Characterization of Single-Nucleotide-Polymorphism Markers for Plasmopara viticola, the Causal Agent of Grapevine Downy Mildew

F. Delmotte, V. Machefer, X. Giresse, S. Richard-Cervera, M. P. Latorse and R. Beffa


Published Ahead of Print 16 September 2011.

Updated information and services can be found at:
http://aem.asm.org/content/77/21/7861

These include:

**REFERENCES**
This article cites 12 articles, 2 of which can be accessed free at:
http://aem.asm.org/content/77/21/7861#ref-list-1

**CONTENT ALERTS**
Receive: RSS Feeds, eTOCs, free email alerts (when new articles cite this article), [more»](http://aem.asm.org/content/77/21/7861#ref-list-1).

Information about commercial reprint orders: [http://aem.asm.org/site/misc/reprints.xhtml](http://aem.asm.org/site/misc/reprints.xhtml)

To subscribe to another ASM Journal go to: [http://journals.asm.org/site/subscriptions/](http://journals.asm.org/site/subscriptions/)
Characterization of Single-Nucleotide-Polymorphism Markers for
Plasmopara viticola, the Causal Agent of Grapevine Downy Mildew

F. Delmotte,¹*, V. Machefer,¹ X. Giresse,¹ S. Richard-Cervera,¹ M. P. Latorse,² and R. Beffa²

INRA, UMR1065 SAVE, F-33883 Villenave d’Ornon Cedex, France,¹ and Bayer CropScience SA, La Dargoire Research Center, 1720 Rue Pierre Baizet, BP 9163, F-69263 Lyon Cedex 09, France²

Received 9 June 2011/Accepted 4 September 2011

We report 34 new nuclear single-nucleotide-polymorphism (SNP) markers that have been developed from an expressed sequence tag library of Plasmopara viticola, the causal agent of grapevine downy mildew. This newly developed battery of markers will provide useful additional genetic tools for population genetic studies of this important agronomic species.

Grapevine downy mildew, a disease caused by the oomycete Plasmopara viticola, causes substantial losses of yields in vineyards worldwide. P. viticola is an invasive species accidentally introduced into Europe in the late 1870s, probably with American vine stocks used to graft the European varieties that were replenished after the phylloxeric crisis. Today, fungicide treatment is the only available method to control this biotrophic pathogen on Vitis vinifera. However, the systematic use of chemicals has led to fungicide resistance in P. viticola populations, thereby reducing the efficiency of a growing number of products. To establish long-term management of fungicide resistance in natural populations of P. viticola, the underlying evolutionary mechanisms that drive the appearance, propagation, and maintenance of resistance need to be elucidated (2). The use of population genetics allows evaluation of the major determinants of fungicide resistance, i.e., selection, mutation, recombination, genetic drift, and gene flow (7).

Conducting population genetic studies of obligate endoparasites, such as P. viticola, requires the development of specific and codominant markers. The species specificity of the marker is especially important, as it allows high-throughput genotyping of isolates directly from sporulating lesions collected from host leaves, avoiding the need for labor-intensive isolate subculture. Eleven microsatellite markers are available to assess the genetic structure within populations of P. viticola (3, 9). Among the 11 markers developed, 3 of them were difficult to score and the remaining 8 markers had a low number of alleles (mean number of alleles/locus of 3.7 ± 1.1). Expressed sequence tag (EST)-derived single-nucleotide-polymorphism (SNP) markers that have polymorphisms for point mutations and insertions or deletions provide promising molecular markers for species in which microsatellites are difficult to isolate and which have low levels of polymorphism (5). Confirming this view, SNP markers have already been successfully used to describe the genetic structure of other plant pathogens (6), including downy mildew species such as Plasmopara halstedii (4, 8). With the aim of increasing the available number of species-specific markers for P. viticola, we present the development of single-nucleotide polymorphisms (SNP) derived from an EST library from Bayer CropScience.

