EST-derived markers highlight genetic relationships among *Plasmopara halstedii* French races

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ABSTRACT

Twelve EST-derived markers were isolated from *Plasmopara halstedii* (Oomycetes), the causal agent of sunflower downy mildew. A total of 25 single nucleotide polymorphisms (SNPs) and five indels were detected by single-strand conformation polymorphism (SSCP) analysis and developed for high-throughput genotyping of 24 isolates. These markers reveal a good level of genetic diversity and were useful in detecting genotypic variability of French populations. A deficit in heterozygotes indicated that *P. halstedii* is a selfing species. For the first time, these markers allowed to reveal genetic relatedness among 14 races characterized over a 19-year period of *P. halstedii* monitoring in France.

Keywords: evolution – pathotype – SNP – sunflower downy mildew – virulence profile.

INTRODUCTION

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).

Our understanding of the recurrent breakdown of sunflower major resistance genes can be improved based on new findings concerning the key processes governing the evolution of *P. halstedii* populations. Indeed, knowledge of the evolutionary potential of plant pathogen species has improved the management of resistance genes and maximized their durability in space and time (McDonald and Linde, 2002). The population genetics approach can be used to evaluate the major forces driving pathogen evolution, i.e. selection, mutation, recombination, genetic drift and gene flow. Previous genetic studies of *P. halstedii* in Europe have reported low levels of genetic and genotypic diversity in this species, with no clear genetic structure revealed with RAPD markers (Komjati et al., 2004; Roeckel-Drevet et al., 1997, 2003), ISSR (Intelmann and Spring, 2002) or ITS sequences (Spring et al., 2006). This precluded any reliable conclusions on the mode of reproduction, genetic structuring or the extent to which pathotypes are related in this species. Single nucleotide polymorphisms (SNPs) are promising molecular markers for population genetics as they are widespread throughout the genome, co-dominant, specific and have a high resolving power. The development of new methods for screening for DNA polymorphism has rendered possible the extensive development of such makers for plant pathogen species. 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 EST-derived markers that may be used for future population genetic studies. Here we report the characterization of 12 polymorphic markers based on SNPs and size variations (insertion-deletion) in Expressed Sequence Tags (ESTs) of *P. halstedii* and the development of high-throughput genotyping methods for 10 of these markers. We used these 12 EST-derived markers to perform a genetic analysis of the "reference races" of *P. halstedii* characterized over a 19-year period of monitoring in France (1988-2006).

MATERIALS AND METHODS

Sampling. We analyzed 24 isolates of *P. halstedii* collected in France between 1966 and 2006. Fourteen of these isolates are "references isolates", corresponding to the first description of the pathotype concerned in France (Table 1). The other 10 isolates (Table 1) were obtained from the French Plant Protection Service monitoring program (Moinard et al., 2006).

Virulence profile determination. Downy mildew pathotypes are defined on the basis of virulence profiles on a set of differential hosts carrying different *Pl* resistance genes. Resistance tests were performed as described by Cohen and Sakston (1974), with the modifications proposed by Mouzeyar et al. (1993): 15 days after inoculation, plants were incubated for 48 h in a saturated atmosphere. Plants were scored as susceptible if sporulation was observed in cotyledons and leaves, and as resistant if no sporulation or only light sporulation was seen on cotyledons. Pathotypes were named according to the international nomenclature of *P. halstedii* pathotypes proposed by Gulya et al. (1998) (Table 2).

Table 1. Race (pathotype), collection site and date of isolation in France for the 24 isolates of *Plasmopara halstedii*. The star (*) indicates isolates corresponding to the “reference pathotype”.

