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PRIMER NOTE

Characterization of microsatellite markers in the interspecific hybrid *Phytophthora alni* ssp. *alni*, and cross-amplification with related taxa

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Abstract

Phytophthora alni ssp. *alni* is an interspecific hybrid oomycete causing a large-scale decay of alders throughout Europe. In this study we developed a set of 10 microsatellite markers that shows promise for population studies and for studying hybridization events between the parental species of the hybrid. Moreover, the genotype and the ploidy of the different subspecies of *P. alni* might be inferred from the quantitative ratio of amplified genome-specific alleles. Nine primer pairs cross amplified with the related species *Phytophthora cambivora* and *Phytophthora fragariae* and yielded distinct alleles.

Keywords: genotyping, oomycete, polyploidy

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Phytophthora alni ssp. *alni* (*Paa*) is a recently described interspecific hybrid oomycete causing a lethal disease of alder throughout Europe (Brasier *et al.* 2004). Two other taxa close to *Paa* are isolated from diseased alders or from soil beneath alders: *Phytophthora alni* ssp. *uniformis* (*Pau*) and *Phytophthora alni* ssp. *multiformis* (*Pam*). All three taxa are phylogenetically very close to *Phytophthora cambivora* (*Pc*) and *Phytophthora fragariae* (*Pf*) (Brasier *et al.* 1999). Previously hypothesized to originate from genetic breakdown of *Paa* (Brasier *et al.* 1999), *Pam* and *Pau* were actually suggested to represent distinct species and to have generated *Paa* through interspecific hybridization (Ioos *et al.* 2006). In addition, Brasier *et al.* (1999) reported that *Paa* was a near tetraploid species ($4n + 2$) in respect with the typical diploid ($2n$) status of the *Phytophthora* genus, and that *Pam* and *Pau* were aneuploid species with $2n + 4$ to $2n + 7$, and $2n + 2$ chromosomes, respectively. However, Ioos *et al.* (2006) demonstrated that three divergent alleles for four unlinked single copy nuclear genes were observed within the *Paa* genome. Two of them matched the two alleles also found in *Pam* and the third one corresponded to the single allele found in *Pau*. As these results contrasted with the ploidy levels reported, and as little is known about the genetic polymorphism within *Paa*

and the two other taxa, we isolated and characterized microsatellite loci in *Paa*. These codominant markers are especially useful to address questions relating to origin and genetic diversity of fungi (Weising *et al.* 1995).

Microsatellite markers were isolated from *Paa* using enrichment libraries. DNA was extracted from *Paa* isolate PAA130 using DNeasy Plant Mini Kit (QIAGEN). Approximately 500 ng μL^{-1} DNA were recovered and used for the construction of five enrichment libraries using biotinylated oligoprobes [(AC)₁₃, (AG)₁₃, (ACG)₆, (AAC)₆ and (AAG)₆] and streptavidin-coated magnetic beads, according to Dutch *et al.* (2000). The libraries were cloned using a TOPO TA Cloning kit (Invitrogen). For each of the five enrichments, 288 clones were screened by colony blot following a protocol developed by Estoup *et al.* (1993). A total of 172 positive clones were subsequently size-screened by polymerase chain reaction (PCR) using vector's primers and 43 clones with insert between 400 and 1000 bp were retained for sequencing. Eighteen primer pairs could be designed from the sequences, either manually or using PRIMER 3 software (Rozen & Skaletski 2000). The primer pairs were tested by PCR with a panel of 6 *Paa*, 4 *Pam*, 8 *Pau*, 2 *Pc* and 2 *Pf* isolates, from various geographical origins in Europe and displaying different mitotypes according to Ioos *et al.* (2006) (Table 1). PCR conditions were optimized for each primer pair. PCR was conducted in a 20- μL volume containing 15–20 ng of

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Table 1 List of the isolates used in the study. Mitotypes of the isolates determined as described in Ios *et al.* (2006)

