h at 28°C . Two loops of bacterial cultures were washed in 1 M NaCl and then processed using the Wizard genomic DNA purification kit (Promega, Charbonnières, France) following the manufacturer's instructions.

Exploitation of genomic resources for VNTR locus extraction. A bioinformatic pipeline (<http://www.biopred.net/VNTR/>) for the prediction of VNTR loci was applied to 10 *X. oryzae* genome sequences with the following parameters: algorithm, TRF; region length, 30 to 1,000 bp; unit length, 5 to 12 bp; and at least 6 tandem repeats with a similarity of at least 80% among the repeats (45). Four complete publicly available genome sequences were screened: three *X. oryzae* pv. oryzae strains from Asia, KACC 10331 (GenBank accession number NC_006834), MAFF 311018 (accession no. NC_007705), and PXO99^A (accession no. NC_010717), as well as one Asian *X. oryzae* pv. oryzicola strain, BLS256 (accession no. NC_017267). In addition, we included six draft genome sequences: Asian *X. oryzae* pv. oryzae (PXO86), African *X. oryzae* pv. oryzae (BAI3, MAI1, and NAI8; accession no. AYSX00000000), and *X. oryzae* pv. oryzicola (GXO1 and MAI10; accession no. AYSY00000000). The pipeline first predicts VNTR loci for each individual genome sequence. In a second step, the VNTR loci are grouped by homology based on the conservation of flanking sequences. In the last step, genomes for which a certain VNTR locus was not predicted in step 1 (e.g., because they do not match the search criteria, such as the minimal number of repeats) are searched for homologous loci, as revealed by the presence of conserved flanking sequences. Finally, amplicon sizes were predicted for the 10 sequenced genomes at the website (http://minisatellites.u-psud.fr/ASPSamp/base_ms/blast/blast_primers_multi.php) in order to estimate if the loci are polymorphic for a given genetic lineage.

Four sets of PCR primers were designed for quadruplex PCRs with an optimal size range of the amplicons and minimal overlap in size, as shown in Table 1. The primers were designed based on a sequence alignment of

the 500-bp flanking regions next to the VNTR loci, and the designed primers were subsequently tested (<http://www.thermoscientificbio.com/webtools/multipleprimer/>) for the optimal annealing temperature and avoidance of dimer formation.

MLVA genotyping. Genotyping was performed based on the method described for *Xanthomonas citri* pv. *citri* (46). Basically, four VNTR loci were amplified in a quadruplex PCR with four primers 5' labeled with 6-carboxyfluorescein (6-FAM), NED, PET, and VIC fluorescent dyes (Applied Biosystems). Conditions were optimized based on test runs (Table 1). One microliter of diluted amplicons (10- to 50-fold dilution, determined from test runs) was mixed with 0.3 μl of the GeneScan -500 LIZ internal size standard (Applied Biosystems) and 10.7 μl of Hi-Di formamide (Applied Biosystems). Capillary electrophoresis was performed on an ABI Prism-3130xl genetic analyzer.

Amplicon sizes were scored with GeneMapper 4.0 (Applied Biosystems) and then converted to a number of tandem repeats (TRs) per lineage as described in Data Set S1 in the supplemental material. As a standard, when a TR copy was truncated, the TR number was rounded to the next higher number.

Bioinformatic analyses. Hunter-Gaston Discriminatory Index (HGDI) scores were calculated (via http://insilico.ehu.es/mini_tools/discriminatory_power/) in order to evaluate the discriminatory power for each locus (47). Allelic richness was calculated for different group combinations using the rarefaction method with HP-Rare v6.6.6 (48). The genetic differentiation between geographic and genetic groups was estimated as fixation indices (F_{ST} values) calculated by Arlequin v.3.5.1.3 (49). As recombination can impact the apparent phylogeny of the strains, we quantified the degree of linkage disequilibrium by estimating the multi-locus indices of association, I_A , and the r_D (corrected index of association) using Multilocus v1.3b (50) with 1,000 randomized data sets. To investigate the correlation between VNTR data and previously published typing data, Mantel tests were performed on pairwise distance matrices with 999 permutations using GenAIEx 6.5 (51). Two data sets were used: MLSA data for 35 individuals (11 African *X. oryzae* pv. oryzae strains, 15 Asian *X.*

oryzae pv. *oryzae* strains, and 9 *X. oryzae* pv. *oryzicola* strains) (34) and RFLP data (Southern blots hybridized with a portion of the *avrXa7* gene encoding a type III effector of the transcription activator-like family) for 56 *X. oryzae* pv. *oryzicola* strains (35).

Phylogenetic relationships within each lineage were determined using different approaches. The patterns of evolutionary descent were inferred using minimum spanning trees (MST) (categorical values or Manhattan distances). Manhattan neighbor-joining (NJ) phylogenetic trees using Jacquard correlation were constructed using BioNumerics v7.2 (Applied Maths, Sint-Martens-Latem, Belgium). Clonal complexes were analyzed with eBURST v3 to find descent patterns between the allelic types (sequence types [STs]) and founder types (52). Groups of strains differing by less than three loci, i.e., consisting of identical types, single-locus variants (SLVs), and double-locus variants (DLVs), were defined as clonal complexes. Discriminant analysis of principal components (DAPC), a clustering method without *a priori* assumptions, was performed using R version 3.0.1 (2013-05-16) with the “ade4” package (53).

