Development of a Variable Number of Tandem Repeats Typing Scheme for the Bacterial Rice Pathogen *Xanthomonas oryzae* pv. *oryzicola*

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ABSTRACT

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Xanthomonas oryzae pv. *oryzicola* is an important bacterial pathogen responsible for outbreaks of bacterial leaf streak (BLS) on rice, mostly occurring in Asia and parts of Africa. To better monitor epidemics and assess population structures, efficient tools that allow the precise identification and diagnosis of pathogenic populations are needed. In this study, we explored variable numbers of tandem repeats (VNTR) as a fast, reliable, and cost-effective molecular typing tool. Screening of three *X. oryzae* pv. *oryzicola* genome sequences (Philippine strain BLS256,

Chinese strain GX01, and Malian strain MAI10) predicted 28 candidate VNTR loci. Primer pairs for polymerase chain reaction (PCR) amplification of all 28 loci were designed and applied to a panel of 20 *X. oryzae* pv. *oryzicola* strains originating from Asia and Africa. Sequencing of PCR amplicons revealed 25 robust and polymorphic VNTR loci that are shared among Asian and African *X. oryzae* pv. *oryzicola* strains. A dendrogram constructed from 25 VNTR loci indicated that most Asian strains are clearly discriminated from African strains. However, in agreement with previous reports, one strain from Mali is related to Asian strains, pointing to a possible introduction of Asian strains to the African continent. The new VNTR-based tool described here is useful for studies of population structures and epidemiological monitoring of *X. oryzae* pv. *oryzicola*.

Additional keyword: MLVA.

Bacterial leaf streak (BLS) of rice is caused by the gram-negative plant-pathogenic bacterium *Xanthomonas oryzae* pv. *oryzicola*. Since it was first discovered in the Philippines in 1918, BLS has become a widespread and economically important disease in the rice-growing areas in Asia and in parts of Africa (12,20,35). The importance of BLS is increasing due to large-scale cultivation of susceptible hybrid rice cultivars worldwide, especially in Southeast China, and due to efficient measures against other rice-infecting microbes (20).

Few studies have been conducted on control measures against BLS. No single resistance gene against *X. oryzae* pv. *oryzicola* has been identified in rice thus far (38). Only a dominant gene, *Rxo1* from maize, has been found to mediate a non-host defense response to *X. oryzae* pv. *oryzicola* (37). *Rxo1* restricts multiplication of rice pathogens harboring the recognized effector (*avrRxo1*) when expressed as a transgene in rice (39). The search for sources of natural resistance against *X. oryzae* pv. *oryzicola* in rice revealed that several loci might contribute to resistance in a quantitative manner (i.e., as quantitative trait loci) (30).

Sustainable control measures against BLS will critically depend on a deeper insight into the structure and dynamics of *X. oryzae* pv. *oryzicola* populations in rice-growing areas. Unfortunately, genotyping techniques for deciphering the epidemiology of *X. oryzae* pv. *oryzicola* have been of very limited use to date. The first comprehensive molecular and pathotypic characterization of African *X. oryzae* pv. *oryzicola* strains from Mali was performed only recently by restriction fragment length polymorphism (RFLP), repetitive sequence-based polymerase chain reaction (rep-PCR), and fluorescent amplified fragment length polymorphism (fAFLP) analysis (12). Further, a set of *X. oryzae* pv. *oryzicola* from three different regions of Burkina Faso and Mali in Africa were characterized by multi-locus sequence analysis (MLSA) of seven housekeeping genes and analysis of the repertoires of type III secretion system effector genes, including two discriminatory genes, *avrRxo1* and *xopW* (13,35). In Asia, *X. oryzae* pv. *oryzicola* strains from the Philippines with high diversity were observed (26). More recently, PCR-based DNA fingerprint analyses were used to evaluate the genetic diversity and population structure of *X. oryzae* pv. *oryzicola* strains from southern China using three specific primers: enterobacterial repetitive intergenic consensus (ERIC), BOX, and J3 (21).

Several methods have been developed to investigate the epidemiology of pathogens but many of them suffer from tech-

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nical limitations, such as being laborious, time-consuming, and marginally reproducible, and with limited comparability between different laboratories. Consequently, better tools for detection, typing, and tracing of the pathogens are needed. More recently, multiple loci variable number of tandem repeat (VNTR) analysis (MLVA) was introduced as a new promising technology for epidemiological surveillance of microorganisms (33). Since then, MLVA has become increasingly popular for molecular typing of bacteria (32,34). Powerful MLVA schemes are available for several human pathogens, such as *Bacillus anthracis*, *Escherichia coli*, *Mycobacterium tuberculosis*, *Pseudomonas aeruginosa*, and *Yersinia pestis*, among others (19,32). MLVA has several advantages over other bacterial genotyping methods, such as ease of performance and portability, high reproducibility and discriminatory power, rapidity, and low costs (18). However, because some VNTR loci may evolve too fast in bacterial genomes, it is necessary to evaluate several candidate loci in order to identify those which are most informative at a given temporal and geographical scale.

In 2001, shortly after the availability of the genome sequence (27), the first VNTR study of a bacterial plant pathogen, *Xylella fastidiosa*, was performed (9). Since then, MLVA became well established for typing of *X. fastidiosa* (6–8). The first MLVA scheme for the genus *Xanthomonas* has been developed for *X. citri* subsp. *citri* (4). Using the same scheme, the population structure of the closely related bacterium *X. citri* pv. *mangiferaeindicae* has been analyzed (24).