Race	Collection site ("*département*")	Year of collection
100*	Unknown	1966
100	Charente-Maritime	1993
100	Cher	1993
710*	Indre	1988
710	Cher	1993
710	Unknown	2000
710	Maine-et-Loire	2004
710	Deux-Sèvres	2006
703*	Lot-et-Garonne	1989
703	Tarn	1993
703	Lot-et-Garonne	2001
703	Haute-Garonne	2004
703	Gers	2006
300*	Aude	1995
700*	Haute-Garonne	1995
304*	Aude	2000
314*	Manche	2001
307*	Haute-Garonne	2002
704*	Deux-Sèvres	2002
714*	Gers	2002
334*	Charente	2004
707*	Lot-et-Garonne	2004
717*	Gers	2004
730*	Tarn	2005

Table 2. Name of race (pathotype), date of first isolation in France and virulence profiles for the 14 French reference isolates of *Plasmopara halstedii*.

Race name[1]	Isolation year	Virulence profiles according to differential hosts[2]								
		D1	D2	D3	D4	D5	D6	D7	D8	D9
100	1966	S	R	R	R	R	R	R	R	R
710	1988	S	S	S	S	R	R	R	R	R
703	1989	S	S	S	R	R	R	S	S	R
300	1995	S	S	R	R	R	R	R	R	R
700	1995	S	S	S	R	R	R	R	R	R
304	2000	S	S	R	R	R	R	R	R	S
314	2001	S	S	R	S	R	R	R	R	S
307	2002	S	S	R	R	R	R	S	S	S
704	2002	S	S	S	R	R	R	R	R	S
714	2002	S	S	S	S	R	R	R	R	S
334	2004	S	S	R	S	S	R	R	R	S
707	2004	S	S	S	R	R	R	S	S	S
717	2004	S	S	S	S	R	R	S	S	S
730	2005	S	S	S	S	S	R	R	R	R

[1] according to Gulya et al. (1998)
[2] S: susceptible (compatible interaction); R: resistant (incompatible interaction)

Molecular markers. A total of 124 ESTs of *P. halstedii* were screened for their polymorphism by polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP). 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 MgCl2, 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 program: an initial denaturation step 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. For each of the different profiles of polymorphic EST markers, five alleles were sequenced in order to determine the mutations responsible for the polymorphism.

Statistical analyses. Genepop version 3.2a was used to calculate expected and observed heterozygosities, unbiased estimates of F_{IS} and F_{ST} and to perform exact tests for departure from Hardy-Weinberg equilibrium. The phylogenetic relationships between French pathotypes were investigated by building a neighbor-joining (NJ) tree based on allele shared distance (D_{AS}), using Populations 1.2.28 software. Bootstrap support for the nodes was calculated over 10,000 replications, using the same program. To describe the genetic structure of *P. halstedii* pathotypes, we also applied a Bayesian approach of genetic mixture analysis using the software Structure v2.2.

RESULTS

Molecular markers. 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 3). 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 2 to 63. For the marker Pha42, the deletion and SNP were linked so there were only two alleles at this locus.

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. Finally, two markers were screened by PCR-SSCP since no enzyme discriminating the alleles could be found. The following protocol was used for CAPS markers: 1 µl of PCR product digested by 0.1 U restriction enzyme in 10X enzyme buffer for 1 hour at the appropriate temperature.

Table 3. Characterization and description of 12 EST-derived markers for *Plasmopara halstedii*: locus name, Genbank accession number, homology of sequences, primer sequences, polymorphism type, annealing temperature of primers and genotyping method used are shown.