MLVA database. MLVA data were deposited in the public database MLVAbank hosted at Institut de Recherche pour le Développement (IRD) Montpellier (<http://www.biopred.net/MLVA/>), which allows sharing and comparing of information with other scientists in an interactive manner and provides access to a few phylogenetic-analysis tools (46).

RESULTS

In silico analysis. Using 10 available genome sequences (four Asian *X. oryzae* pv. *oryzae* strains, three African *X. oryzae* pv. *oryzae* strains, and three *X. oryzae* pv. *oryzicola* strains), 92 VNTR loci with 5-bp to 12-bp repeats were identified. Surprisingly, only five VNTR loci were conserved and polymorphic among the three genetic lineages previously defined by Gonzalez and coworkers (20). Thirty-seven additional VNTR loci were conserved and polymorphic among two different genetic lineages: 5 loci among Asian *X. oryzae* pv. *oryzae* and African *X. oryzae* pv. *oryzae* strains, 6 loci among African *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola* strains, and 26 loci among Asian *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola* strains. All 50 of the other VNTR loci were specific to one lineage (either predicted to be monomorphic or absent in the other lineages). Promising VNTR loci were selected according to their presence and polymorphism in at least two lineages. PCR amplicons that were generated on a set of 20 strains (test panel) per genetic lineage were sequenced (see Table S2 in the supplemental material). The test panel included strains that represent the worldwide diversity of the pathovar (to assess the conservation of loci), as well as a few strains that are geographically closely related (to assess the discriminatory power). DNA sequencing of two predicted loci, Xo_G25 and Xo_G53, among 40 Asian and African *X. oryzae* pv. *oryzae* strains revealed that they were located next to each other in the genome (at position 1434114 of strain PXO99^A) (see Fig. S1 in the supplemental material). Xo_G25 (repeat size, 5 bp) was found to be polymorphic for Asian *X. oryzae* pv. *oryzae* (2 to 15 TRs) and monomorphic for African *X. oryzae* pv. *oryzae* (2 TRs). In contrast, Xo_G53 (repeat size, 8 bp) was found to be polymorphic for African *X. oryzae* pv. *oryzae* (5 to 10 TRs) and monomorphic for Asian *X. oryzae* pv. *oryzae* (2 TRs). Thus, the two loci were combined into a single VNTR locus called Xo_G2553.

In order to implement a universal MLVA scheme, 16 loci were selected that maximized the resolution between and within the different lineages of *X. oryzae* (Fig. 1 and Table 1). Importantly, all 16 loci were predicted to be present in all three lineages and polymorphic in at least two lineages. To ensure that the loci are well distributed along the bacterial chromosome, the 16 VNTR loci

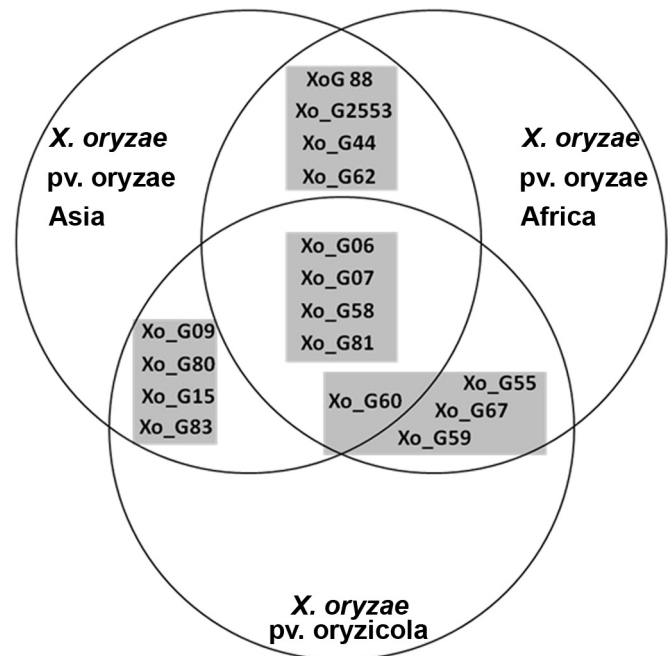


FIG 1 Venn diagram of VNTR loci that were included in the general typing scheme for *X. oryzae*. The positions of loci in the diagram correspond to their predicted polymorphism within each lineage. The four gray squares represent the four quadruplex-PCR pools.

were mapped on the complete genome sequences of Asian *X. oryzae* pv. *oryzae* strains KACC10331, MAFF311018, and PXO99^A and *X. oryzae* pv. *oryzicola* strain BLS256 (see Fig. S1 in the supplemental material). The loci were distributed over the genomes with good synteny between strains. All loci from Asian *X. oryzae* pv. *oryzae* strains were syntenic to each other, except for Xo_2553, which mapped at a different position in PXO99^A. In contrast, BLS256 showed limited synteny with Asian *X. oryzae* pv. *oryzae*, which is in agreement with previously published genome comparisons (5).

Multiplexing in a 4-by-4 scheme, consisting of four quadruplex PCRs, was organized to include three highly discriminatory sets per lineage, while one set was predicted to include monomorphic loci that would serve as diagnostic markers for the respective lineages. For two loci, Xo_G07 and Xo_G44, large PCR amplicons were predicted due to the presence of insertion (IS) elements next to the VNTR locus for Asian *X. oryzae* pv. *oryzae* strains and *X. oryzae* pv. *oryzicola* strains, respectively, thus preventing their use for these lineages in further analyses.

