Applied and Environmental Microbiology

Microsatellite Markers for Characterization of Native and Introduced Populations of Plasmopara viticola, the Causal Agent of Grapevine Downy Mildew

Mélanie Rouxel, Daciana Papura, Marilise Nogueira, Virginie Machefer, Damien Dezette, Sylvie Richard-Cervera, Sébastien Carrere, Pere Mestre and François Delmotte
Appl. Environ. Microbiol. **2012, 78(17):6337. DOI: 10.1128/AEM.01255-12.**
Published Ahead of Print 15 June 2012.

AEM
Journals.ASM.org

Microsatellite Markers for Characterization of Native and Introduced Populations of *Plasmopara viticola*, the Causal Agent of Grapevine Downy Mildew

Mélanie Rouxel,[a,b] Daciana Papura,[a,b] Marilise Nogueira,[a,b] Virginie Machefer,[a,b] Damien Dezette,[a,b] Sylvie Richard-Cervera,[a,b] Sébastien Carrere,[c] Pere Mestre,[d,e] and François Delmotte[a,b]

INRA, ISVV, UMR1065 Santé et Agroécologie du Vignoble, Villenave d'Ornon, France[a]; Université de Bordeaux, ISVV, UMR1065 Santé et Agroécologie du Vignoble, Villenave d'Ornon, France[b]; INRA, Laboratoire des Interactions Plantes Microorganismes (LIPM), Castanet-Tolosan, France[c]; Université de Strasbourg, UMR1131 Santé de la Vigne et Qualité du Vin, Colmar, France[d]; and INRA, UMR1131 Santé de la Vigne et Qualité du Vin, Colmar, France[e]

We reported 31 microsatellite markers that have been developed from microsatellite-enriched and direct shotgun pyrosequencing libraries of *Plasmopara viticola*, the causal agent of grapevine downy mildew. These markers were optimized for population genetics applications and used to characterize 96 *P. viticola* isolates from three European and three North American populations.

Grapevine (*Vitis vinifera*) is cultivated worldwide, and wine-making plays an important role in the economy of many countries. Grapevine downy mildew is considered one of the most important grapevine diseases in temperate climates. *Plasmopara viticola* ([Berk. & Curt.] Berl. and de Toni), the causal agent of downy mildew, is a diploid heterothallic oomycete native to North America. Conducting population genetic studies on this obligate endoparasite requires the development of species-specific markers, such as microsatellites (SSRs) or single nucleotide polymorphisms (SNPs). These markers allow high-throughput genotyping of isolates through extraction of DNA directly from plant lesions, avoiding time-consuming subculture of isolates on leaves. To date, 11 microsatellites and eight SNP markers have been described in *P. viticola* (5, 6, 8). Related to the economic importance of this pathogen species, the low number of genetic markers available in *P. viticola* reflects the difficulties that were previously encountered by research groups to isolate microsatellites using traditional methods (5, 7, 8). Recent reports showed the relevance of combining the use of high-throughput sequencing technologies with bioinformatics to isolate microsatellite markers in non-model species (1, 11). So far, two methods based on shotgun pyrosequencing have been applied to microsatellite marker discovery: (i) direct shotgun pyrosequencing (DSP) by randomly sequencing the genome and searching *a posteriori* for microsatellite sequences (1, 10) and (ii) microsatellite enriched-library pyrosequencing (MEP) that uses a microsatellite-enriched library as a basis for shotgun pyrosequencing (11). Based on these two approaches, we report the development of 31 microsatellite markers for *P. viticola* that will increase the genotyping capacity for this major pathogen of grapevine. The new species-specific markers developed here provide the possibility of using a common set of microsatellites from local to continental geographic scales, opening the door for new genetic studies addressing *P. viticola* dispersal processes.