To date, only one complete genome sequence of *X. oryzae* pv. *oryzicola* is publicly available, corresponding to strain BLS256 from the Philippines (2). Our laboratories have access to two more *X. oryzae* pv. *oryzicola* genome sequences at draft level, belonging to strain MAI10 from Mali (R. Koebnik and V. Verdier, unpublished data) and strain GX01 from China (Y.-Q. He, unpublished data). These genomic resources were used to explore 28 candidate VNTR loci and to develop a high-resolution MLVA typing scheme for *X. oryzae* pv. *oryzicola*. Application to a panel of 20 strains of different levels of genetic relatedness originating from Asia and Africa revealed a high resolute power of the

MLVA scheme, useful for studies of population structures and epidemiological surveillance.

MATERIALS AND METHODS

Bacterial strains, growth condition, and DNA extraction.

The bacterial strains used in this study are listed in Table 1, including those whose genomes have been sequenced: *X. oryzae* pv. *oryzicola* Philippine strain BLS256 (2), Malian strain MAI10 (R. Koebnik and V. Verdier, unpublished data), and Chinese strain GX01 (Y.-Q. He, unpublished data). All the sequenced strains were used as controls for PCR. *X. oryzae* pv. *oryzicola* strains were grown in peptone-sucrose agar (PSA) medium (10 g of peptone, 10 g of sucrose, 1 g of glutamic acid, and 16 g of agar per liter of H₂O) at 28°C. Genomic DNA was extracted from bacterial colonies on the PSA plates cultured for 48 h using the Wizard genomic purification kit from Promega (Promega, Paris). The quality and quantity of DNA were evaluated by agarose gel electrophoresis and spectrophotometry (Nanodrop ND-1000; Nanodrop Technologies, Wilmington, DE).

Prediction of VNTR loci and primer design. The complete genome sequence of the Philippine *X. oryzae* pv. *oryzicola* strain BLS256 (GenBank accession number CP003057) and two draft genome sequences from Malian *X. oryzae* pv. *oryzicola* strain MAI10 and Chinese strain GX01 were scrutinized for the presence of the candidate VNTR loci using a web-based prediction pipeline (<http://www.biopred.net/VNTR/>). Parameters were set as follows: Algorithm TRF (tandem repeat finder) (1), region length of 30 to 1,000 bp, unit length of 5 to 9 bp, at least six copies, and an identity of a least 80% between adjacent repeats. Predicted VNTR loci were grouped according to their shared 500-bp flanking regions and dubbed with the first letter of the strain designation, followed by a number reflecting the order along the genome sequence.

Homologous 500-bp regions next to the predicted VNTR loci were extracted from all three *X. oryzae* pv. *oryzicola* genome sequences and aligned using MUSCLE (<http://www.ebi.ac.uk/>)

TABLE 1. Bacterial strains (*Xanthomonas oryzae* pv. *oryzicola*) used in this study

Strains	Country, location	Year	Host	Haplotype ^a								Resources ^b
				RFLP				rep-PCR	Eric-PCR	AFLP	VNTR	
				IS1112	avrXa10	IS1113	Xoo1762					
Diversity panel												
BLS256 (CFBP7109)	Philippine	1985	<i>Oryza sativa</i>	...	...	...	...	...	...	...	1	C. Vera Cruz
LMG797 (CFBP2286)	Malaysia	1964	<i>O. sativa</i>	25	25	17	6	19	14	3	2	CFBP
NCPBP2921 (CFBP2287)	Malaysia	1973	<i>O. sativa</i>	26	26	17	6	20	14	...	3	CFBP
UPB497	Malaysia	...	<i>O. sativa</i>	...	26	...	...	26	20	3	4	C. Bragard
GX01	Guangxi, China	2005	<i>O. sativa</i> sp. <i>indica</i>	...	...	...	...	...	...	...	5	Y. Q. He
GX07	Guangxi, China	2008	<i>O. sativa</i> var. Teyou 838	...	...	...	...	...	...	...	6	J. X. Feng
FJ15	Fujian, China	2008	<i>O. sativa</i> var. Yongyou 6	...	...	...	...	...	...	...	7	J. X. Feng
MAI3 (CFBP7326)	Niono, Mali	2004	<i>O. sativa</i> var. Wassa	27	27	18	7	21	15	3	8	V. Verdier
MAI4 (CFBP7327)	Niono, Mali	2004	<i>O. sativa</i> var. Nonioka	28	28	19	8	22	16	...	9	V. Verdier
MAI5 (CFBP7328)	Niono, Mali	2004	<i>O. sativa</i> var. Jamajigi	29	28	19	9	23	16	3	10	V. Verdier
MAI7 (CFBP7334)	Niono, Mali	2004	<i>O. sativa</i> var. Jamajigi	30	29	17	10	24	16	3	11	V. Verdier
MAI8 (CFBP7330)	Niono, Mali	2004	<i>O. sativa</i> var. Nonioka	31	28	17	8	22	17	3	12	V. Verdier
MAI10 (CFBP7331)	Niono, Mali	2004	<i>O. sativa</i> var. Nonioka	27	30	17	8	22	17	3	12	V. Verdier
MAI11 (CFBP7335)	Niono, Mali	2004	<i>O. sativa</i> var. Jamajigi	27	30	18	8	21	18	3	13	V. Verdier
Stability panel												
FJ11	Fujian, China	2008	<i>O. sativa</i> var. Yongyou 6	...	...	...	...	...	...	...	14	J. X. Feng
FJ12	Fujian, China	2008	<i>O. sativa</i> var. Yongyou 6	...	...	...	...	...	...	...	15	J. X. Feng
FJ13	Fujian, China	2008	<i>O. sativa</i> var. Yongyou 6	...	...	...	...	...	...	...	16	J. X. Feng
MAI18 (CFBP7338)	Kogoni, Mali	2009	<i>O. longistaminata</i>	...	...	...	...	...	...	...	17	V. Verdier
MAI19	Kogoni, Mali	2009	<i>O. longistaminata</i>	...	...	...	...	...	...	...	17	I. Wonni
MAI21	Kogoni, Mali	2009	<i>O. longistaminata</i>	...	...	...	...	...	...	...	18	35

^a Haplotypes based on restriction fragment length polymorphism (RFLP), repetitive sequence-based polymerase chain reaction (rep-PCR), enterobacterial repetitive intergenic consensus (ERIC)-PCR, and amplified fragment length polymorphism (AFLP) analyses were taken from Gonzalez et al. (12). Same numbers indicate the same haplotype. VNTR = variable number of tandem repeats.