MLVA-16 on a worldwide collection of *Xanthomonas oryzae*. The multilocus VNTR analysis scheme based on the 16 loci (MLVA-16) was used to genotype 338 strains of *X. oryzae*. This scheme allowed us to distinguish 279 haplotypes. HGDI scores were calculated for all strains and ranged from 0.49 for Xo_G62 to 0.93 for Xo_G09 (Table 2). As expected from *in silico* analyses, one multiplexed set per lineage contained loci with low HGDI scores (Table 2). For example, Xo_G09, Xo_G15, and Xo_G80 were monomorphic for 59 African *X. oryzae* pv. *oryzae* strains. Likewise, Xo_G2553, Xo_G88, and Xo_G62 showed only low diversity for 152 *X. oryzae* pv. *oryzicola* strains. Only one locus, Xo_G59, which was predicted to be monomorphic based on the four avail-

TABLE 2 Genetic diversity of *X. oryzae* lineages estimated with the MLVA-16 scheme

Locus	<i>X. oryzae</i> pv. <i>oryzae</i> Africa (<i>n</i> = 59)			<i>X. oryzae</i> pv. <i>oryzae</i> Asia (<i>n</i> = 127)			<i>X. oryzae</i> pv. <i>oryzicola</i> Africa (<i>n</i> = 70)			<i>X. oryzae</i> pv. <i>oryzicola</i> Asia (<i>n</i> = 82)			Total (<i>n</i> = 338)	
	No. of alleles	Allelic range ^a	HGDI score	No. of alleles	Allelic range	HGDI score	No. of alleles	Allelic range	HGDI score	No. of alleles	Allelic range	HGDI score	No. of Alleles	HGDI score
Xo_G06	4	6–9	0.58	8	7–18	0.69	6	5–10	0.74	7	5–12	0.79	10	0.81
Xo_G07	8	4–18	0.70	– ^b	–	–	4	5–8	0.55	8	5–13	0.65	13	0.83
Xo_G58	6	2–7	0.64	8	3–13	0.77	10	6–16	0.83	6	6–11	0.32	14	0.81
Xo_G81	4	7–15	0.60	6	6–25	0.28	13	11–26	0.85	12	3–20	0.81	20	0.84
Xo_G2553	5	26–32	0.55	12	38–52	0.70	1	5	0	2	4–5	0.02	19	0.74
Xo_G88	7	4–10	0.64	13	2–16	0.62	1	1	0	1	1	0	15	0.72
Xo_G44	4	3–7	0.57	7	5–11	0.74	3	4–6	0.67	–	–	–	9	0.70
Xo_G62	3	4–14	0.63	4	2–5	0.53	1	3	0	2	2–3	0.02	6	0.49
Xo_G55	8	12–24	0.75	1	4	0	13	16–30	0.89	15	9–25	0.90	22	0.83
Xo_G59	5	4–9	0.56	3	3–5	0.53	4	4–13	0.47	5	4–8	0.51	8	0.75
Xo_G60	5	3–7	0.62	3	1–3	0.51	1	2	0	4	2–5	0.23	7	0.71
Xo_G67	2	10–11	0.49	1	7	0	9	13–21	0.85	22	13–41	0.95	28	0.81
Xo_G09	1	2	0	22	2–32	0.95	14	2–20	0.70	24	2–32	0.93	30	0.93
Xo_G15	1	3	0	31	10–45	0.92	11	3–15	0.82	12	4–27	0.85	38	0.92
Xo_G80	1	3	0	5	3–14	0.49	3	4–7	0.15	8	6–13	0.78	11	0.75
Xo_G83	2	1–2	0.03	3	1–3	0.08	2	1–3	0.03	3	2–4	0.26	4	0.66

^a Alleles with the smallest number and with the largest number of repeats.

^b –, locus was not amplified due to the insertion of an IS element.

able genome sequences, turned out to be polymorphic when 127 Asian *X. oryzae* pv. *oryzae* strains were analyzed, resulting in an HGDI score of 0.56. As suggested by the analysis of the four genome sequences, the locus Xo_G07 did not lead to PCR amplicons of less than 500 bp (which was the cutoff size for the electrophoretic analysis) for Asian *X. oryzae* pv. *oryzae*. It was impossible to design a primer between the IS element and the repeat array. Similarly, no signal was detected for almost all *X. oryzae* pv. *oryzicola* strains, except for seven African *X. oryzae* pv. *oryzicola* strains at the locus Xo_G44, because of amplicon sizes greater than 500 bp.