Species	Isolate	Mitotype	Host	Geographical origin	Year isolated	
<i>Phytophthora alni</i> ssp. <i>alni</i>	PAA129	U	<i>Alnus glutinosa</i>	SW France	2003	
	PAA130*	M	<i>Alnus glutinosa</i>	NE France	2003	
	PAA143	M''	<i>Alnus glutinosa</i>	Poland	2002	
	PAA151	U	<i>Alnus glutinosa</i>	NE France	2004	
	PAA162	U	<i>Alnus glutinosa</i>	Germany	2004	
	PAAa2'	U	<i>Alnus glutinosa</i>	NE France	2005	
	PA Ae3	M	<i>Alnus glutinosa</i>	NE France	2005	
	PAA151	U	<i>Alnus glutinosa</i>	NE France	2004	
	PAA70	M	<i>Alnus glutinosa</i>	the Netherlands	Unknown	
	PAAa2	M	<i>Alnus glutinosa</i>	NE France	2005	
	PAA38	M	<i>Alnus glutinosa</i>	N France	2002	
	PAA74	U	<i>Alnus glutinosa</i>	Scotland	2000	
	PAA75	M	<i>Alnus viridis</i>	Scotland	2000	
	PAA81	U	<i>Alnus glutinosa</i>	England	1997	
	PAA82	M	<i>Alnus glutinosa</i>	England	1996	
	PA Ae1	M	<i>Alnus glutinosa</i>	NE France	2005	
	PAA85	M	<i>Alnus glutinosa</i>	England	Unknown	
	PAA86	M	<i>Alnus glutinosa</i>	Belgium	1999	
	PAA88	M	<i>Alnus glutinosa</i>	Belgium	2001	
	PAA91	M'	<i>Alnus glutinosa</i>	Hungary	2001	
	PAA112	M	<i>Alnus glutinosa</i>	NW France	2003	
	PAA114	M	<i>Alnus glutinosa</i>	NE France	2002	
	PAA134	M	<i>Alnus glutinosa</i>	Germany	2000	
	PAA141	U'	<i>Alnus glutinosa</i>	Austria	Unknown	
	PAA144	M''	<i>Alnus glutinosa</i>	Poland	2003	
	PAA178	M''	<i>Alnus glutinosa</i>	Poland	2003	
	PAA185	M	<i>Alnus glutinosa</i>	NE France	2004	
	PAA194	M	<i>Alnus glutinosa</i>	NW France	2005	
	PA Ad6'	U	<i>Alnus glutinosa</i>	NW France	2005	
	PAAa10	M	<i>Alnus glutinosa</i>	N France	2005	
	PA Ac10	M	<i>Alnus glutinosa</i>	N France	2005	
	PA Ad2'	M'	<i>Alnus glutinosa</i>	NE France	2005	
	PA Ae2'	M	<i>Alnus glutinosa</i>	NE France	2005	
	PAAa11'	M	<i>Alnus glutinosa</i>	Germany	2005	
<i>Phytophthora alni</i> ssp. <i>multiformis</i>	PAM54	M	<i>Alnus glutinosa</i>	NW France	2000	
	PAM71	M	<i>Alnus glutinosa</i> soil	the Netherlands	1995	
	PAM90	M	<i>Alnus glutinosa</i> soil	the Netherlands	1995	
	PAM186	M'	<i>Alnus glutinosa</i>	Belgium	2001	
	PAM73	nd	<i>Alnus glutinosa</i>	England	1996	
<i>Phytophthora alni</i> ssp. <i>uniformis</i>	PAU60	U''	<i>Alnus glutinosa</i>	NE France	1999	
	PAU84	U'	<i>Alnus glutinosa</i>	Sweden	1999	
	PAU87	U	<i>Alnus glutinosa</i>	Belgium	2001	
	PAU89	U	<i>Alnus cordata</i>	Italy	2000	
	PAU142	U	<i>Alnus glutinosa</i>	Slovenia	2000	
	PAU188	U	<i>Alnus incana</i>	Belgium	2001	
	PAUb3	U''	<i>Alnus glutinosa</i>	NE France	2005	
	PAUc6'	U	<i>Alnus glutinosa</i>	NE France	2005	
	PAU187	U	<i>Alnus glutinosa</i>	Belgium	2001	
	PAU96	U	<i>Alnus glutinosa</i>	Hungary	1999	
	PAU97	U	<i>Alnus glutinosa</i> soil	Hungary	1999	
	PAU98	U	<i>Alnus glutinosa</i> soil	Hungary	1999	
	PAUb3	U'	<i>Alnus glutinosa</i>	NE France	2005	
	<i>Phytophthora cambivora</i>	PCJC17	C2	<i>Quercus</i> sp. soil	NE France	1999
		PC643	C1	<i>Castanea sativa</i> soil	SW France	2000
PC463		C2	<i>Castanea sativa</i>	SW France	1994	
PCga1		C2	<i>Quercus</i> sp. soil	NE France	1999	