Two complementary methods were used to isolate new microsatellite markers in *P. viticola*. First, we searched for microsatellites in sequences generated by direct pyrosequencing of DNA and cDNA of *P. viticola* using the 454 genome sequencer FLX Titanium. cDNA was prepared using zoospores from strains SC and SL (see Table S1 in the supplemental material) with the Clontech SMARTer PCR cDNA synthesis kit (Saint-Germain-en-Laye, France). Genomic DNA was prepared using zoospores from the strain Pv221 (see Table S1 in the supplemental material) with the DNeasy plant minikit (Qiagen Inc., Chatsworth, CA). Half a run of 454 was performed for each strain, yielding 369,105 reads (69 Mb) for SC, 419,725 reads (139.5 Mb) for SL, and 391,760 reads (130 Mb) for Pv221 (see Table S2 in the supplemental material). Perfect di- to hexanucleotide microsatellite markers with a minimum of six repeat copies were searched with SciRoKo (9), resulting in the identification of 131 nonredundant loci: 88 resulted from the direct shotgun pyrosequencing of genomic DNA of isolate Pv221, and 53 resulted from the direct shotgun pyrosequencing of cDNA of both SL and SC isolates. Primers were successfully designed for 52 of these loci using the Perl script DesignPrimer (9). Second, we applied the high-throughput method developed by Malausa et al. (11) that is based on coupling multiplex microsatellite enrichment and next-generation pyrosequencing to isolate microsatellites in the strain Pv221 (see Table S1 in the supplemental material). Briefly, an adapted biotin enrichment protocol was applied using eight biotin-labeled oligonucleotides—$(AG)_{10}$, $(AC)_{10}$, $(AAC)_8$, $(AGG)_8$, $(ACG)_8$, $(AAG)_8$, $(ACAT)_6$, and $(ATCT)_6$—and the resulting library was sequenced by 454. This led to the identification of 2,092 microsatellite motifs among 33,057 reads (6.6 Mb) (see Table S2 in the supplemental material). The QDD pipeline (12) was used to analyze the sequences and design primers for amplification of the detected microsatellite motifs. Retaining sequences greater than 80 bp with perfect and compound microsatellites presenting a minimum of six repetitions led to the selection of 66 nonredundant loci. Therefore, the two approaches yielded a total of 118 microsatellite loci. None of the loci were common to the two approaches, and we did not

Received 18 April 2012 **Accepted** 5 June 2012
Published ahead of print 15 June 2012
Address correspondence to Mélanie Rouxel, melanie.rouxel@bordeaux.inra.fr.
Supplemental material for this article may be found at http://aem.asm.org/.

doi:10.1128/AEM.01255-12

TABLE 1 Characteristics of the 35 microsatellite loci developed for *Plasmopara viticola*