The allelic richness following a rarefaction procedure was calculated for three scenarios, considering two (*X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola*), three (*X. oryzae* pv. *oryzae* from Africa, *X. oryzae* pv. *oryzae* from Asia, and *X. oryzae* pv. *oryzicola*, corresponding to the previously defined lineages), or four (*X. oryzae* pv. *oryzae* from Asia, *X. oryzae* pv. *oryzicola* from Asia, *X. oryzae* pv. *oryzae* from Africa, and *X. oryzae* pv. *oryzicola* from Africa) groups of strains (Table 3; see Table S3 in the supplemental material). In the last scenario, the allelic richness (*A*) was greater for *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola* strains from Asia, with *A* values of 9.35 and 8.66, respectively, than for *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola* strains from Africa, with *A*

TABLE 3 Average allelic richness and private allelic richness calculated by the rarefaction method on different possible population groups^a

Genetic group	4 groups (<i>n</i> = 59)		3 groups (<i>n</i> = 59)		2 groups (<i>n</i> = 152)	
	<i>A</i>	<i>A_p</i>	<i>A</i>	<i>A_p</i>	<i>A</i>	<i>A_p</i>
<i>X. oryzae</i> pv. <i>oryzae</i> Africa	4.19	1.04	4.19	1.04	9.33	4.95
<i>X. oryzae</i> pv. <i>oryzae</i> Asia	9.35	3.80	9.35	3.80		
<i>X. oryzae</i> pv. <i>oryzicola</i> Africa	7.52	1.53	9.12	4.76	9.12	4.76
<i>X. oryzae</i> pv. <i>oryzicola</i> Asia	8.66	1.56				

^a Four groups corresponding to the two pathovars and two continents, three groups corresponding to the three genetic lineages, and two groups corresponding to the two pathovars. *n*, number of strains retained for rarefaction calculation.

values of 4.19 and 7.52, respectively. Asian strains of *X. oryzae* pv. *oryzae* showed a relatively high private allelic richness (*A_p*) of 3.80 in comparison to African *X. oryzae* pv. *oryzae* (*A_p* = 1.04).

Monomorphic loci in each lineage tended to have a low number of repeats and to correspond to private alleles (see Table S3 in the supplemental material). A distinct combination of four alleles (Xo_G09, 2 TRs; Xo_G15, 3 TRs; Xo_G80, 3 TRs; and Xo_83, 2 TRs) was found to be unique to African *X. oryzae* pv. *oryzae*. *X. oryzae* pv. *oryzicola* was found to have two private monomorphic alleles (Xo_G25, 5 TRs, and Xo_G88, 1 TR). For Asian *X. oryzae* pv. *oryzae*, two alleles (Xo_G55, 4 TRs, and Xo_G67, 7 TRs) were lineage specific (see Table S3 in the supplemental material).

The association indices were highly significant within each lineage and at the country level, supporting strong linkage disequilibrium (Table 4). Additionally, the correlation of MLVA-16 and MLSA data sets (34) tested on a collection representative of the

TABLE 4 Linkage disequilibrium per lineage, with the number of individuals, *I_A*, and *r_D*

Genetic group	<i>n</i> ^a	<i>I_A</i> ^b	<i>r_D</i> ^b
<i>X. oryzae</i> pv. <i>oryzicola</i> Africa	70	0.56	0.06
Mali	36	1.28	0.14
Burkina Faso	34	0.91	0.10
<i>X. oryzae</i> pv. <i>oryzicola</i> Asia	82	1.25	0.12
China	32	3.34	0.32
Philippines	48	1.10	0.10
<i>X. oryzae</i> pv. <i>oryzae</i> Africa	62	6.43	0.56
Burkina Faso	23	3.62	0.46
Mali	25	5.16	0.48
<i>X. oryzae</i> pv. <i>oryzae</i> Asia	127	1.323	0.10
Philippines	69	2.57	0.20

^a *n*, number of individuals.

^b *P* value < 0.001.

TABLE 5 Pairwise F_{ST} values between populations

Genetic group	F_{ST}	
	<i>X. oryzae</i> pv. <i>oryzae</i> Africa	<i>X. oryzae</i> pv. <i>oryzae</i> Asia
<i>X. oryzae</i> pv. <i>oryzae</i> Africa	0	
<i>X. oryzae</i> pv. <i>oryzae</i> Asia	0.41624 ^a	0
<i>X. oryzae</i> pv. <i>oryzicola</i>	0.33466 ^a	0.41889 ^a

^a P value < 0.001.

three lineages ($n = 35$) showed significant congruence (Mantel $R = 0.86$; $P = 0.001$). On the other hand, the congruence between MLVA-16 and RFLP *avrXa7* data sets (35) was only slightly significant (Mantel $R = -0.12$; $P = 0.056$) on a collection of 56 African *X. oryzae* pv. *oryzicola* strains (see Fig. S2 in the supplemental material).

The three genetic lineages proposed by Gonzalez and coworkers were clearly differentiated ($P < 0.001$) (Table 5; see Fig. S3 in the supplemental material), and *X. oryzae* pv. *oryzicola* was less differentiated from African *X. oryzae* pv. *oryzae* ($F_{ST} = 0.33466$) than from Asian *X. oryzae* pv. *oryzae* ($F_{ST} = 0.41889$) (20).

***X. oryzae* pv. *oryzae* from Africa.** As illustrated by the MST (Fig. 2) and the Manhattan NJ tree (see Fig. S4 in the supplemental material), strains from Niger and Burkina Faso were genetically relatively close to each other while strains from Cameroon and Mali were more distinct. Strains from two distant Burkina Faso regions, Bagre and Sourou, constituted a clonal group, and two Burkina Faso strains isolated from Bagre in 2004 clustered apart. Two strains originating from two different countries (BAI1 and NAI8), which were isolated in the same year (2004), shared the same haplotype. Strain BAI4, which was isolated from *Oryza glaberrima* and corresponds to race A2, was well separated from the other strains of the Burkina Faso-Niger complex (>4 locus differ-

ences and 1 to 5 repeat differences per locus), confirming previous results (20).