^b CFBP, Collection Française de Bactéries Phytopathogènes, Institut National de la Recherche Agronomique (INRA), Angers, France; LMG, Belgian Coordinated Collection of Microorganisms, University of Gent, Belgium; NCPBP, National Collection of Plant Pathogenic Bacteria, United Kingdom.

Tools/msa/muscle/) (10). PCR primers matching conserved segments were designed using the Finnzymes website (http://www.finnzymes.fi/tm_determination.html) (Table 2). To allow multiplexing, primers were designed to have similar annealing temperatures at $69.5 \pm 2.5^\circ\text{C}$ at 200 nM primer concentration and

50 mM salt concentration. Designed primer sequences were queried against the *X. oryzae* pv. *oryzicola* genome sequences using the MFEprimer website (<http://biocompute.bmi.ac.cn/MFEprimer/>) (25) to confirm that the primer pairs will only amplify one locus per genome.

TABLE 2. Oligonucleotide primers, polymerase chain reaction (PCR) conditions, and characteristics of variable numbers of tandem repeats (VNTR) analyzed in this study

VNTR ^a	TRS ^b	Position ^c	Open reading frames ^d	Primers (5'–3')	PCR conditions			
					T (°C) ^e	Amplicon (bp) (number of repeats) ^f		
						BLS256	GX01	MAI10
B01	TTCCCAA	208464–208544	XOC_0222: SEC-C motif protein	GCACAGCAGCCACGGCAA/ GCGTGGACGGATGCCAGC	55	366 (11)	338 (7)	352 (12)
B02	CGCACAG	351012–351077	NO	AGGCGGCGTTGCATCGTG/ AACAGCGCGTGCCGATG	55	502 (9)	467 (4)	467 (4)
B03	TCCCTCGAA	446122–446194	NO	GGCTCGTCGCTACGGAGC/ TGGAACACGGCTGCGTCG	60	405 (8)	NC	NC
B04	CGATTGCC	825999–826047	XOC_0825: two-component system sensor protein KdpD	CGTCGGCCGCCAAGTACG/ GTCTCGGACACGCGCACG	60	949 (6)	941 (5)	946 (6)
B05	CGATAC	1450149–1450186	XOC_1467: conserved hypothetical protein	GCAGACGGATGGGCGTTG/ CGCTGTCGGCAGGGTTTG	55	343 (6)	361 (9)	369 (7)
B06	AATCGGG	1453245–1453400	XOC_1471: acetyltransferase	GGAATGCCGAAGCGCTCG/ TGCTCGACCTGGAAGGCG	50	916 (21)	881 (16)	790 (3)
B07	GGCAGT	1523859–1523924	XOC_1550: conserved hypothetical protein	TAATCGGCACCGGCGAGC/ AAGGCGATGCTGCTGGGC	55	623 (10)	631 (6)	581 (3)
B08	TGAGCCT	1666816–1666862	XOC_1684: hypothetical protein	GCGGTGGGCGAGCGATGTC/ ATGGTTGTGGTGCGGCGG	50	705 (6)	729 (5)	698 (5)
B09	CGGGAAT	1775575–1775625	NO	CGCGCCGATCACGCCCTTG/ GGCCACAAACGCAACCCG	55	511 (7)	497 (5)	497 (5)
B10	AATCCCG	1838952–1838993	NO	GCGCTACACCGGCTTCGC/ ATCGGAACGTGGCTGCGC	55	284 (6)	277 (5)	277 (3)
B11	CCTGGA	2154494–2154537	XOC_2159: hypothetical protein	CTGCCGGCCTTGCCCAAG/ ATGCTTGCGCGTCCACGG	55	614 (7)	590 (3)	596 (4)
B12	CAGGCC	2159999–2160079	XOC_2163: N-acetylmuramoyl-L-alanine amidase	TGACCCGCATACGACAGCG/ AAGCGGTCATCGCTGCGG	58	448 (13)	400 (5)	406 (6)
B13	TGGCAG	2574214–2574251	XOC_2531: FHA domain protein	TGGATCAGCGTGACCGGC/ TCGCCACCTCGTTCACGC	65	499 (6)	517 (11)	526 (13)
B14	GGCGGAT	2857319–2857385	XOC_2789: Smr domain protein	GGATCTGCACGCGGCCAC/ CGCCTTGTCGATCCGCA	55	789 (9)	754 (4)	789 (10)
B15	GCAGGT	3164085–3164120	XOC_3096: putative secreted protein	CCGACCAATGCCGAGCAG/ CCAGTCTTCGGCCACGCG	55	168 (6)	162 (5)	150 (3)
B16	CGAGATT	3243409–3243450	NO	GCTCGGCCACGAAGCTGC/ GCGCATGAAGCAGGTCGG	55	391 (6)	384 (5)	384 (5)
B17	CTGCTG	3605916–3606009	NO	AGGCAGGCGAAATCGGCG ACGCCGAGGAAGCCGGTG	60	826 (15)	937 (34)	856 (25)
B18	CCGATTC	3731758–3731807	XOC_3625: proton glutamate symport protein	ACGGATGGCGTTGGCCAG TCGGCATGATCCTGGGCG	55	395 (7)	367 (3)	374 (4)
B19	TGCTGTTGC	3919919–3919977	XOC_3795: general secretion pathway protein N	GAAACAGGCGCGGCGTCA CTGACCGGCGTGCTGCTG	55	576 (6)	609 (10)	600 (10)
B20	TGGCTAT	3942348–3942533	XOC_3810: conserved hypothetical protein	GCTTGGCGGGTCACATCG TGGATCGACGCCGACTG	55	483 (26)	364 (9)	406 (17)
B21	TGATTGGC	4317966–4318022	XOC_4162: hypothetical protein	ACCCGGCAACTCGCAACC GGCACGAGCAAGCGGCAT	55	318 (7)	358 (12)	366 (13)
G01	GGCGGT	797716–797740	NO	TCAAGCGCGTCGCATTGC CAAGACACGCCGCATGC	67	626 (4)	674 (12)	620 (3)
G04	TCTCT	1812443–1812462	NO	ACAGGCATCGGCCGATTG GGGGTCTGCTGGGTCGAC	55	272 (4)	282 (6)	277 (5)
G05	CGCGTG	1796631–1796682	XOC_1822: ABC transporter ATP-binding protein	CAGCACAGCGATCGCCTG CAGGGGCTGCGCAGCTAC	53	367 (8)	361 (7)	355 (6)
G09	CGGCAA	712624–712868	XOC_0712: rod shape-determining protein	GGACACCATGCCCGTCGG GGACACCATGCCCGTCGG	65	339 (5)	386 (6)	386 (8)
G11	CCGCGT	2578487–2578508	XOC_2535: transposase	AGCCCCACCAATCACGC ACGCGGAGCAAAAGGCGT	55	401 (3)	416 (6)	328 (3)
M01	TCGGGAT	2792163–2792193	NO	CGCAGATGCTCGTTGCCG CGATGCGACGCTGGATGC	60	554 (4)	561 (5)	568 (6)
M08	GGGATTC	3518302–3518341	NO	CAGGGCGAACGCGATGAG GCTCCATGGTGCCGAGAG	58	427 (5)	427 (5)	441 (7)