An EST library was constructed using material isolated from 5-day-old infected leaves of Vitis vinifera inoculated with a strain of P. viticola provided by Bayer CropScience. Total RNA was extracted from the leaf sample, cDNA was synthesized and cloned, and a total of 3,500 reads were generated by Sanger sequencing. Reads were trimmed and assembled using the Staden Package software program, resulting in 438 contigs and 1,887 singletons. Grapevine sequences were discarded by performing BLAST analyses on the Vitis vinifera genome (10), and the remaining cDNA sequences were compared to the transcripts of P. sojae and P. ramorum (12) using TBLASTX and to the Swiss-Prot amino acid database using BLASTX. This led to the unambiguous identification of 974 P. viticola sequences.

We screened these 974 P. viticola sequences for new specific markers, such as single-sequence repeats (SSRs) and SNPs. First, we “mined” the cDNA/expressed sequence tag (EST) database for potentially polymorphic SSRs by performing an in silico search for tandem repeat patterns of ≥10 bp using the sputnik program (http://expressosoftware.com/sputnik/index.html). SSRs of ≥10 bp are likely to be monomorphic in such a plant pathogen species; therefore, we did not include shorter SSRs in this analysis (5). Among the 974 cDNA clones, we found eight sequences that had an SSR of ≥10 bp. Of these, seven sequences were excluded because no primer pairs could be designed around the repeated motif (located at the extremities of the clone sequence). The other SSR was in a sequence that gave a significant BLAST hit with a Phytophthora species tRNA gene. This locus was not retained for further genetic analysis because of its mitochondrial origin. Second, we studied polymorphisms of 28 EST sequences with highly significant similarity (E values < 10⁻²⁰) to known protein sequences. We designed primers to screen the sequences for SNPs. We detected polymorphisms using a panel of 42 isolates collected in two different vineyards in France (Latresne in the Bordeaux vineyards, Nîmes in the Côte du Rhône vineyards).

We extracted the DNA from P. viticola isolates using freeze-dried infected plant tissue as previously described by Delmotte...
et al. (3). All PCR amplification reactions were carried out in a final volume of 25 μL containing 10 ng of genomic DNA, 2 mM MgCl₂, 150 μM each deoxynucleoside triphosphate (dNTP), 4 pmol of each primer, and 0.2 U Taq Silverstar DNA polymerase (Eurogentec) in reaction buffer. Thermal cycling was performed under the following conditions: an initial denaturation step of 5 min at 94°C, followed by 38 cycles of 50 s denaturation at 94°C, 50 s annealing at 50°C, and 60 s elongation at 72°C, and a final elongation step of 10 min at 72°C. SNPs were detected by PCR-single-strand conformation polymorphism (PCR-SSCP): conformational differences due to the mutations were revealed on a 6% nondenaturing polyacrylamide gel with migration at 4°C at 10 W overnight. The polyacrylamide gels were silver stained as described by Benbouza et al. (1). GenePop version v4 (11) was used to calculate allelic frequencies, observed and expected heterozygosities, and fixation index (Fₛ) and to perform exact tests for genotype linkage disequilibrium and deviation from Hardy-Weinberg equilibrium.

Among the 28 sequences evaluated by PCR-SSCP, 17 were polymorphic (61%). However, 9 of these 17 DNA sequences were excluded from further population genetic analysis because they had limited genetic diversity (i.e., an expected heterozygosity lower than 0.1). For each of the remaining markers (n = 8), all of the alleles detected by PCR-SSCP were sequenced to determine the exact position of the mutation responsible for the observed polymorphism. The markers gave a total of 34 SNPs, including 24 transitions and 10 transversions (Table 1). The mean (± standard error [SE]) number of alleles per marker was 2.4 (± 0.18), and the frequency of the rarest allele ranged from 0.06 to 0.35, with a mean (± SE) frequency of 0.21 (± 0.1) (Table 2). The expected heterozygosity for each locus ranged between 0.20 and 0.66, demonstrating the presence of genetic diversity in these markers. Pairwise tests revealed that these EST-derived markers were not in linkage disequilibrium. Significant deviation from the Hardy-Weinberg equilibrium was observed for Pvi2, Pvi3, and Pvi5, due to a deficit in expected heterozygotes (Table 2). This might result from the Wahlund effect, i.e., reduction of heterozygosity caused by subpopulation structure. On this basis, the estimated genetic differentiation between the two *P. viticola* populations analyzed was significantly different from zero (Fₛ = 0.01, P < 0.01).