DAPC identified three groups within the African *X. oryzae* pv. *oryzae* strains. DAPC-XooAf1 contained Malian strains and DAPC-XooAf2 contained strains from Cameroon and the Burkina Faso strain BAI4, while DAPC-XooAf1 comprised all the other strains from Burkina Faso and Niger. Using DAPC with four clusters, Malian strains could be further separated into two distinct clusters that correspond to *X. oryzae* pv. *oryzae* strains collected in Mali (i) between 1979 and 2009 (from Office du Niger and Kayes) and (ii) between 2010 and 2012 in Office du Niger. The second cluster formed a closely related genetic complex composed of 12 haplotypes (Fig. 2B; see Table S4 in the supplemental material). Eleven of the 12 haplotypes belonged to strains from Niono, while one haplotype belonged to two strains from Sélingué, a site that is located far from Niono (see Fig. S5 in the supplemental material).

***X. oryzae* pv. *oryzicola*.** Based on the categorical minimum spanning tree, almost all Asian and African *X. oryzae* pv. *oryzicola* strains were well separated into two groups (Fig. 3), except for two Chinese strains, HN-DA-1 and Xoc-China, which clustered with the African strains. Strain HN-DA-1 shared the same allele at 10 loci with the African *X. oryzae* pv. *oryzicola* strain BAI11, and Xoc-China had eight alleles in common with HN-DA-1. No haplotype was shared between strains from different countries.

No clear clustering was revealed by the DAPC analysis following the k -means analysis, suggesting that there is no evident population structure (53). However, assuming two clusters (k -mean = 2), as supported by the MST and NJ tree, DAPC analysis grouped all African strains into one cluster (DAPC-Xoc1) and most Asian strains into the other cluster (DAPC-Xoc2). Only a few strains were excluded from this grouping: strains Xoc-China, GXO1, and HN-DA-1 from China and BLS103, BLS419, and BLS489 from

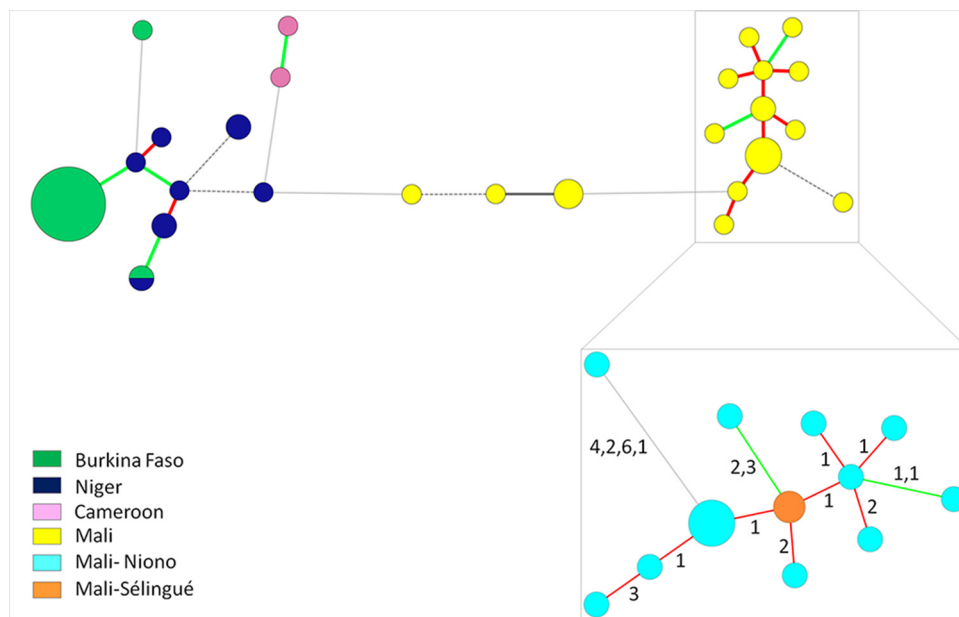


FIG 2 Minimum spanning tree of African *X. oryzae* pv. *oryzae* strains using BioNumerics 7.1 and detailed view of the Malian clonal complex of strains from Niono and Sélingué (inset). The differences in repeat numbers per locus between two haplotypes is given between the circles. The circle sizes are proportional to the number of strains per haplotype. The red lines correspond to single-locus variants, green lines correspond to double-locus variants, black lines correspond to triple-locus variants, dashed lines correspond to quadruple-locus variants, and light-gray lines correspond to more than quadruple-locus variants.

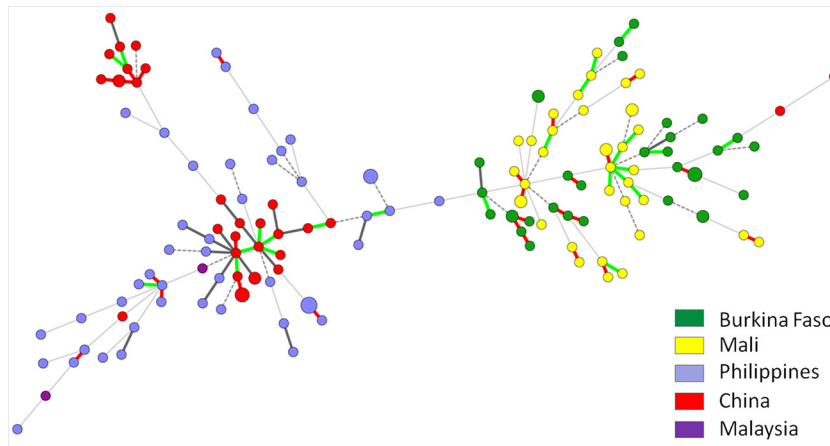


FIG 3 Minimum spanning tree of *X. oryzae* pv. *oryzicola* strains using BioNumerics 7.1. For details, see the legend to Fig. 2.

