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PRIMER NOTE

Twelve polymorphic expressed sequence tags-derived markers for *Plasmopara halstedii*, the causal agent of sunflower downy mildew

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Abstract

Twelve expressed sequence tags-derived markers were isolated from *Plasmopara halstedii* (Oomycetes), the causal agent of sunflower downy mildew. A total of 25 single nucleotide polymorphisms and five indels were detected by single-strand conformation polymorphism analysis and developed for high-throughput genotyping of 32 isolates. There was a high level of genetic diversity (H_E = 0.484). Observed heterozygosity ranged from 0 to 0.143 indicating that *P. halstedii* is probably a selfing species. These markers were also useful in detecting significant genetic variations among French populations (F_{ST} = 0.193) and between French and Russian populations (F_{ST} = 0.23). Cross-amplification tests on three closely related species indicated that no loci amplified in other Oomycete species.

Keywords: Oomycetes, pathotype evolution, physiological race, SNP, SSCP

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Sunflower downy mildew due to *Plasmopara halstedii* (Berlese & de Toni) is potentially one of the most damaging diseases in sunflower. *P. halstedii* is a homothallic Oomycete that alternates one sexual generation with several asexual generations. It is an obligate endoparasite that cannot be cultivated independently from its plant host. *P. halstedii* develops gene–for–gene interactions with its host *Helianthus annuus* and presents several physiological races known as pathotypes. Genetic resistance in cultivated sunflower varieties is the most efficient control method against the disease but the efficiency of major resistance genes is regularly challenged. To date, at least 20 different pathotypes have been described in different parts of the world (Tourvieille de Labrouhe *et al*. 2000).

Previous studies have failed to describe the genetic structure of *P. halstedii* populations, probably because the molecular markers used were non-specific, dominant and insufficiently polymorphic at the intraspecific level (Roeckel-Drevet *et al*. 2003; Spring *et al*. 2006). With a total of 174 nucleotide sequences available in the international nucleotide sequence database, *P. halstedii* is a typical example of a non-model organism for which genomic resources are very scarce. We used the 145 cDNA sequences available to design a set of expressed sequence tags (EST)-derived markers that may be used for future population genetic studies. Here we report the characterization of 12 polymorphic markers based on single nucleotide polymorphisms (SNP) and size variations (insertion/deletion) in ESTs of *P. halstedii* and the development of high-throughput genotyping methods for 10 of these markers.

A total of 124 ESTs of *P. halstedii* were screened for their polymorphism by polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP). At this stage, a panel of 16 individuals belonging to different pathotypes originating from one location in Russia (N = 8) and another in France (N = 8) were used to discover polymorphic sites.

DNA extraction was performed on infected plant tissue as previously described for *Plasmopara viticola* by Delmotte *et al*. (2006). Marker amplification reactions were carried out in a final volume of 25 µL containing 10 ng of genomic DNA, 2 mM of $MgCl_2$, 150 µM of each dNTP, 4 pmol of each primer and 0.2 U *Taq* Silverstar DNA polymerase (Eurogentec) in reaction buffer. Reactions were performed with the following programme: an initial denaturation step

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Table 1 Characterization and description of 12 EST-derived markers for *Plasmopara halstedii*: locus name, GenBank Accession no., homology of sequences, primer sequences, annealing temperature of primers and polymorphism description (type of polymorphism, localization, nucleotide substitution or number of inserted nucleotides) and sequence sizes (with/without deletion) are shown. For SNP identity, the most common allele is written first. For each locus, summary statistics for allelic richness, frequency of the rarest allele, expected heterozygosity (H_E) and observed heterozygosity (H_O) are given

Locus name	GenBank Acc. no.	Homology	Primer sequences (5′–3′)	SNP		Indel		Size (bp)	Allele number	Rare allele frequency	Heterozygosity	
				Position	Identity	Position	Size				H_E	H_O
Pha6	CB174585	Transportin	F: GTCGCTGATTTTATGTTTATGTGC R: TACTACCTCAGTCACATCATCACC	246	T/C	—	—	387	2	0.429	0.508	0
Pha39	CB174648	Hypothetical protein	F: GATTGGGTTCCTTGTTTGGA R: ATCTTCGCTGCCAGCTTCT	—	—	78	11	226/237	2	0.214	0.349	0
Pha42	CB174650	Hypothetical protein	F: GGATGTTGCTCGTCAAGTAGC R: ACGCATCCTACGCATTCAAC	66	A/G	137	4	252/256	2	0.321	0.452	0.071
Pha43	CB174680	Hypothetical protein	F: ACTCAGGACTGGGCAACAAT R: CGACATCCTTGTGAGCTTGT	—	—	77	4	275/279	2	0.429	0.508	0.143
Pha54	CB174708	Hypothetical protein	F: ATTTGGCAACGTCTCAGAGC R: CCATCGTAATAACATTCTTTAAAGTCC	294	T/C	—	—	580	2	0.071	0.133	0
Pha56	CB174714	40S ribosomal protein S2	F: GCGGTACTGGTCTATGTGCTG R: TTCAAGAAGTTTGATTTTTTCATGC	183	G/A	—	—	473	2	0.357	0.476	0
Pha74	CB174642	Hsp 90	F: ACCTCGCATGGTTGCTTTAC R: TTGCTATTTCGGCCTACTGG	—	—	338	63	366/429	2	0.357	0.476	0
Pha79	CB174692	Hypothetical protein	F: GACGCCCCACTTAGCTTTC R: TTCGGGAGTAAGTGATTGAGC	18 mutations		—	—	346	4	0.071	0.677	0
Pha82	CB174573	MMSDH*	F: ACTCGATCCATGCAGTAAGTAAG R: AGGAGGCTTTGCAGATTGAA	261	C/T	—	—	365	2	0.357	0.476	0
Pha99	CB174703	Hypothetical protein	F: CTCGCATTCAAACGGAAAAT R: CAAGCCAACTGTGCATGAAT	222	G/A	—	—	244	2	0.357	0.476	0
Pha106	CB174676	Hypothetical protein	F: TTGACGTTTATGCGAAGTGC R: CAAAGGAAGTTGTGATGGTGAG	—	—	215	2	296/298	2	0.321	0.452	0.071
Pha120	CB174660	Hypothetical protein	F: CTATTTAAAGGGGCCCGAAC R: CGGGTTTCCTCCATTAATCC	269	A/T	—	—	360	2	0.357	0.476	0