Table 1 Continued

Species	Isolate	Mitotype	Host	Geographical origin	Year isolated
	PC99428	C2	<i>Castanea sativa</i>	France	1999
	PCst3r1	C2	<i>Quercus petraea</i>	SW France	1999
	PC627	C2	<i>Castanea sativa</i>	Italy	1999
	PC1a21	C2	<i>Quercus</i> sp. soil	NW France	1999
	PC4n444	C2	<i>Castanea sativa</i>	NW France	2004
	PC4n1125	C2	<i>Castanea sativa</i>	NW France	2004
	PC051422	C2	<i>Castanea sativa</i>	NW France	2005
<i>Phytophthora fragariae</i> var. <i>fragariae</i>	PFF309	FF	<i>Fragaria</i> × <i>ananassa</i>	Great Britain	1962
	PFFCSL	FF	<i>Fragaria</i> × <i>ananassa</i>	Unknown	Unknown
	PFF209	FF	<i>Fragaria</i> × <i>ananassa</i>	England	1946
<i>Phytophthora fragariae</i> var. <i>rubi</i>	PFR109	FR	<i>Rubus</i> sp.	Great Britain	1991
	PFR163	FR	<i>Rubus</i> sp.	France	Unknown
	PFR59	FR	<i>Rubus</i> sp.	Scotland	Unknown
	PFRCSL	FR	<i>Rubus</i> sp.	England	Unknown
	PFR96795	FR	<i>Rubus</i> sp.	Scotland	1985
<i>Phytophthora cactorum</i>	CAC4810	nd	Unknown	France	Unknown
<i>Phytophthora cinnamomi</i>	DSF970060	nd	<i>Quercus suber</i>	France	1999
<i>Phytophthora citricola</i>	AUL045	nd	<i>Alnus glutinosa</i>	France	1999
<i>Phytophthora citrophthora</i>	2 N1021	nd	<i>Rosa</i> sp.	France	2002
<i>Phytophthora cryptogea</i>	990675	nd	<i>Actinidia sinensis</i>	France	1999
<i>Phytophthora erythroseptica</i>	960713	nd	<i>Polygonum oberti</i>	France	1999
<i>Phytophthora europaea</i>	AL5	nd	<i>Quercus</i> sp. soil	France	1998
<i>Phytophthora gonapodyides</i>	Gonap4	nd	<i>Quercus</i> sp. soil	France	1998
<i>Phytophthora humicola</i>	3 N1245j	nd	<i>Alnus glutinosa</i> soil	France	2003
<i>Phytophthora inundata</i>	9500802	nd	<i>Alnus glutinosa</i> soil	France	1998
<i>Phytophthora lateralis</i>	98093–1 PV	nd	<i>Chamaecyparis</i> sp.	France	1998
<i>Phytophthora megasperma</i>	BK1	nd	<i>Quercus</i> sp. soil	France	1998
<i>Phytophthora nicotianae</i>	960579	nd	<i>Nicotiana tabacum</i>	France	1996
<i>Phytophthora</i> taxon forest soil	8CARPPOC1	nd	<i>Quercus</i> sp. soil	France	1998
<i>Phytophthora palmivora</i>	970423	nd	<i>Hedera</i> sp.	France	1997
<i>Phytophthora parasitica</i>	970029	nd	<i>Lycopersicon</i> sp.	France	1997
<i>Phytophthora</i> taxon Pgchlamydo	Haye,3,1	nd	<i>Quercus</i> sp. soil	France	1998
<i>Phytophthora pseudosyringae</i>	EW5	nd	<i>Quercus</i> sp. soil	France	1998
<i>Phytophthora psychrophila</i>	FF20	nd	<i>Quercus</i> sp. soil	France	1998
<i>Phytophthora quercina</i>	FNA	nd	<i>Quercus</i> sp. soil	France	1999
<i>Phytophthora ramorum</i>	2 N083	nd	<i>Rhododendron</i> sp.	France	2002
<i>Phytophthora sojae</i>	443	nd	<i>Glycine max</i>	Unknown	Unknown
<i>Phytophthora syringae</i>	2JZ2	nd	<i>Quercus</i> sp. soil	France	1999