Philippines clustered with the African strains in DAPC-Xoc1 (see Table S4 in the supplemental material). Chinese strains sampled in 2011 displayed two different haplotypic groups, with only SLVs and DLVs that were amenable to eBURST analysis (see Fig. S5 in the supplemental material). The first haplotypic group comprised 10 strains with eight haplotypes, and the second haplotypic group comprised 8 strains with seven haplotypes. On the other hand, Philippine *X. oryzae* pv. *oryzicola* strains that were sampled over 30 years from various sites showed very few SLVs or DLVs and thus no visible structuring into clonal complexes.

The minimum spanning tree of African *X. oryzae* pv. *oryzicola* strains did not reveal close links among strains from Burkina Faso

and/or Mali. Fifteen small clonal complexes, consisting of two to four STs, were found. Another, larger complex from Mali with eight STs, originating from two close sites, Kogoni and Niono, corresponded mainly to DLVs (Fig. 3). Importantly, isolates originating from the same field (MAI17 to MAI25 from Kogoni, MAI26 to MAI35 from Macina, and MAI40 to MAI45 from Sélingue) could be differentiated into up to three clonal complexes consisting of SLVs and DLVs.

***X. oryzae* pv. *oryzae* from Asia.** Asian *X. oryzae* pv. *oryzae* strains in our collection were found to be very diverse. No haplotypes belonging to different countries were connected as SLVs or DLVs in the minimum spanning tree (Fig. 4). We also studied two

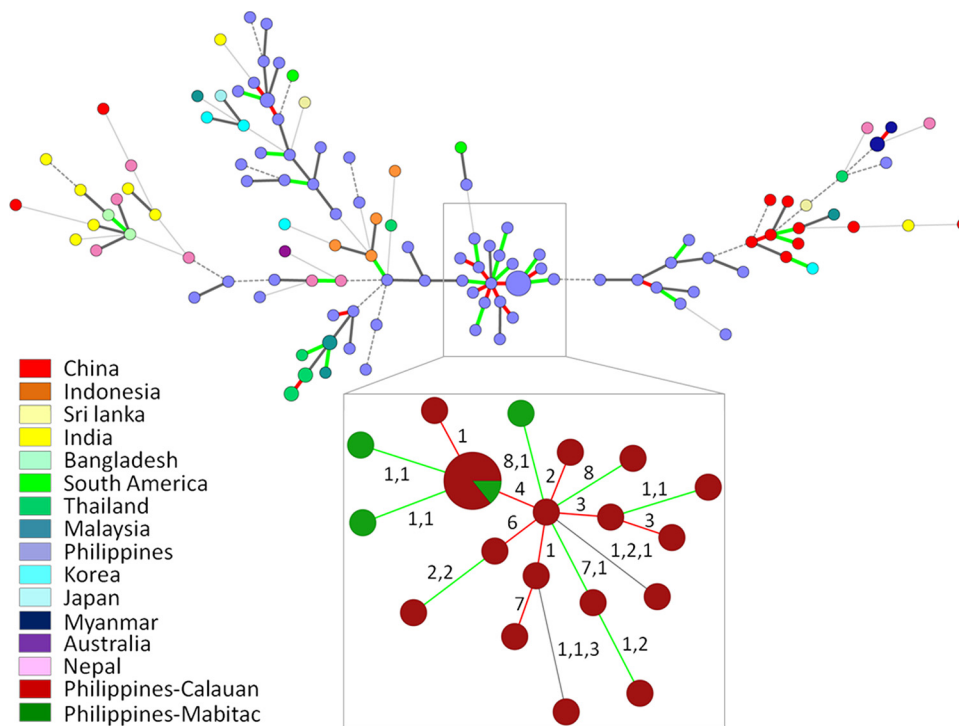


FIG 4 Minimum spanning tree of Asian *X. oryzae* pv. *oryzae* strains using BioNumerics 7.1 and detailed view of a Philippine clonal complex of strains from Calauan and Mabitac (inset). The differences in repeat numbers per locus between two haplotypes are given between the circles. For details, see the legend to Fig. 2.

strains from South America, FXO 27 (LMG 9585) from Bolivia and CIAT1185 from Colombia. Both strains appeared to be related to Philippine strains, although they were distinct at five and three loci, respectively. A clonal complex of Philippine strains with SLVs and DLVs grouped 18 haplotypes corresponding to 24 strains that were sampled between 1994 and 2003 in the Laguna Bay region (Calauan and Mabitac) within a radial distance of about 20 km (Fig. 4B; see Fig. S5 in the supplemental material). The other 12 strains sampled in the same region over the same period and a strain from Los Baños, a neighboring Calauan village, that was sampled in 1979, were not related to this clonal complex. These findings could be explained by the fact that BLB is endemic, i.e., established for a long time in Philippines (54).