^a VNTR loci.

^b Tandem repeat sequence.

^c Position in strain BLS256.

^d Annotated function in strain BLS256.

^e Annealing temperature.

^f Expected amplicon size and number of repeats for strain. NC indicates that the locus is present on different contigs and the amplicon sizes thus cannot be calculated.

PCR amplification, agarose gel electrophoresis, and DNA sequencing. PCR amplifications were performed using genomic DNA of *X. oryzae* pv. *oryzicola* strains, including the reference strains BLS256, MAI10, and GX01 as the template. Each PCR reaction was carried out in a final volume of 25 µl and containing 5 to 10 ng of genomic DNA, 2.5 mM MgCl₂, 40 nM PCR primers, 2 mM dNTP, and 0.5 units of *Taq* DNA polymerase (Promega Corp., Madison, WI) in 1× buffer. All reactions were run for 35 cycles, each consisting of 30 s at 94°C, 30 s at 50 to 67°C (depending on the primer pair), and 60 s at 72°C, with an initial activation step of 5 min at 94°C and a final extension step of 10 min at 72°C.

PCR products were separated on 1.2% agarose gels and visualized under UV upon staining with ethidium bromide. PCR-amplified VNTR loci from the panel of 20 *X. oryzae* pv. *oryzicola* strains were adjusted to the required DNA concentration (at least 30 ng/µl) and sent for custom DNA sequencing (Beckman Coulter Genomics, UK).

Description and analysis of VNTR profiles. The number of repeats of each VNTR allele was counted upon alignment of DNA sequences using the MUSCLE software. Repeat numbers were rounded to the closest integer number. The allelic profile of a given strain was defined as the repeat numbers at each VNTR locus included in the MLVA scheme. The discriminatory power of single and combined (MLVA-25) VNTR loci, MLSA, RFLP, and rep-PCR data were calculated using the Hunter-Gaston discriminatory index (HGDI), using the following formula:

$$D = 1 - \left[\frac{1}{N(N-1)} \sum_{j=1}^s x_j(x_j - 1) \right]$$

where D is the numerical index of discriminatory power, N is the total number of strains in the typing scheme, s is the total number of different strain types, and x_j is the number of strains belonging to the j th type (14).

Phylogenetic trees were derived from repeat numbers, based on neighbor joining using the continuous Manhattan dissimilarity index of the software package DARwin, version 5.0.158, from CIRAD France (<http://darwin.cirad.fr/>) (22).

RESULTS

Exploitation of genomic resources for development of an MLVA scheme for *X. oryzae* pv. *oryzicola*. Complete and draft *X. oryzae* pv. *oryzicola* genome sequences were used to predict VNTR loci with at least six tandem repeats of 5 to 9 bp in length. For the completely sequenced Philippine strain BLS256, 21 loci were predicted, whereas, for the two draft genome sequences of strains GX01 and MAI10, only 11 and 10 loci were predicted, respectively (Fig. 1; Table 2). Nine loci from BLS256 were also predicted for GX01 (B06), for MAI10 (B12, B14, and B19), or for both of these strains (B01, B05, B13, B17, and B20). Based on the conservation of flanking regions, all 42 predicted loci could be grouped to 28 distinct VNTR loci (Fig. 1A). BLAST analyses revealed that all predicted loci were also present in the other strains, even if they were not predicted due to smaller number of repeats or higher variability among individual repeats than required by our prediction parameters. All predicted loci were mapped onto the genome sequence of strain BLS256 and were found to be randomly distributed along the chromosome (Fig. 1B).