Finally, we evaluated the potential use of primers for these

<table>
<thead>
<tr>
<th>Locus</th>
<th>Accession no.</th>
<th>Homology</th>
<th>Primers (5’–3’)</th>
<th>Ta (°C)</th>
<th>Size (bp)</th>
<th>nₐ</th>
<th>Hₑ</th>
<th>Hₒ</th>
<th>Observed frequency</th>
<th>Hardy-Weinberg</th>
<th>Allelic frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pvi1</td>
<td>JF897856</td>
<td>Hypothetical protein Ribosomal protein Hsp 60</td>
<td>F: CCCTGACTCCCTGTTGTTC  R: ACGAATAGGTTGCGAGTGGGA  F: TGAGTAGGGCAAAGATCAGC  R: CGATACACGCTATACCCAC  F: CTCAGGGGAGGATGCAAT  R: CAAATCCGTAGGGTTCATGC  F: CTACATCTCGTCCGAGAAAGG  R: AACGAATAGGGTGCGTAGGA</td>
<td>50</td>
<td>494</td>
<td>2</td>
<td>0.45</td>
<td>0.34</td>
<td>0.17</td>
<td>0.96</td>
<td>0.66</td>
</tr>
<tr>
<td>Pvi2</td>
<td>JF897857</td>
<td>HSP 90</td>
<td>F: AAAGGGGCAAGATCAGC  R: CGATACACGCTATACCCAC  F: CTCAGGGGAGGATGCAAT  R: CAAATCCGTAGGGTTCATGC  F: CTACATCTCGTCCGAGAAAGG  R: AACGAATAGGGTGCGTAGGA</td>
<td>50</td>
<td>450</td>
<td>3</td>
<td>0.66</td>
<td>0.25</td>
<td>0</td>
<td>1</td>
<td>0.40</td>
</tr>
<tr>
<td>Pvi3</td>
<td>JF897858</td>
<td>Manganese superoxide dismutase</td>
<td>F: CTCAGGGGAGGATGCAAT  R: CAAATCCGTAGGGTTCATGC  F: CTACATCTCGTCCGAGAAAGG  R: AACGAATAGGGTGCGTAGGA</td>
<td>50</td>
<td>299</td>
<td>3</td>
<td>0.54</td>
<td>0.32</td>
<td>0</td>
<td>1</td>
<td>0.63</td>
</tr>
<tr>
<td>Pvi4</td>
<td>JF897859</td>
<td>Annexin</td>
<td>F: CCCTGACTCCCTGTTGTTC  R: ACGAATAGGTTGCGAGTGGGA  F: TGAGTAGGGCAAAGATCAGC  R: CGATACACGCTATACCCAC  F: CTCAGGGGAGGATGCAAT  R: CAAATCCGTAGGGTTCATGC  F: CTACATCTCGTCCGAGAAAGG  R: AACGAATAGGGTGCGTAGGA</td>
<td>50</td>
<td>366</td>
<td>2</td>
<td>0.20</td>
<td>0.15</td>
<td>0.27</td>
<td>0.98</td>
<td>0.89</td>
</tr>
<tr>
<td>Pvi5</td>
<td>JF897860</td>
<td>Hypothetical protein</td>
<td>F: GGACATTCGGCGTTGTG  R: CGCAGCTCTCCCTTCATATT  F: GGAATTTGGAACCAGAAGGTC  R: TAAATGAGTAAACCGGGTTTG  F: CTCAGGGGCAAGGACCTTAC  R: GAACACACGAGCAACACT  F: CCAAGTGCAAGCAAGTAAA  R: GCCAAAAAGGGAAAAATAAAGCA</td>
<td>50</td>
<td>278</td>
<td>3</td>
<td>0.55</td>
<td>0.14</td>
<td>0</td>
<td>1</td>
<td>0.61</td>
</tr>
<tr>
<td>Pvi6</td>
<td>JF897861</td>
<td>HSP 90</td>