Locus	GenBank accession no.	F and R primer sequences (5′–3′)	Repeat motif	Annealing temp (°C)	Size range of alleles (bp)	Method of identification[a]
Pv61	JQ219983	TCTTCAGGTAGATGCGACCA; GGTGACTCCTCGGACGAATA	$(CA)_9$	54	181–187	MEP
Pv65	JQ219972	CTTTGGCCCACGTCATAGTT; CGCTTTCGGTAGGTCCATTA	$(TC)_9$	57	196–202	MEP
Pv67	JQ219973	GCATTGAGCAGACACCTTGA; GAGCGATAAGACCACAAATAGTGA	$(AC)_9$	54	348–368	MEP
Pv74	JQ219984	GCAACGTTGTGCAAGCTTTA; GCATTATGATGGAGCTCACG	$(AG)_7$	54	176–182	MEP
Pv76	JQ219974	CTGGTTGCTGATGCACTGAC; GGCGGTGACTAAGTCGTTGT	$(TC)_7$	57	136–140	MEP
Pv83	JQ219985	TGCAGCATTGTTTCATCCAT; ACACGGTACTTTGCGTTCCT	$(TG)_6$	54	238–242	MEP
Pv87	JQ219986	CGTGCAATTCAAACAACAGG; CTCACAAGGACGACTGGACA	$(CT)_6$	54	152–154	MEP
Pv88	JQ219987	AATACCAAAAATGGCCGTCA; ACTCTCTTGCCAGCACCATC	$(GT)_6$	54	202–208	MEP
Pv91	JQ219975	ACCAGCCTTTGCGAAGATAA; TGAAAGTTACGTGTCGCACC	$(TG)_6$	54	142–146	MEP
Pv93	JQ219976	TAGCACCGGACTAGGCGTAT; TGTACCCTGTTGCCCTCTTC	$(GT)_6$	54	147–151	MEP
Pv96	JQ219977	TAGTCTTCAGATTTCGCCGT; ATCATTGTAAGGCCAAGAAA	$(CA)_6$	54	172	MEP
Pv100	JQ219978	TGATAAGATACCGCACAGGC; TTGTTTGAAGCACTGAACGC	$(TA)_6$	54	231	MEP
Pv101	JQ219979	AACACGGCGCCAAAGTATTA; GGGCATTAACGTGCAAATTC	$(CTT)_6$	54	263–266	MEP
Pv102	JQ219980	GATCGCCTTTTGCAATGTCT; AAAGGAGTCAACATGCTCGC	$(TC)_6$	54	273	MEP
Pv103	JQ219981	TGACCTACCACCCATTTACCA; ACGGTCAGGTCAAAAGCAGT	$(TG)_6$	54	277–299	MEP
Pv104	JQ219982	CTACGCTCGAGGATGACACA; GACATTGCCGCACCTAAGAT	$(CA)_6$	54	321–324	MEP
Pv124	JQ219988	AACGACAGACGGATTTCTGC; GACCTCGAGCGCTTTGAC	$(AGG)_6$	57	139–142	MEP
Pv126	JQ219989	GCTCTCTGCAGGACGTTTTT; GCCGTTCTTCACGTTCTAGC	$(GAC)_{10}$	50	182–206	MEP
Pv127	JQ219990	TTGAAAACGCGGATAGGAAC; GAACGTCCAGTTCGGATTGT	$(CA)_9$	54	213–223	MEP
Pv133	JQ219991	AACGACAGACGGATTTCTGC; CGACCTCGTCTTCACTTTCC	$(AGG)_6$	54	178–181	MEP
Pv134	JQ219992	CATGCTCACGTAGACCTCCA; AATGCAGAGCTCCCATAACG	$(AG)_6$	54	220–226	MEP
Pv135	JQ219993	GGTGCTCTGCTTCGACACTT; CGCCACACAAGTCAACTTTC	$(TTC)_{10}$	57	217–220	DSPg
Pv136	JQ219994	GTTTCGCTGAAACAGAAGGC; ATCGTCCTGCCAGAAATGAC	$(CTT)_{10}$	57	161–164	DSPg
Pv137	JQ219995	AAGTGGGACACATCAAGCGT; TGGCAATAAGTTTATGCCTCG	$(AT)_9$	57	243–256	DSPg
Pv138	JQ219996	CGTGGATCATGACGTTTGTC; CGACGAATCAGGGACAAGAT	$(TA)_9$	57	225–235	DSPg
Pv139	JQ219997	GACCCGGACAATGGACTCTA; CCGCCATGTATTGAACAGTG	$(AC)_8$	57	126–133	DSPg
Pv140	JQ219998	GCTTGAGAAGAATGGAACGC; CCCAGAAGGGTGATACGAGA	$(TA)_9$	57	172–201	DSPg
Pv141	JQ219999	ACGACGACATGAGCTGTACG; GAAGGTGGTGTCATGGGTTT	$(TC)_9$	57	190–192	DSPg
Pv142	JQ220000	TTATGCCACGCAAATCTCTG; AGGGCGAAATACGAGAGTGA	$(CT)_{11}$	57	209–219	DSPg
Pv143	JQ220001	CCTGAATAAAGCAACACGCA; TTGGCAGCAAATTGTACGAC	$(AT)_8$	57	121–135	DSPg
Pv144	JQ220002	ACCAAGAATCGCACCTAACG; GTCTGCCTGTTTGTCGGTTA	$(AT)_{12}$	57	161–192	DSPg
Pv145	JQ220003	GACTTGAAGGAAGCCATCCA; CTCTCTCCAAAGTTCGTCGG	$(AGA)_6$	57	204	DSPc
Pv146	JQ220004	CTCGGACCTTGAAGAACGAC; ACGTGGCCTAGGTTCACAAG	$(GAG)_7$	57	242–245	DSPc
Pv147	JQ220005	TCGACTACGAGTCCGAGAGG; TTCTAGCTCGACGAAGACCG	$(TCGACT)_8$	57	189–219	DSPc
Pv148	JQ220006	CGACCTATGTTTCGCCATTT; GAGTCGTCGTAGAAGGCGTC	$(ACA)_6$	57	134–137	DSPc