Multiple sequence alignment of flanking regions allowed design of universal PCR primers for Asian and African strains of *X. oryzae* pv. *oryzicola* (Table 2). In silico PCR predicted that the amplified DNA fragments of all 28 loci should be polymorphic among the three sequenced strains of *X. oryzae* pv. *oryzicola* (Table 2).

VNTR analyses of a test panel of *X. oryzae* pv. *oryzicola* strains. To assess the utility of the 28 candidate VNTR loci for

Fig. 1. Predicted variable numbers of tandem repeats (VNTR) loci in three sequenced *Xanthomonas oryzae* pv. *oryzicola* strains. **A**, Number and name of predicted VNTR loci among three *X. oryzae* pv. *oryzicola* reference strains; **B**, location of candidate VNTR loci on the circular genome map of *X. oryzae* pv. *oryzicola* strain BLS256.

the development of an MLVA typing scheme, we selected 20 strains from our collection as a test panel (Table 1). Fourteen strains representing worldwide diversity, including seven Asian isolates (three strains from China, three strains from Malaysia, and the Philippine reference strain BLS256) and seven African strains (all from Mali), served as a diversity panel. Twice, three supposedly closely related strains originating from China and Mali were included in the study to elucidate the resolution of the VNTR loci (Table 1, stability panel). These strains had been collected in the same year, in the same region, and from the same cultivar.

Upon PCR amplification and confirmation by analytical agarose gel electrophoresis, DNA fragments were sequenced using one of the PCR primers, thus allowing an estimate of the number of tandem repeats for every VNTR locus (Table 3). Among the 28 candidate VNTR loci, 25 yielded amplification products for most *X. oryzae* pv. *oryzicola* strains and allowed unambiguous assignment of the number of tandem repeats. However, three loci (B07, B12, and G01) provided unsatisfactory results (Table 3). For B07 and B12 loci, sequences from nearly half of the strains were entirely different from those of the reference strains. For G01, only poor amplification was observed for several strains, suggesting that this locus is not widespread in *X. oryzae* pv. *oryzicola* strains from Asia (UBP497) and Africa.

For the remaining 25 unambiguous VNTR loci, repeat numbers were polymorphic among the set of 20 strains. Altogether, 18 unique VNTR patterns (haplotypes) were resolved (Table 4). Repeat numbers varied between 2 (B06 and B08) and >40 (B17 and B20). On average, 5.6 haplotypes were observed per locus. Some loci had only two to three haplotypes (B10, B16, G11, and M01) and appeared to be stable. In contrast, other loci consisting of high repeat numbers appeared to evolve rapidly, thus resulting in up to 13 haplotypes (B06, B17, B20, and B21).

For some loci (B06 in strain BLS256; B06, B07, and B17 in strain GX01; and B01, B10, B14, B17, B20, and G19 in strain MAI10), discrepancies were observed between our experimentally derived amplicon sizes and the sizes predicted from the available genome sequences (Table 5). For instance, B06 was

predicted to be 923 bp in BLS256, whereas it was observed to be 916 bp, corresponding to a difference of a single 7-bp repeat unit. Likewise, B06 was predicted to be 1,413 bp in GX01, whereas it was observed to be 881 bp, corresponding to a difference of 76 7-bp repeat units; and B17 was predicted to be 856 bp in MAI10,

TABLE 4. Hunter-Gaston discriminatory index (HGDI) scores for individual or combined (MLVA-25) variable numbers of tandem repeats (VNTR) loci calculated for 20 *Xanthomonas oryzae* pv. *oryzicola* strains

VNTR loci	Range of repeat number ^a	Haplotypes	HGDI scores
B01	7–14	7	0.795
B02	3–9	7	0.779
B03	3–8	5	0.653
B04	5–8	4	0.658
B05	5–9	5	0.721
B06	2–32	9	0.795
B07	Unrelated		
B08	2–6	5	0.695
B09	4–7	4	0.363
B10	3–6	3	0.511
B11	3–8	6	0.737
B12	Unrelated		
B13	5–10	5	0.706
B14	3–11	7	0.847
B15	3–6	4	0.623
B16	5–6	2	0.526
B17	13–47	8	0.795
B18	3–11	6	0.737
B19	3–14	7	0.795
B20	6–49	13	0.953
B21	5–17	9	0.858
G01	Poor		
G04	3–6	4	0.647
G05	4–9	5	0.712
G09	4–10	5	0.816
G11	3–6	3	0.484
M01	4–6	3	0.700
M08	5–10	5	0.726
MLVA-25	2–49	18	0.974

^a Unrelated = unrelated loci and poor = poorly conserved.

TABLE 3. Repeat numbers of 28 variable numbers of tandem repeats (VNTR) loci in 20 *Xanthomonas oryzae* pv. *oryzicola* strains