<td>F: GGACATTCGGCGTTGTG  R: CGCAGCTCTCCCTTCATATT  F: GGAATTTGGAACCAGAAGGTC  R: TAAATGAGTAAACCGGGTTTG  F: CTCAGGGGCAAGGACCTTAC  R: GAACACACGAGCAACACT  F: CCAAGTGCAAGCAAGTAAA  R: GCCAAAAAGGGAAAAATAAAGCA</td>
<td>50</td>
<td>200</td>
<td>2</td>
<td>0.45</td>
<td>0.48</td>
<td>0.77</td>
<td>0.52</td>
<td>0.66</td>
</tr>
<tr>
<td>Pvi12</td>
<td>JF897862</td>
<td>Ubiquitin</td>
<td>F: TGAGTAGGGCAAAGATCAGC  R: CGATACACGCTATACCCAC  F: CTCAGGGGAGGATGCAAT  R: CAAATCCGTAGGGTTCATGC  F: CTACATCTCGTCCGAGAAAGG  R: AACGAATAGGGTGCGTAGGA</td>
<td>50</td>
<td>372</td>
<td>2</td>
<td>0.55</td>
<td>0.42</td>
<td>0.15</td>
<td>0.88</td>
<td>0.40</td>
</tr>
<tr>
<td>Pvi13</td>
<td>JF897863</td>
<td>Peptidyl-prolyl isomerase</td>
<td>F: GGACATTCGGCGTTGTG  R: CGCAGCTCTCCCTTCATATT  F: GGAATTTGGAACCAGAAGGTC  R: TAAATGAGTAAACCGGGTTTG  F: CTCAGGGGCAAGGACCTTAC  R: GAACACACGAGCAACACT  F: CCAAGTGCAAGCAAGTAAA  R: GCCAAAAAGGGAAAAATAAAGCA</td>
<td>50</td>
<td>638</td>
<td>2</td>
<td>0.33</td>
<td>0.28</td>
<td>0.32</td>
<td>0.93</td>
<td>0.79</td>
</tr>
</tbody>
</table>

*Polymorphisms were detected using a panel of 42 isolates collected in two different vineyards in France. Summary statistics for the number of alleles (nₐ), expected and observed heterozygosities (Hₑ and Hₒ, respectively), the probability of heterozygote deficit or heterozygote excess (compared with Hardy-Weinberg proportions), and the frequency of alleles are given for each locus.*
SNPs in three closely related oomycetes species. We did not detect cross-amplification of the EST-derived markers in *Bremia lactucae* (lettuce downy mildew), *Phytophthora infestans* (potato late blight), or *Plasmopara halstedii* (sunflower downy mildew).

So far, no nuclear SNPs in *Plasmopara viticola* have been reported in the literature (but see reference 2 for the characterization of mitochondrial SNPs). The EST-derived markers described here, combined with the previously described SSRs, will increase our capacity to study the fine-scale spatial genetic structure of *P. viticola* populations.

This work was supported by Bayer CropScience under research program no. 22000150.

We thank I. Haeuser-Hahn (BCS) for providing the EST library, C. Dutech for valuable comments on previous versions of the manuscript, and S. Ahmed for English corrections.

**REFERENCES**