*, isolate used for microsatellite isolation; nd, not determined.

template DNA, 2 µL of 10× polymerase buffer, 1.5 mM MgCl₂, 0.2 mM dNTP, 0.2 µM of each primer, 0.5 U of *Taq* polymerase (Sigma) and water was added to 20 µL. Touch-down PCR conditions were first five cycles at 94 °C for 30 s, 66–62 °C (see initial annealing temperatures, Table 2) for 30 s and 72 °C for 1 min; followed by 8 cycles at 94 °C for 30 s, 66–55 °C minus 0.5 °C per cycle for 30 s, and 72 °C for 1 min; 22 cycles at 94 °C for 30 s, 62–51 °C for 30 s, and 72 °C for 1 min; and a final elongation step at 65 °C for 30 min. All retained forward primers were labelled to allow size and dye multiplexing. Allele sizes were determined on a CEQ 8000 Genetic Analysis System (Beckman Coulter). Eight out of the 18 primer pairs tested yielded no polymorphism or

unclear pattern and were therefore discarded. The remaining 10 primer pairs were tested by PCR on a larger collection of *Paa*, *Pau*, *Pam*, *P. cambivora* and *P. fragariae* isolates as well as with 23 other *Phytophthora* spp. (Table 1).

Except PA12-F/R that were specific to *Paa* and *Pam* but gave no size polymorphism, all the primers retained yielded an amplicon with *Paa*, *Pau*, *Pam*, *Pc* and *Pf*, confirming their close kinship. In *Paa*, the number of alleles for the nine polymorphic loci ranged from two to five indicating a low level of allelic variation, consistent with this hybrid species being of recent origin (Brasier *et al.* 2004; Ios *et al.* 2006). In addition, all the alleles observed in *Paa* were those encountered either in *Pam* or in *Pau* (Table 2), strengthening the

Table 2 Primer sequences and characteristics for the nine polymorphic microsatellite loci isolated from *Phytophthora alni* ssp. *alni*. All reverse PCR primers were fluorescently labelled