*MMSDH: methylmalonic semialdehyde dehydrogenase.

of 4 min at 96 °C, followed by 38 cycles of 40 s denaturation at 96 °C, 50 s annealing at 57 °C, 90 s elongation at 72 °C, and a final elongation step of 10 min at 72 °C. Sequence polymorphism was revealed on a 6% non-denaturing polyacrylamide gel with migration at 4 °C at 10 W overnight. The gel was silver-stained as described by Benbouza *et al*. (2006). For each of the different profiles of polymorphic EST markers, five alleles were sequenced in order to determine the mutations responsible for the polymorphism (Table 1).

Finally, with the 12 markers developed, we genotyped a total of 32 isolates originating from two locations in France ($N = 24$) and from another in Russia ($N = 8$). Five SNPs were transformed into cleaved amplified polymorphism sequence (CAPS) markers. Four indel polymorphisms were automated on a Beckman Coulter Ceq8000 capillary sequencer using the manufacturer's recommendations and one was directly visualized on agarose gel. The two remaining markers were screened by PCR-SSCP since no enzyme discriminating the alleles could be found (Table 2). The following protocol was used for CAPS markers: 1 μL of PCR product digested by 0.1 U restriction enzyme in 10× enzyme buffer for 1 h at the appropriate temperature (Table 2). GENEPOP version 3.2a (Rousset & Raymond 1997) was used to calculate for each marker the rarest allele frequencies, the *F*-statistic, expected and observed heterozygosities, and to perform the exact test for genotypic linkage disequilibrium and departures from Hardy–Weinberg proportions (Table 1).

Among the 124 ESTs tested by PCR-SSCP, only 12 were found to be polymorphic (9.6%). A total of five indels and 25 SNPs were revealed, one locus (Pha79) presenting 18 SNPs among the 25 (Table 1). The frequency of SNPs in coding regions was 0.52 SNP per kb and was 0.15 when the most polymorphic locus Pha79 was excluded. Five markers presented size polymorphism, with the number of inserted or deleted nucleotides varying in a range from two to 63. For the marker Pha42, the deletion and SNP were linked so there were only two alleles at this locus.

The markers were all di-allelic except Pha79, which presented four alleles. The frequency of the rarest allele ranged from 0.071 to 0.429, with a mean (± SE) of 0.325 (± 0.031). The combination of the 12 genomic markers revealed 21 different multilocus genotypes (16 in France and 5 in Russia) among the 32 isolates analysed. The expected heterozygosity per locus ranged from 0.133 to

Locus name	Genotyping techniques	Allele 1		Allele 2	
		Identity	Size	Identity	Size
Pha6	CAPS (T*sp*45I)	T	209/85/78/14	C	287/85/14
Pha39	Sequencer	—	226	—	237
Pha42	Sequencer	—	252	—	256
Pha43	Sequencer	—	279	—	275
Pha54	CAPS (*Fau*I)	C	293/286	T	579
Pha56	CAPS (*Oli*I)	G	473	A	292/181
Pha74	Agarose	—	366	—	429
Pha79	SSCP	—	—	—	—
Pha82	CAPS (B*sp*MI)	C	268/96	T	364
Pha99	CAPS (B*sr*DI)	G	217/26	A	243
Pha106	Sequencer	—	296	—	298
Pha120	SSCP	A	360	T	360

Table 2 Genotyping method, allele identity and size are described for each locus. For CAPS markers, the corresponding endonucleases are written in brackets and fragment sizes originating from the digestion are listed. Pha79 is not detailed in the table because it presented 18 SNPs that resulted in four different alleles

0.677, indicating a high level of genetic diversity. Mean observed heterozygosity was dramatically low and a significant heterozygosity deficit was found for all markers ($H_O = 0.024$). No linkage disequilibrium was detected between the markers. These results indicate that *P. halstedii* is a highly selfing species. Significant genetic differentiation was found between the two French populations ($F_{ST} = 0.193$, $P < 10^{-3}$) and between the French and Russian populations ($F_{ST} = 0.234$, $P < 10^{-3}$). These findings illustrate the value of these markers for future studies of population genetic structure.

Cross-amplifications of all polymorphic markers were carried out on three closely related Oomycetes: *Bremia lactucae* (lettuce downy mildew), *Phytophtora infestans* (potato late blight) and *Plasmopara viticola* (grapevine downy mildew). However, none of the amplifications of the 12 EST-derived markers succeeded in these species.

This set of 12 EST-derived markers will allow high-throughput genotyping of isolates directly from sporulating lesions. This could improve basic knowledge of population genetics and evolution in *P. halstedii*.

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