Loci ^a	Country	B01	B02	B03	B04	B05	B06	B07	B08	B09	B10	B11	B12	B13	B14	B15	B16	B17	B18	B19	B20	B21	G01	G04	G05	G09	G11	M01	M08
Minimum		7	3	3	5	5	2	4	2	4	3	3	3	5	3	3	5	13	3	3	6	5	3	3	4	4	3	4	5
Maximum		14	9	8	8	9	32	10	6	7	6	8	13	10	11	6	6	47	11	14	49	17	12	6	9	10	6	6	10
Haplotype		7	7	5	4	5	9	6	5	4	3	6	6	5	7	4	2	8	6	7	13	9	5	4	5	5	3	3	5
I																													
FJ13	Fujian, China	13	8	5	8	5	14	9	3	5	5	3	8	7	9	4	6	13	5	5	33	16	3	4	4	4	4	4	8
FJ12	Fujian, China	12	7	5	7	6	19	6	3	5	5	3	6	7	9	5	6	13	5	5	36	17	3	4	4	4	4	4	8
GX07	Guangxi, China	13	7	5	7	6	15	8	3	5	5	3	7	7	10	5	6	13	5	5	33	14	3	4	4	5	4	4	8
FJ15	Fujian, China	14	9	5	8	6	15	8	3	5	5	3	7	7	4	5	6	13	5	5	28	16	3	4	4	4	4	4	8
CFBP2287	Malaysia	11	6	5	6	7	11	–	3	4	3	3	–	–	8	5	6	13	6	3	49	9	7	4	4	5	3	4	8
CFBP2286	Malaysia	12	7	6	7	6	21	–	5	6	3	6	–	6	11	6	6	20	11	5	24	5	5	4	–	5	3	4	5
BLS256	Philippine	11	9	8	6	6	21	10	6	7	6	7	13	6	9	6	6	15	7	6	26	7	4	4	8	5	3	4	5
II																													
FJ11	Fujian, China	9	4	3	5	7	32	8	4	5	5	3	5	10	5	5	5	47	3	9	24	12	4	6	7	6	6	5	5
GX01	Guangxi, China	7	4	3	5	9	16	6	5	5	5	3	5	9	4	5	5	34	3	10	9	12	12	6	7	6	6	5	5
MAI3	Niono, Mali	8	3	4	5	5	11	–	5	5	3	3	–	5	8	3	5	28	4	14	11	11	4	5	9	5	3	5	6
III																													
MAI10	Niono, Mali	12	4	3	6	7	3	–	5	5	3	4	6	7	10	3	5	25	4	10	17	13	–	5	6	8	3	6	7
MAI8	Niono, Mali	12	4	3	6	7	3	–	5	5	3	4	–	7	10	3	5	25	4	10	17	13	–	5	6	8	3	6	7
MAI4	Niono, Mali	12	4	3	6	7	3	–	5	5	3	4	–	7	10	3	5	20	4	10	17	13	3	5	6	8	3	6	7
MAI21	Niono, Mali	12	4	3	6	7	3	–	5	5	3	4	–	6	11	3	5	20	4	10	10	12	3	5	6	8	3	6	7
MAI5	Niono, Mali	12	4	3	6	7	3	–	5	5	3	4	–	7	11	3	5	20	4	10	10	12	3	5	6	8	3	6	7
MAI11	Niono, Mali	11	4	3	6	7	3	–	5	5	3	4	6	7	11	3	5	20	4	10	15	12	–	5	–	8	3	6	7
MAI19	Niono, Mali	7	5	3	6	8	3	–	4	5	3	5	–	5	9	3	6	20	4	9	14	12	4	5	6	10	3	5	7
MAI18	Niono, Mali	7	5	3	6	8	3	–	4	5	3	5	–	5	9	3	6	20	–	9	14	12	–	5	6	10	3	5	7
MAI7	Niono, Mali	12	4	3	6	6	3	–	5	5	3	4	4	–	9	3	6	31	4	7	10	14	–	5	6	6	3	5	7
IV																													
UBP497	Malaysia	–	3	6	7	7	2	–	2	6	3	8	3	5	3	3	–	20	3	5	6	11	–	3	4	4	3	5	10

^a Loci in clusters I, II, III, and IV. Minimal and maximal numbers of repeats are given for each locus. Haplotypes = number of different VNTR patterns given for each locus; – indicates that polymerase chain reaction (PCR) amplification failed or DNA sequence of the PCR amplicon was not related to the VNTR locus.

whereas it was observed to be 886 bp, corresponding to a difference of five 6-bp repeat units.

Discriminatory indices (HGDI) were calculated for all VNTR loci except for B07, B12, and G01 (Table 4). HGDI scores varied significantly from 0.363 for B09 to 0.953 for B20. As described previously (28), VNTR loci were classified into highly (>0.6), moderately (0.3 to 0.6), and poorly (<0.3) discriminating based on the HGDI scores. Of the 25 loci, 22 loci showed high discriminatory power. Only three loci (B09, B10, and G11) were found to be only moderately discriminatory (0.336, 0.511, and 0.484, respectively) (Table 4). Combining all 25 VNTR loci into an MLVA-25 scheme resulted in an HGDI score of 0.974, thus demonstrating their suitability for typing of *X. oryzae* pv. *oryzicola* strains.

New diagnostic markers specific to strains from China and Mali. Some haplotypes were clearly distinct when comparing Asian strains with African strains. Except for strain MAI3, which shares several features with Asian strains and might have been introduced to Africa recently (13), 11 loci were not polymorphic among the African *X. oryzae* pv. *oryzicola* strains (Table 3). Locus B06 contains only three repeats in strains from Mali

(except for MAI3), while most Asian strains have repeat arrays with >10 copies. Locus B20 is another striking example where most Asian strains exhibit a higher number of repeat units (24 to 49, except for strains UBP497 and GX01) than the African strains (10 to 17) (Table 3; Fig. 2).

In *sensu stricto*, not taking into account a possible Asian origin of strain MAI3, 19 of 25 VNTR loci were polymorphic in Malian strains (except for B09, B10, B15, B18, G04, and G11) while 22 of 25 were polymorphic in Chinese strains from Guangxi and Fujian provinces (except for B09, B10, and B11). Therefore, one could consider the nonpolymorphic loci as diagnostic markers for strains from China and Mali. Specifically, a VNTR profile with five, five, and three repeats for B09, B10, and B11, respectively (called VNTR-C profile of 5-5-3) might serve as a marker for Chinese strains. Likewise, a VNTR-M profile of 5-3-3-4-5-3 for B09, B10, B15, B18, G04, and G11 would indicate an origin from Mali.