The DAPC analysis using a *k*-mean of 2 clustered a subset of Philippine strains together with Chinese, Malaysian, Indonesian, Thai, Burmese, and most Korean and Nepalese strains, as well as with the Colombian strain CIAT1185 (DAPC-XooAs1). The other cluster, DAPC-XooAs2, contained another subset of Philippine strains (mainly strains sampled before the 1990s), along with eight Indian strains, two Bangladeshi strains, two Nepalese strains, and one Korean strain, as well as the Bolivian strain FXO 27 (see Table S4 in the supplemental material).

DISCUSSION

We developed a new MLVA scheme that identifies the different lineages of *X. oryzae*, i.e., pathovars and those of continental origin, and shows high discriminatory power on small scales in space and time.

On a global scale, the MLVA-16 scheme confirmed the lineage differentiation that was largely described in previous typing and phylogenetic studies (20, 33, 34, 55). However, on a smaller scale, the MLVA-16 scheme was shown to be more discriminatory than other previously used molecular-typing tools. The MLVA-16 scheme could discriminate strains from the same region and even strains originating from the same field. Interestingly, epidemiologically related strains kept a signature of their relationship by descent on this small spatiotemporal scale.

Recombination does not strongly bias the phylogenetic signal. Even though the association indices are quite low for some lineages, linkage disequilibrium was highly significant. Moreover, distance matrices obtained from MLVA-16 and from a different genotyping technique (MLSA) were significantly correlated. These lines of evidence support low levels of recombination, as has also been suggested for other *Xanthomonas* species (56). However, one needs to be cautious, because these observations could result from sampling biases. For instance, geographical isolation might have occurred in our collections, consequently promoting high linkage disequilibrium (57). To test this hypothesis, the locus association should be tested on a strongly sampled population, i.e., more strains isolated on a small spatiotemporal scale. Nevertheless, our results support the usefulness of the 16 VNTR markers to investigate demographic or epidemiological patterns.

As a special case, a distance matrix obtained from a *tal* gene-based RFLP data set was not correlated with an MLVA-16-based distance matrix when applied to a set of Malian and Burkina Faso *X. oryzae* pv. *oryzicola* strains. *tal* genes, which consist of tandem repeats with a large repeat unit (58), contribute to the colonization of host plants in a compatible interaction or trigger specific defenses in an incompatible interaction. Hence, *tal* gene-based markers are expected to be under selection. Moreover, the RFLP

band sizes of *tal* genes do not always reflect their functional relatedness, i.e., identical *tal* RFLP bands can belong to functionally unrelated *tal* genes while different *tal* RFLP bands can correspond to functionally analogous *tal* genes. Together, these considerations may explain why a matrix generated by this marker is not correlated with a matrix obtained from neutral markers, such as most VNTRs.

MLVA-16 as a tool to identify lineages. The MLVA-16 scheme produced some private and monomorphic alleles for each genetic lineage. Consequently, the different lineages of *X. oryzae* can be distinguished. In addition, other lineage-polymorphic loci also contribute to this differentiation. Hence, MLVA-16 directly identifies the pathogen responsible for a disease, i.e., BLB or BLS. Since BLB and BLS symptoms can be confused in the field at very early and late stages of the diseases, and also in cases of double infection, this information is important for efficient disease management. In the context of prior knowledge about prevalent BLB and BLS pathogens in a certain geographic area, the ability to distinguish between the two pathogens helps in deployment of disease-resistant rice cultivars and/or application of specific antibacterial agents. Moreover, the ability to distinguish between BLS and BLB might be helpful for quarantine purposes, especially with the increase in rice seed trade between countries.

F_{ST} indices of diversity suggest that African *X. oryzae* pv. *oryzae* strains are closer to *X. oryzae* pv. *oryzicola* than to Asian *X. oryzae* pv. *oryzae*, as shown previously by Gonzalez and coworkers using RFLP, AFLP, and rep-PCR. However, these results are challenged by an MLSA of the three housekeeping genes *gyrB*, *rpoD*, and *glnA* at a lower resolution, where the two *X. oryzae* pv. *oryzae* lineages group together and are more distant from *X. oryzae* pv. *oryzicola* (34). Interestingly, another MLSA using a different set of housekeeping genes, *fusA*, *gyrB*, and *gapA*, rather supported our scenario, i.e., that African *X. oryzae* pv. *oryzae* strains are closer to *X. oryzae* pv. *oryzicola* than to Asian *X. oryzae* pv. *oryzae* (33). Apparently, the choice of markers (with only a few polymorphisms) used for such analysis plays an important role in the discrimination of lineages. To resolve this problem, a genome-wide SNP analysis or an MLSA of the core genome could be performed (55). In conclusion, MLVA-16 differentiates lineages well on a global scale.

MLVA-16 has limited value for large-scale epidemiology. First reported in Asia, *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola* were described decades later in Africa (4, 20). It has been assumed that BLB and BLS have been introduced accidentally from Asia to Africa. The fact that both African *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola* showed less allelic richness than Asian *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola* supports an Asian ancestor for African strains. However, even though this study was conducted on a large strain collection, additional and more extensive sampling will be necessary to confirm an Asian origin of the African populations of *X. oryzae*.