[a] MEP, microsatellite-enriched pyrosequencing; DSPg, direct shotgun pyrosequencing of genomic DNA; DSPc, direct shotgun pyrosequencing of cDNA.

identify in our sequence data set the loci previously described by Gobbin et al. (8) and Delmotte et al. (5).

An initial PCR amplification was performed on a panel of 21 *P. viticola* isolates and on a negative control (*V. vinifera*). DNA extractions were performed as described in Delmotte et al. (5). PCR amplifications were carried out in a final 15-μl reaction volume including 1.5 μl of 10× buffer (Eurogentec, Belgium), 0.45 μl of 50 mM $MgCl_2$, 0.4 μl of 10 mM deoxynucleoside triphosphates (dNTPs), 0.3 μl of a dye-labeled forward primer and an unlabeled reverse primer (10 mM), and 0.2 U of *Taq* Silverstar DNA polymerase (Eurogentec, Seraing, Belgium). PCR cycles were performed in an Eppendorf Mastercycler ep gradient with the following conditions: an initial denaturation at 94°C for 4 min and 38 cycles of 30 s at 94°C, 30 s at the appropriate annealing temperature (Table 1), and 35 s at 72°C, ending with a 5-min extension at 72°C. PCR products were migrated on agarose gel (1%) with a Gene Ruler 100-bp DNA ladder (Fermentas), and those presenting irregular amplification or multiple banding patterns were discarded. The remaining 44 loci were analyzed as follows: 1 μl of PCR products (diluted at 1:100) was mixed with 8.86 μl of formamide and 0.14 μl of GeneScan 600 LIZ (internal lane size standard) and analyzed in an Applied Biosystems 3130 capillary sequencer. Alleles were scored using the GeneMapper v4.0 software (Applied Biosystems, Foster City, CA). Finally, 35 primer pairs with clearly interpretable PCR products were retained (Table 1).

In order to assess the polymorphism of these loci, we genotyped 96 *P. viticola* isolates from Europe and North America (see Table S1 in the supplemental material). The European samples included isolates from two French regions (Gironde, $n = 16$; Vaucluse, $n = 16$) and from the Rhine Valley in Germany ($n = 16$), while samples from North America originated in Michigan ($n = 16$), New York ($n = 16$), and Virginia ($n = 16$). DNA extractions, PCR amplifications, and automated genotyping at the 35 microsatellite loci were performed as described previously. The total number of alleles per loci (N_a) and the observed and expected heterozygosity (H_O and H_E) were estimated for each locus using Genetix v4.05 (2). Tests for deviation from the Hardy-Weinberg equilibrium (HWE) were conducted using the same software, and the significance threshold was determined using Bonferroni's correction for multiple tests. The number of alleles per locus ranged from 1 to 16, with a mean (± standard deviation [SD]) allele number per locus of 3.9 (± 2.8) (Table 2). In Europe, 12 loci

TABLE 2 Population genetic analysis of 35 SSR loci in European and North American populations of *Plasmopara viticola* based on the genotyping of 96 isolates[a]