Locus name	Genbank acc. no.	Homology	Primer sequences (5'-3')	Polymorphism	T_a (°C)	Genotyping method
Pha6	CB174585	Transportin	F: GTCGCTGATTTTATGTTTATGTGC R: TACTACCTCAGTCACATCATCACC	SNP	57	CAPS (T*sp*45I)
Pha39	CB174648	Hypothetical protein	F: GATTGGGTTCCTTGTTTGGA R: ATCTTCGCTGCCAGCTTCT	Indel	57	Sequencer
Pha42	CB174650	Hypothetical protein	F: GGATGTTGCTCGTCAAGTAGC R: ACGCATCCTACGCATTCAAC	indel	57	Sequencer
Pha43	CB174680	Hypothetical protein	F: ACTCAGGACTGGGCAACAAT R: CGACATCCTTGTGAGCTTGT	indel	57	Sequencer
Pha54	CB174708	Hypothetical protein	F: ATTTGGCAACGTCTCAGAGC R: CCATCGTAATAACATTCTTTAAAGTCC	SNP	57	CAPS (F*au*I)
Pha56	CB174714	40S ribosomal protein S2	F: GCGGTACTGGTCTATGTGCTG R: TTCAAGAAGTTTGATTTTTCATGC	SNP	57	CAPS (O*li*I)
Pha74	CB174642	Hsp 90	F: ACCTCGCATGGTTGCTTTAC R: TTGCTATTTCGGCCTACTGG	indel	57	Agarose
Pha79	CB174692	Hypothetical protein	F: GACGCCCCACTTAGCTTTC R: TTCGGGAGTAAGTGATTGAGC	SNP	57	SSCP
Pha82	CB174573	MMSDH[1]	F: ACTCGATCCATGCAGTAAGTAAG R: AGGAGGCTTTGCAGATTGAA	SNP	57	CAPS (B*sp*MI)
Pha99	CB174703	Hypothetical protein	F: CTCGCATTCAAACGGAAAAT R: CAAGCCAACTGTGCATGAAT	SNP	57	CAPS (B*sr*DI)
Pha106	CB174676	Hypothetical protein	F: TTGACGTTTATGCGAAGTGC R: CAAAGGAAGTTGTGATGGTGAG	Indel	57	Sequencer
Pha120	CB174660	Hypothetical protein	F: CTATTTAAAGGGGCCCGAAC R: CGGGTTTCCTCCATTAATCC	SNP	57	SSCP

[1]MMSDH: methylmalonic semialdehyde dehydrogenase.

Genotypic structure. Based on combinations of the 12 genomic markers, we identified 11 different multilocus genotypes among the 24 isolates analyzed. Three multilocus genotypes were found in multiple copies. A combination of eleven EST markers was sufficient to discriminate all the multilocus genotypes in the dataset, demonstrating the high resolving power of the markers (Fig. 1). Three pathotypes were represented by more than one isolate (race 100 represented by 3 isolates, races 703 and 710 each represented by 5 isolates). Isolates of the same pathotype presented an identical multilocus genotype, indicating that the three pathotypes may correspond to three clonal lineages. Conversely, two multilocus genotypes included more than one pathotype: the first multilocus genotype comprised pathotypes 100, 300 and 304 and the second comprised pathotypes 307 and 703.

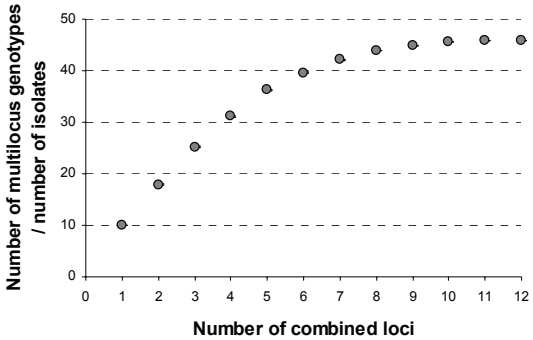

Fig. 1. Number of genotypes discriminated (number of multilocus genotypes/total number of isolates) as a function of the number of loci combined to discriminate isolates of *Plasmopara halstedii.*

Population genetic structure. The expected heterozygosity level was consistent with high levels of genetic diversity across loci ($0.349<H_E<0.677$). However, observed heterozygosity levels were much lower, with a mean H_O of 0.026. Only three of the 11 distinct multilocus genotypes were heterozygous: two at two loci and one at one locus. Tests of deviation from Hardy-Weinberg equilibrium revealed significant strong heterozygote deficits with respect to expectations under the assumption of random mating. All loci presented significant and positive F_{IS} values, with an overall F_{IS} of 0.948.

Bayesian assignment analyses showed three genetic groups of isolates: the first cluster was constituted by a single multilocus genotype including 3 pathotypes: 100, 300 and 304. The second cluster included 4 multilocus genotypes and 5 pathotypes: 703, 307, 700, 730, 707. The third cluster included 6 pathotypes with different multilocus genotypes: 710, 334, 314, 714, 717 and 704. Pathotypes 334, 707 and 730 were clearly 'mixed' genotypes that presented alleles belonging to two different clusters (Fig. 2).