Phylogenetic analysis. To study the genetic relationships among Asian and African *X. oryzae* pv. *oryzicola* strains, an unweighted pair group with arithmetic means cluster analysis based on Manhattan genetic distances was performed (Fig. 2). In

TABLE 5. Comparison of observed and expected sizes of variable numbers of tandem repeats (VNTR) markers in *Xanthomonas oryzae* pv. *oryzicola* reference strains^a

VNTR marker	BLS256				GX01				MAI10			
	Expected		Observed		Expected		Observed		Expected		Observed	
	Size (bp)	RN	Size (bp)	RN	Size (bp)	RN	Size (bp)	RN	Size (bp)	RN	Size (bp)	RN
B06	923	22	916	21	1,413	92	881	16	...	...	...	...
B07	...	...	...	...	625	5	631	6	...	...	...	...
B17	...	...	...	...	889	26	937	34	856	20	886	25
B01	...	...	...	...	...	...	...	...	352	9	373	12
B10	...	...	...	...	...	...	...	...	277	5	263	3
B14	...	...	...	...	...	...	...	...	789	9	796	10
B19	...	...	...	...	...	...	...	...	600	9	609	10
B20	...	...	...	...	...	...	...	...	406	15	420	17

^a Size = amplicon size and RN = repeat number.

Fig. 2. Dendrogram of 20 *Xanthomonas oryzae* pv. *oryzicola* strains obtained by neighbor joining using the continuous Manhattan dissimilarity index. Bootstrap values (for 1,000 replicates) are given at the nodes.

the resulting dendrogram, Asian strains were clearly separated from African strains, except for MAI3. All *X. oryzae* pv. *oryzicola* strains were grouped into four clusters which widely reflected their geographic origin. Cluster I was composed of Asian strains, including four Chinese strains (GX07, FJ12, FJ13, and FJ15), two Malaysian strains (CFBP2286 and CFBP2287), and the Philippine strain BLS256. Cluster III exclusively consisted of strains from Mali. Strikingly, cluster II contains two Chinese strains and one African strain, MAI3, further supporting a possible Asian origin of strain MAI3. One Malaysian strain, UPB497, was clearly separated from all other strains and made up cluster IV.

DISCUSSION

MLVA has become an important and popular typing tool to study the population structure of many human and animal pathogens because this technique is highly discriminatory, portable, robust, and fast (23,32,34). Although the first VNTR study of a bacterial plant pathogen, *X. fastidiosa*, dates back to 2001 (9), VNTR has not yet received much attention in the field of bacterial phytopathology, probably owing to limited access to (multiple) genome sequences. Only recently, VNTR analyses were used to characterize other plant pathogens, such as ‘*Candidatus Liberibacter* spp.’, *Pseudomonas syringae*, and *X. citri* (5,11,16,24). Here, we took advantage of the availability of three genome sequences of *X. oryzae* pv. *oryzicola* to develop the first MLVA typing scheme for rice-pathogenic xanthomonads.

Most PCR primers allowed unambiguous amplification of only one specific VNTR-related DNA fragment, demonstrating that the method is robust and useful for large-scale analyses. In a few cases, however, amplification failed despite several attempts (B01 and B16 for strain UPB497; G05 for CFBP2286 and MAI11; B13 for CFBP2287 and MAI7; and B18 for MAI18). Either these VNTR loci are not present in these strains; they have been disrupted by IS elements, preventing amplification due to the large size; or they suffer from mutations at the primer binding sites. With the available sequence information generated within this project, we will evaluate new PCR primers to amplify the few missing loci.

In this study, a few discrepancies appeared between our experimentally derived amplicon sizes and the sizes predicted from the available genome sequences (Table 5). We assume that these discrepancies are due to mistakes in the genome assemblies,

especially in the cases of next-generation draft genome sequencing of strains MAI10 (454 technology) and GX01 (Illumina technology). However, we cannot rule out that VNTR loci evolved new repeat numbers during strain propagation, a scenario which might have occurred for strain BLS256; in this case, we only found one difference by one single repeat.

Compared with the previously described typing methods such as rep-PCR, ERIC-PCR, RFLP, and AFLP, our data suggest that MLVA is at least equally powerful to discriminate *X. oryzae* pv. *oryzicola* strains. For instance, nine strains from the diversity panel (CFBP2286, CFBP2287, MAI3, MAI4, MAI5, MAI7, MAI8, MAI10, and MAI11), originating from Mali and Malaysia, were clearly resolved into eight haplotypes by VNTR markers (HGDI = 0.972), whereas only six and five haplotypes were obtained by rep-PCR (HGDI = 0.889) and ERIC-PCR analysis (HGDI = 0.861), respectively (Table 6) (12). RFLP analysis with insertion sequence elements (IS1112 and IS1113) or type III effector genes (*avrXa10* and *Xoo1762*) resulted in seven, three, seven, and five haplotypes, respectively, with HGDI scores of 0.667 to 0.917. Only combination of all four RFLP probes allowed resolution of all nine strains (Table 6) (12). On the other hand, our MLVA scheme did not allow resolution of strains MAI8 and MAI10 that had distinct profiles in two RFLP schemes (IS1112 and *avrXa10*) (Table 6) (12). Yet, compared with RFLP, VNTR has the advantage of higher sensitivity and better reproducibility. Its high resolution is also supported by the fact that all three Chinese strains and two of the Malian strains from the stability panel had distinct haplotypes, thus demonstrating its suitability for *X. oryzae* pv. *oryzicola* population typing (Table 1).