Locus	Total N_a	Europe						North America					
		Global			H_O for Gironde (France)	H_O for Rhine Valley (Germany)	H_O for Vaucluse (France)	Global			H_O for Michigan	H_O for Virginia	H_O for New York
		N_i	N_a	H_O				N_i	N_a	H_O			
Pv61	4	41	3	0.073	0.062	0.125		43	4	0.349	0.071	0.333	0.643
Pv65	5	42	2	0.523	0.562	0.437	0.562	25	4	0.24*	0.333	0.429	0.133*
Pv67	3	40	2	0.275	0.2	0.25	0.467	36	3	0.111	0.187	0.1	
Pv74	4	38	2	0.026*	0*	0.071		17	3	0.059*	0.25		
Pv76	4	37	1					37	3	0.027*		0.071*	
Pv83	4	41	2	0.073	0.067	0.125		38	4	0.211*	0.111	0.333	0.143
Pv87	2	42	1					45	2	0*			
Pv88	4	40	2	0.15	0.286	0.062	0.062	44	3	0.25		0.267	0.467
Pv91	3	42	2	0.261	0.125	0.562		45	3	0.178		0.25	0.267
Pv93	4	41	3	0.317	0.133	0.562	0.125	33	2	0.333		0.25	0.467
Pv96	1	42	1					32	1				
Pv100	1	31	1					0	0				
Pv101	3	42	2	0.595	0.562	0.5	0.875	27	3	0.259*		0.091	0.75
Pv102	1	30	1					0	0				
Pv103	4	42	2	0.309	0.312	0.5		46	3	0.283*	0.312	0.067	0.467
Pv104	2	42	1					39	2	0.051*		0.2	
Pv124	2	42	1					47	2	0.17		0.437	0.067
Pv126	6	42	2	0.024	0.062			42	6	0.547*	0.429	0.6	0.615
Pv127	5	39	3	0.051	0.143			35	5	0.657	0.692	0.6	0.667
Pv133	2	41	1					42	2	0.167		0.467	
Pv134	4	42	1					43	4	0.349*	0.333	0.467	0.231
Pv135	2	42	2	0.048		0.125		45	2	0.467	0.867	0.2	0.333
Pv136	2	41	1					38	2	0*			
Pv137	6	41	3	0.585	0.625	0.533	0.562	28	5	0.393*	0.143	0.571	0.286
Pv138	6	40	5	0.25*	0.312	0.312*		38	6	0.263*	0.154	0.467	0.1
Pv139	4	41	3	0.146		0.375		45	3	0.156		0.133	0.357
Pv140	9	41	8	0.683	0.867	0.437	0.75	44	7	0.454*	0.333	0.467	0.571
Pv141	2	42	2	0.548	0.562	0.375	0.875	45	2	0.511		0.733	0.8
Pv142	5	42	2	0.238	0.375	0.125	0.312	13	5	0.308*	1	0.333	
Pv143	5	42	3	0.548	0.687	0.625	0.312	44	4	0.25*	0.214	0.067	0.467
Pv144	16	42	11	0.667*	0.75	0.812	0.375*	44	11	0.204*	0.4	0.214*	0*
Pv145	1	42	1					45	1				
Pv146	2	42	1					34	2	0.206	0.111	0.429	
Pv147	6	42	5	0.476	0.562	0.562	0.125	46	2	0.196*	0.067	0.187	0.333
Pv148	2	42	2	0.286	0.187	0.25	0.5	43	2	0.372	0.357	0.429	0.333

[a] For each population, 16 isolates were genotyped. N_i, number of isolates amplified; N_a, number of alleles; H_O, observed heterozygosity; *, significant deviation from the Hardy-Weinberg equilibrium test after Bonferroni's correction for multiple tests ($P < 0.0014$).

(34.2%) were monomorphic, while only two loci (5.7%) were monomorphic in North America (Pv96 and Pv145). The reduced allelic polymorphism found in the European population may likely illustrate the founder effect and the demographic bottleneck resulting from the introduction of grapevine downy mildew in Europe in the late 1870s (4). It is worth noting that two loci, found to be monomorphic in Europe, could not be successfully amplified in American isolates (Pv100 and Pv102). This result might reflect the fact that microsatellite discovery was performed on sequences obtained from European strains of the pathogen.

