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PRIMER NOTE

Microsatellite DNA markers for *Plasmopara viticola*, the causal agent of downy mildew of grapes

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Abstract

Microsatellite loci were isolated from *Plasmopara viticola* (Oomycetes), the causal agent of downy mildew of grape, one of the most damaging fungal diseases of grapevine worldwide. Seven polymorphic loci were obtained from an enriched partial genomic library. A low genetic diversity was observed at all loci, with a mean observed allele number of 3.75 and an observed heterozygosity ranging from 0.074 to 0.547. Cross-amplification tests on three closely related taxa indicated that two loci could be used in other Oomycetes species. These microsatellite loci were proved to be useful for population genetic analysis.

Keywords: enriched library, grape downy mildew, microsatellite, *Plasmopara viticola*

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Grape downy mildew is one of the most damaging fungal diseases of grapevine (*Vitis* spp.) worldwide. It is caused by the heterotallic diploid *Plasmopara viticola* (Berk. & Curt. *ex*. de Bary), a biotrofic Oomycetes (Stramenopiles) native of North America. In the late 1870s, *P. viticola* was accidentally introduced to Europe, probably when American vine stocks resistant to grape phylloxera (*Daktulosphaira vitifoliae*) were used to graft the European varieties (Viennot-Bourgin 1949). The solution to control this devastating pathogen was found by Alexis Millardet who discovered in 1882 the prophylactic effect of lime and copper and developed the 'Bordeaux mixture', which became the first successful fungicide to be used in vineyards. Today, grape downy mildew control in vineyards is ensured by effective fungicide spray programme. However, the control of grape downy mildew by chemicals has led to fungicide resistance development in *P. viticola* populations. Managing fungicide resistance, i.e. controlling the occurrence and the spread of resistant strains in vineyards, requires a good knowledge of population genetics of the pathogen (reproductive mode, gene flow, effective population size). Gobbin *et al*. (2003a, b, 2005) have carried out the first genetic study on *P. viticola* using four microsatellite markers. However, these studies only assessed the genetic structure at the very fine scale (vineyard) and three of the markers used either presented low polymorphism or unclear banding pattern. In this context, we present the results of a new genomic library enriched for microsatellites on *P. viticola*.

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Genomic DNA was extracted from fresh sporangia following Sambrook *et al*. (1989) and digested with *Rsa*I restriction enzyme. A 500–900-bp fraction of the digested DNA was selected on agarose gel, purified and ligated to *Rsa* linkers. The enrichment procedure followed the protocol from Kijas *et al*. (1994) based on streptavidin-coated magnetic particles (Magnesphere, Promega) with slight modifications. A 5′-biotinylated $(CT)_{10}$ and $(GT)_{10}$ oligonucleotide were used as probes. The polymerase chain reaction (PCR) products were purified and ligated into pGEM-T Easy vector (Promega), and the plasmid transformed into *Escherichia coli* supercompetent cells (XL1 blue, Stratagene). In total for both motives (CT and GT), 8714 recombinant clones were cultured onto LB agar/ampicillin Petri dishes at 37 °C overnight. Individual *E. coli* colonies were transferred to a positively charged Hybond-N^+ membrane (Amersham). DNA was fixed on the membrane by baking at 80 °C for an hour. Positive colonies were identified by hybridization with $(CT)_{10}$ and $(GT)_{10}$ probes labelled with digoxigenin using the DIG Nucleic Acid Detection Kit (Boehringer Manheim). A total of 389 positive clones were

Table 1 Primer sequences of seven variable microsatellite loci in *Plasmopara viticola*. N_i, number of individual; N_a, number of alleles; H_O, observed heterozygosity; H_E, expected heterozygosity. The forward primers were labelled with Beckman dyes D2 (Pv13, Pv16), D3 (Pv14, Pv39) and D4 (Pv7, Pv17, Pv31)