On a global scale, the MLVA-16 scheme revealed hardly any shared or related haplotypes (SLVs and DLVs) among strains from different countries. Similar findings were obtained with a microsatellite-based MLVA-14 of *X. citri* (46). Generally, no link can be drawn from a vast number of strains on a really large geographic scale or over a long time based on microsatellites that evolve rapidly. Therefore, the development of another MLVA scheme based on TRs with a slower molecular clock, i.e., minisatellites, where repeat units are longer, would be more appropriate

for large-scale epidemiology. Indeed, as shown by N'Guessan and coworkers for *Ralstonia solanacearum*, the number of alleles in a large collection of strains decreases with an increase in the repeat unit size (59). Similarly, Pruvost and coworkers demonstrated the superiority of a minisatellite-based scheme over a microsatellite-based scheme for large-scale epidemiology of *X. citri* (46). Preliminary analyses have shown that no useful VNTR loci with repeat unit sizes above 12 bp are shared among the three *X. oryzae* lineages, thus preventing the development of a universal scheme. In the future, lineage-specific minisatellite schemes will be developed for global epidemiological monitoring.

Despite these concerns, MLVA-16 provided some important information on a larger geographical scale. For instance, we identified two Chinese *X. oryzae* pv. *oryzicola* strains (Xoc-China and HN-DA-1) that differed greatly from the other Asian *X. oryzae* pv. *oryzicola* strains (only 7 common alleles with the closest Asian *X. oryzae* pv. *oryzicola* strain). Our results suggest that these strains are rather related to African *X. oryzae* pv. *oryzicola* strains (10 common alleles with the closest African *X. oryzae* pv. *oryzicola* strain). This finding coincides with the recent increase of the seed-borne BLS disease in Africa and with tighter commercial links between Africa and China. Moreover, the two strains from South America, which are related to two Philippine haplotypes, support a scenario with distinct events of introduction in South America, perhaps from Philippines. Finally, two African *X. oryzae* pv. *oryzae* strains from different countries, BAI3 from Burkina Faso and NAI8 from Niger, shared the same haplotype. This finding may reflect the exchange of material between western African countries.

In conclusion, even if the MLVA-16 scheme is not ideal for large-scale epidemiology with slow migration, it is still a valuable tool for more modern situations where migration is very fast due to human transport. To demonstrate the power of this tool, we would need a larger set of strains representative of the total diversity. Currently, access to strains or even genomic DNA of *X. oryzae* is limited due to quarantine restrictions or regulatory issues concerning biodiversity in the different countries. The MLVAbank database, which makes our data accessible for analyses and comparisons, will help to coordinate international efforts to understand the epidemiology of *X. oryzae*.

MLVA-16 as a new tool for local epidemiological monitoring. Our results show that the MLVA-16 scheme is very useful for local epidemiological surveillance on a regional or country-wide scale for all three *X. oryzae* lineages. First, African *X. oryzae* pv. *oryzae* strains sampled from Malian regions (Niono and Sélingue) in the same period (2010 and 2012) showed relatively close haplotypes. Their allelic profiles reveal an expansion that has occurred clonally from an unidentified founder. However, the Malian collection showed a dynamic population structure, since Malian strains sampled in 2010 and 2012 are distant from Malian strains sampled up to 2009. However, a phylogenetic signature for relatedness remained while epidemiological information was lost, because strains of both complexes differ by at least nine alleles. Second, on a slightly larger scale, a set of *X. oryzae* pv. *oryzicola* strains sampled in southern China in 2011 shared identical or similar haplotypes, as shown by the minimum spanning tree and eBURST analysis (Fig. 4; see Fig. S5 in the supplemental material). Third, 24 Philippine *X. oryzae* pv. *oryzae* strains sampled over 9 years in Laguna Bay are linked in a clonal complex. It would be interesting to clarify how such a limited number of haplotypes was main-

tained for many years in this region. Moreover, since several very different haplotypes of Philippine *X. oryzae* pv. *oryzae* were found to coexist within this region, further investigation by extensive sampling would be of high interest.

Our results show that the MLVA-16 scheme holds great potential for small-scale reconstruction of population dynamics. Such an improved understanding of microevolution will be key for short- and medium-term management of control strategies for *X. oryzae*, particularly by deciphering the pathways of dispersal both by natural transmission and by human-mediated transmission (including seed transmission).

Conclusions. The MLVA-16 scheme will be useful for high discrimination among *X. oryzae* strains and to identify the lineages and pathovars of these pathogens. MLVA-16 will further allow us to analyze new outbreaks and epidemics of both pathovars of *X. oryzae*. Additional samplings at the population level will be a further step to evaluate the efficiency of MLVA-16 by describing the patterns of descent of the strains and the population structures. Specifically, recently isolated *X. oryzae* pv. *oryzicola* strains from eastern and central Africa could be characterized (22–24).

Directions for use. In cases where the pathovar and geographical origin are known, e.g., upon multiplex PCR (13), one could omit one of the four primer pools that correspond to loci that are largely monomorphic within a lineage. Hence, one would use a subset of primer pools in a multiplex MLVA-12 scheme that is adapted to the pathovars and origins of the isolates. Asian *X. oryzae* pv. *oryzae* strains need to be analyzed with pools 1, 2, and 4 (MLVA-12a); African *X. oryzae* pv. *oryzae* strains need pools 1, 2, and 3 (MLVA-12b); and *X. oryzae* pv. *oryzicola* strains need pools 1, 3, and 4 (MLVA-12c). Results can be analyzed and compared to our and other's data using the public database at IRD Montpellier (<http://www.biopred.net/MLVA/>), which allows sharing of information with other scientists in an interactive manner and provides access to a few phylogenetic-analysis tools (46).

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