Plasmopara viticola reproduces clonally for part of the year, which can result in the spread of identical multilocus genotypes. We found 89 distinct multilocus genotypes (G) among the 96 isolates genotyped (N) ($G/N = 0.93$), indicating limited resampling of clones. The seven repeated multilocus genotypes were found in Vaucluse ($n = 6$) and in New York ($n = 1$). Since clonal amplification of genotypes can affect data interpretation, subsequent Hardy-Weinberg tests were performed using only one copy per multilocus genotype identified ($n = 89$). The expected heterozygosities ranged from 0.024 to 0.888. Among the 31 polymorphic microsatellite markers, only five presented a deviation from the Hardy-Weinberg equilibrium. Significant deficits in observed heterozygotes were detected at only 3 loci (Pv74, Pv138, and Pv144) in Europe (Table 2). For North American populations, 15 loci presented a significant deficit in heterozygotes, but this number fell to 3 (Pv65, Pv76, and Pv144) when the analysis was performed separately on the 3 geographic populations (Table 2). The occurrence of null alleles is the most likely explanation for the within-population heterozygote deficits detected at these loci (3).

The combined use of high-throughput sequencing technologies and bioinformatics led to the isolation and development of 31 new microsatellite markers for *P. viticola*, the causal agent of grapevine downy mildew. Our results suggest that a similar ap-

proach will be successful for the discovery of microsatellites in other non-model plant-pathogenic species. These 31 new microsatellite loci provide a new tool for conducting large-scale population genetic studies that will increase our understanding of the worldwide genetic structure of this invasive plant pathogen.

ACKNOWLEDGMENTS

This research was supported by AIP Bioressources (INRA, France) and the Foundation Jean Poupelain (Javrezac, France).

We thank the following colleagues and institutes for their help with collection of grapevine downy mildew samples: D. Gadoury from Cornell University, A. Baudoin from Virginia Tech, and H. Kassemeyer from Staatliches Weinbauinstitut Freiburg.

REFERENCES

1. **Abdelkrim J, Robertson BC, Stanton JOL, Gemmell NJ.** 2009. Fast, cost-effective development of species-specific microsatellite markers by genomic sequencing. Biotechniques **46**(3):185–191.
2. **Belkhir K, Borsa P, Chikhi L, Raufaste N, Bonhomme F.** 1996-2004. GENETIX 4.05, logiciel sous Windows TM pour la génétique des populations. Laboratoire Génome, Populations, Interactions, CNRS UMR 5000, Université de Montpellier II, Montpellier, France. http://kimura.univ-montp2.fr/genetix/.
3. **Chakraborty R, De Andrade M, Daiger SP, Budowle B.** 1992. Apparent heterozygote deficiencies observed in DNA typing data and their implications in forensic applications. Ann. Hum. Genet. **56**:45–47.
4. **Chen WJ, et al.** 2007. At least two origins of fungicide resistance in grapevine downy mildew populations. Appl. Environ. Microbiol. **73**: 5162–5172.
5. **Delmotte F, et al.** 2006. Microsatellite DNA markers for *Plasmopara viticola*, the causal agent of downy mildew of grapes. Mol. Ecol. Notes. **6**:379–381.
6. **Delmotte F, et al.** 2011. Characterization of single-nucleotide-polymorphism markers for *Plasmopara viticola*, the causal agent of grapevine downy mildew. Appl. Environ. Microbiol. **77**:7861–7863.
7. **Dutech C, et al.** 2007. Challenges of microsatellite isolation in fungi. Fungal Genet. Biol. **44**:933–949.
8. **Gobbin D, Pertot I, Gessler C.** 2003. Identification of microsatellite markers for *Plasmopara viticola* and establishment of high throughput method for SSR analysis. Eur. J. Plant Pathol. **109**:153–164.
9. **Kofler R, Schlötterer C, Lelley T.** 2007. SciRoKo: a new tool for whole genome microsatellite search and investigation. Bioinformatics **23**(13): 1683–1685.
10. **Lepais O, Bacles CFE.** 2011. Comparison of random and SSR-enriched shotgun pyrosequencing for microsatellite discovery and single multiplex PCR optimization in *Acacia harpophylla* F. Muell. Ex Benth. Mol. Ecol. Resour. **11**:711–724.
11. **Malausa T, et al.** 2011. High-throughput microsatellite isolation through 454 GS-FLX titanium pyrosequencing of enriched DNA libraries. Mol. Ecol. Resour. **11**:638–644.
12. **Meglécz E, et al.** 2010. QDD: a user-friendly program to select microsatellite markers and design primers from large sequencing projects. Bioinformatics **26**(3):403–404.