Locus	Repeat motif	GenBank Accession no.	Primer sequence (5'–3')	Size (bp)	Annealing T°C*	Total number of different alleles/no. of alleles per individual (allele sizes)						
						<i>P. alni</i> ssp. <i>alni</i>	<i>P. alni</i> ssp. <i>multiformis</i>	<i>P. alni</i> ssp. <i>uniformis</i>	<i>P. cambivora</i>	<i>P. fragariae</i> var <i>fragariae</i>	<i>P. fragariae</i> var <i>rubi</i>	other species
PA3	(CAA) ₅ GA	DQ665899	F: CTTGGATAGAGCCGTCGTTTC R: TCCTACTGTTGGGAGCAAGG	201	62 °C	3/2+ (198, 212, 216)	2/1–2+ (198, 216)	2/1–2 (198, 212)	4/3 (210, 211, 213, 215)	1/1 (217)	1/1 (217)	–
PA6	(CAA) ₁₁ TAA(CAA)	DQ665900	F: AACACCCGCTTGAAGACG R: GTAGCCACCGCACATGAATC	303	66 °C	3/3+ (278, 284, 287)	1/1 (278)	3/3 (278, 284, 287)	2/2 (278, 281)	2/1–2 (275, 288)	2/2 (275, 288)	–
PA8	(ACA) ₅ T(ACA)	DQ665901	F: GGTCAGCCAAGAGCAAAGAG R: CTGTGAGCTGCAAGAAGCAG	376	66 °C	5/3–5+ (356, 359, 361, 367, 368)	3/2–3+ (356, 359, 368)	2/2 (361, 367)	2/1–2 (367, 369)	2/2 (364, 367)	1/1 (367)	<i>P. europaea</i> (368)
PA11	(CAA) ₅ CAG(CAA)	DQ665902	F: TGCAAACAGTGCCTCTCTTC R: CCTAGATCCAGCGACAGCTC	226	62 °C	2/2+ (226, 232)	2/1–2+ (226, 232)	1/1 (226)	2/1–2 (224, 225)	1/1 (226)	1/1 (225)	–
PA14	(CT) ₇ (...)(TTTC) ₄	DQ665904	F: TGGCAAACAGACACGAAGTC R: GAAACCCAGTCATCCGAGAG	173	62 °C	3/3+ (173, 175, 177)	2/2+ (173, 175)	1/1 (177)	4/2 (174, 177, 179, 181)	2/2 (168, 174)	2/2 (175, 177)	–
PA17	(GTC) ₄ (...)(GC) ₄	DQ665905	F: AGCGACAATGCAGGAAGC R: CTGTCTGGGCATTTCATGTGG	317	62 °C	2/2 (313, 317)	2/2 (313, 317)	1/1 (313)	2/1–2 (309, 313)	2/2 (308, 312)	1/1 (315)	<i>P. europaea</i> (385)
PA23	(GAA) ₇ GGA(GAA) ₃	DQ665906	F: GGAGATAGCCACGAGACACC R: CAAGCATCGCTGTAAACGAC	155	62 °C	2/2+ (133, 148)	2/2 (133, 148)	1/1 (148)	1/1 (148)	1/1 (148)	1/1 (148)	–
PA30	(AC) ₈	DQ665907	F: TAGGGGACTTTAAACCCCA R: GTTGGCCGACTAGAGATTT	127	66 °C	4/4+ (120, 122, 123, 124)	3/3+ (120, 122, 124)	1/1 (123)	2/2 (118, 122)	2/2 (120, 122)	2/2 (120, 122)	–
PA31	(CAA) ₈	DQ665908	F: GCTTCTCACTGCACAGCAAC R: AGGTTATTTGGAGCCTGATGC	195	62 °C	2/1–2+ (183, 192)	1/1 (192)	2/2 (183, 192)	4/3–4 (174, 180, 186, 192)	1/1 (150)	1/1 (150)	–

*, initial annealing temperature for touchdown PCR; †, loci with alleles consistently showing different peak areas.

hypothesis that *Pau* and *Pam* may actually be the progenitors of *Paa*. Due to the uncertain nature of ploidy in these different taxa, expected heterozygosities and linkage disequilibria were not computed. However, depending on the locus, up to three or five different alleles were simultaneously observed in individual *Pam* and *Paa* isolates, respectively. In addition, genotyping of the PCR products obtained with the primer pairs PA3, PA6, PA8, PA11, PA14, PA23, PA30 and PA31 showed that for individual *Paa* and *Pam* isolates, the respective quantities of the different alleles (based on peak areas) were not always equal. The occurrence of unbalanced peak ratios suggests these species are polyploid (Christiansen 2006). The genotype amplification patterns for these loci will be particularly useful to unravel the genotypes of *Paa* and *Pam*.

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