Another approach (MLSA) to type *X. oryzae* pv. *oryzicola* strains relies on partial sequencing of the four housekeeping genes *rpoD* (encoding RNA polymerase sigma-70 factor), *gyrB* (encoding DNA gyrase subunit B), *glnA* (encoding glutamine synthetase I), and *efp* (encoding elongation factor P) (13). However, only very few single-nucleotide polymorphisms (SNPs) were found (i.e., zero SNP in *rpoD*, five SNPs in *gyrB*, one SNP in *glnA*, and one SNP in *efp*) among the *X. oryzae* pv. *oryzicola* strains of the diversity panel (except for the Chinese strains which were not included in that study). Scrutinizing these four genes separately, only one, three, two, and two haplotypes were determined, with HGDI scores of 0, 0.644, 0.2, and 0.2, respectively (13). Combining all four housekeeping genes results in four haplotypes with an HGDI score of 0.694, far lower than that of our MLVA-25 score (0.972).

Interestingly, DNA sequences of all housekeeping genes are identical between the African strain MAI3 and the Asian strains BLS256, CFBP2286, and UPB497 (originating from the Philippines and Malaysia) (13). In contrast, all other analyzed strains from Mali (MAI4, MAI5, MAI7, MAI8, MAI10, and MAI11) share four SNPs in *gyrB*. We sequenced a gene fragment of *gyrB* from all six Chinese strains and found them to be identical to the fragments of BLS256, CFBP2286, and UPB497 (data not shown). Hence, these SNPs appear to be diagnostic for Asian and African strains and support the Asian origin of strain MAI3. Finally, the Asian origin of strain MAI3 is further supported by its ‘Asian’ repertoire of type III effectors. In contrast to most African strains, MAI3 does not suffer from an IS element insertion in *xopW* and this strain contains *xopAJ* (synonym of *avrXa10*), a gene which had hitherto only been detected in Asian *X. oryzae* pv. *oryzicola* strains but not in other strains of *X. oryzae* (13,35).

Our MLVA-derived dendrogram demonstrated that Asian and African *X. oryzae* pv. *oryzicola* strains belong to distinct phylogenetic groups (Fig. 2), a finding which was consistent with previous reports (12,13,35). In line with data from MLSA and type III effector mining, Malian strain MAI3 clustered together with two Chinese strains (cluster II), thus supporting an Asian (maybe Chinese) origin of this isolate. Trans-continental transfer of xanthomonads through contaminated germplasm or seed is not

TABLE 6. Hunter-Gaston discriminatory index (HGDI) scores for combined (MLVA-25) variable numbers of tandem repeats (VNTR) loci, restriction fragment length polymorphism (RFLP), multi-locus sequence analysis (MLSA), and repetitive sequence-based polymerase chain reaction (rep-PCR) profiles calculated for nine *Xanthomonas oryzae* pv. *oryzicola* strains from the diversity panel

Typing methods	Haplotypes	HGDI scores	References
MLVA			
MLVA-25	8	0.972	This study
RFLP			
RFLP-IS1112	7	0.917	12
RFLP-IS1113	3	0.667	12
RFLP- <i>avrXa10</i>	6	0.889	12
RFLP- <i>Xoo1762</i>	5	0.806	12
RFLP	9	1	12
rep-PCR			
rep-PCR	6	0.889	12
ERIC-PCR ^a	5	0.861	12
MLSA			
<i>gyrB</i>	3	0.644	13
<i>rpoD</i>	1	0	13
<i>efp</i>	2	0.2	13
<i>glnA</i>	2	0.2	13
MLSA-4	4	0.694	13

^a ERIC = enterobacterial repetitive intergenic consensus.

without precedents and urges the development of sensitive and efficient tools for detection and high-resolution typing (15,29).

Genetic distances among the African strains (except for MAI3) are smaller than those among the Asian strains, even within the set of Chinese strains. One could speculate that African strains are endemic and started to diversify only recently, concomitant with intensification of rice cultivation on the African continent. We included six Chinese strains in our analyses, originating from the two provinces Guangxi and Fujian. Strains of both provinces are found in two different clusters, hinting at a transmission of contaminated rice seed between these two provinces. Notably, strain UBP497 from Malaysia forms an independent cluster, well separated from the other two Malaysian strains. Unfortunately, detailed information about the year and site of collection is not available for all three Malaysian strains. Altogether, this set of VNTR markers will be useful for population analyses at different spatial scales, suggesting that VNTR typing is a novel, powerful technique for epidemiological monitoring of *X. oryzae* pv. *oryzicola* strains.

Simple diagnostic markers for the discrimination of *X. oryzae* pv. *oryzicola* strains at large geographic scale could supplement high-resolution molecular typing. Genomic-based diagnostic markers have been developed to distinguish *X. oryzae* pv. *oryzae* from *X. oryzae* pv. *oryzicola* strains and to specifically detect U.S. strains related to *X. oryzae* (17,31). However, these markers do not allow researchers to distinguish African from Asian *X. oryzae* pv. *oryzicola* strains. Although analysis of *xopW* and *xopAJ* and detection of specific SNPs in housekeeping genes might allow differentiation of African and Asian *X. oryzae* pv. *oryzicola* strains, specific VNTR profiles, such as the VNTR-C profile (repeat numbers of 5-5-3 for B09, B10, and B11, respectively) and the VNTR-M profile (repeat numbers of 5-3-3-4-5-3 for B09, B10, B15, B18, G04, and G11, respectively), would even allow the identification of strains from China and perhaps Mali (Table 4). Analyses of more strains, including isolates from other African and Asian countries, will be necessary to generalize these conclusions. For instance, BLS has been reported also in Madagascar, Nigeria, Senegal, and Burkina Faso (<http://www.eppo.int>) (3,36) and their analyses will allow researchers to conclude whether or not the Malian strains are representative of African *X. oryzae* pv. *oryzicola* and whether African and Asian *X. oryzae* pv. *oryzicola* strains can be clearly differentiated by our method.

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