Locus	GenBank Accession no.	Primer sequence (5′–3′)	Repeat motif	T_a (°C)	Size range (bp)	N_i	N_a	H_E	H_O
Pv7	DQ217575	L: TCTTCCGAAAAGGGACGTAA R: GCGTCACTGCATCTACGAAA	$(TG)_7$	60	289–297	42	5	0.471	0.223
Pv13	DQ217576	L: CGATGAAGTGGACCCTCATT R: CCGGTAGTCAATTGCACCTT	$(TG)_8$	61	214–220	91	4	0.510	0.195
Pv14	DQ217577	L: CAGAAACGCACAAGGTCTGA R: AATTGCATACTGCAGCAACG	$(TG)_8$	65	120–128	75	5	0.677	0.547
Pv16	DQ217578	L: TAAAAATATGGTGGCGTCAG R: CCAGCAGTCTCCGTCTCATCAG	$(TGC)_2TGT(TGC)_3$	57	248–251	60	2	0.494	0.460
Pv17	DQ217579	L: CAGAGTCGAACAAGTACATTG R: CTTTGTCGCCTTCTAACAAC	$(TC)_{12}$	59	160–172	72	6	0.543	0.462
Pv31	DQ217580	L: TCCCCATGCTGAAGAGTTTC R: TTCTTTCTAAGGCCGTGTGG	$(CA)_9$	60	241–247	14	4	0.592	0.143
Pv39	DQ217581	L: ACGCATGGCGAACACGTAAG R: CAGACGGGAAGAAGTTGCTC	$(CA)_6$	61	174–176	54	2	0.209	0.190

Table 2 Cross-amplification in three closely related plant-pathogens species (Oomycetes): *Plasmopara halstedii*, *Bremia lactucae* and *Phytophthora infestans*. Unsuccessful amplifications are noted as —. When we obtained amplification products, we presented the size of both alleles

	Pv7	Pv13	Pv14	Pv16	Pv17	Pv31	Pv39
Plasmopara halstedii	—	—	124/126	248/251	—	—	171/196
Bremia lactucae	—	—	124/126	243/243	—	—	179/192
Phytophthora infestans	—	—	124/126	248/248	—	—	—

picked and stored in plates containing glycerol at –80 °C. One hundred and ninety-two of them were sequenced, and we obtained 181 sequences, of which 160 containing repeated units. Redundant clones were eliminated leading to a set of 58 unique sequences. For 40 sequences, pairs of primers were successfully designed using the software PRIMER 3 (Rozen & Skaletsky 2000). PCRs were carried out in 15 μL of reaction mixture containing 1.5 μL 10× buffer, 0.45 μL 25 mM $MgCl_2$, 0.2 μL 10 mM dNTPs, 0.2 U of *Taq* Silverstar DNA polymerase (Eurogentec), 0.2 μL of 10 μM of a dye-labelled forward primer and an unlabelled reverse primer. Amplifications were performed on a 9700 thermocycler (PE Applied Biosystems). After an initial denaturing step of 2 min at 94 °C, 35 cycles were performed, each consisting of 30 s at 95 °C, 30 s at the appropriate annealing temperature (Table 1) and 30 s at 72 °C. A final extension step was performed at 72 °C for 2 min.

Among 40 loci tested, 32 either gave nonreproducible amplifications, showed multiband patterns or amplified the host plant (*Vitis vinifera*). Finally, seven loci specific to downy mildew and giving clear amplifications were chosen for population analysis (Table 1). PCR products were sized on a Beckman Coulter Ceq8000 automated sequencer using the manufacturer's fragment detection chemistry.

The seven loci were tested on 150 individuals of *P. viticola* sampled at a large geographical scale in European ($N = 100$) and American vineyards ($N = 50$). They showed a low level of variability with a number of alleles ranging from one to six (mean = 3.75), an averaged observed heterozygosity ranging from 0.143 to 0.547, and an expected heterozygosity ranging from 0.209 to 0.677. Significant heterozygosity deficit was observed at Pv7, Pv13, Pv17 and Pv31 resulting from the reduced polymorphism at these loci in European populations, suggesting a strong bottleneck during the introduction of *P. viticola* in Europe. There were no significant deviations from linkage equilibrium between any pair of loci. The utility of these primers for closely related species was tested for the same PCR and electrophoresis conditions on three related Oomycetes species: *Plasmopara halstedii*, *Phytophtora infestans* and *Bremia Lactucae*. We obtained PCR products for Pv14, Pv16 and Pv39 (Table 2). Finally, microsatellites isolation from *P. viticola* proved to be difficult with a density of nonredundant positive clones of 1.6%. Furthermore, the microsatellite sequences were often short and yielded low variable markers.

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