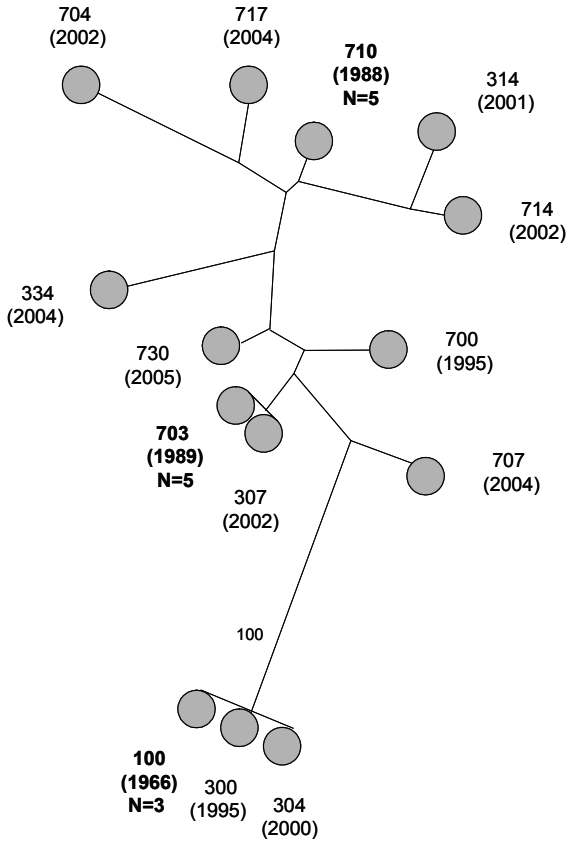

Fig. 2. NJ clustering from the allele shared distance matrix calculated among the 14 pathotypes of *Plasmopara halstedii* based on 12 EST-derived markers. Date of the first description in France of each pathotype is indicated in brackets. Pathotypes 100, 703 and 710 were represented by three to five isolates (N) and isolates belonging to one same pathotype presented an identical multiclocus genotype. Numbers above branches of the tree are bootstrap percentages after 10,000 re-samplings.

DISCUSSION

The EST-derived markers used in this study displayed high levels of intraspecific polymorphism which made it possible to infer the reproduction mode of *P. halstedii* and to assess the relationships among French pathotypes. These markers are specific and could therefore be used for the high-throughput genotyping of isolates directly from sporulating lesions collected from host leaves, avoiding the need for labor-intensive isolate subculture. *P. halstedii* is a homothallic species, and is therefore able both to

outcross and to self. However, our results suggest that *P. halstedii* is mainly a selfing species, with only limited outcrossing.

The finding that *P. halstedii* pathotypes cluster into three genetically differentiated groups, each including one of the first races described in France (i.e. 100, 703, 710), sheds new light on sunflower downy mildew evolution. Races 100, 703 and 710 correspond to three clonal lineages that not only present a strong genetic differentiation but also have very distinct virulence profiles. Given the strong geographic structuration of race distribution in France, we hypothesize that these results reflect (at least) three different introductions of this pathogen in France: the first introduction corresponds to race 100 (before 1966) that is now widely distributed in France, the second to race 710 in the North of France (before 1988) and the third to race 703 in South-West of France (before 1989). From then on, the triple introduction of *P. hasltedii* might have provided the raw genetic materials for more complex evolutionary processes of race emergence such as recombination between pathotypes or accumulation of mutation in clonal lineages (further referred as clonal evolution). These three introductions of *P. halstedii* could have provided the raw genetic materials for more complex evolutionary processes, such as recombination between pathotypes or the accumulation of mutations in clonal lineages (clonal evolution), in the emergence of new races.

ACKNOWLEDGEMENTS

This work was supported by the CETIOM (*Centre Technique Interprofessionnel des Oléagineux Métropolitains*) and partly by MAP (Ministry for Agriculture and Fishing) within the framework of the program CASDAR.

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