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Genetic characterization of the natural hybrid species *Phytophthora alni* as inferred from nuclear and mitochondrial DNA analyses

Renaud Ioos[a,b,*] , Axelle Andrieux[a], Benoît Marçais[a], Pascal Frey[a]

[a] Institut National de la Recherche Agronomique, UMR1136, Pathologie Forestière, 54280 Champenoux, France

[b] Laboratoire National de la Protection des Végétaux, Unité de Mycologie Agricole et Forestière, Domaine de Pixérécourt, 54220 Malzéville, France

* Corresponding author. Fax: +33 03 83 39 40 69. E-mail address: ioos@nancy.inra.fr (R. Ioos).

Abstract

The different subspecies of *Phytophthora alni*, *P. alni* subsp. *alni* (Paa), *P. alni* subsp. *uniformis* (Pau), and *P. alni* subsp. *multiformis* (Pam), are recent and widespread pathogens of alder in Europe. They are believed to be a group of emergent heteroploid hybrids between two phylogenetically close *Phytophthora* species. Nuclear and mitochondrial DNA analyses were performed, using a broad collection of *P. alni* and two closely related species, *P. cambivora* and *P. fragariae*. *Paa* possesses three different alleles for each of the nuclear genes we studied, two of which are present in *Pam* as well, whereas the third matches the single allele present in Pau. Moreover, *Paa* displays common mtDNA patterns with both *Pam* and Pau. A combination of the data suggests that *Paa* may have been generated on several occasions by hybridization between *Pam* and *Pau,* or their respective ancestors. *Pau* might have *P. cambivora* as a species ancestor, whereas *Pam* seems to have either been generated itself by an ancient reticulation or by autopolyploidization.

Keywords: RAS-Ypt; ASF-like; TRP1; GPA1; mtDNA-RFLP; cox1; nadh1; Interspecific hybridization

1. Introduction

The role of natural hybridization in the evolutionary history of many animal and plant species is well recognized today. Numerous examples illustrate that it has contributed also to the evolution of many pests and pathogens (Arnold, 2004). Until recently, only a few natural species hybrids were reported in the Fungi or Stramenipila kingdoms (Burnett, 1983). However, since the 1990s, there have been several reports of hybridization events among true fungi or oomycetes, especially involving plant pathogens (Olson and Stenlid, 2002). Man in't Veld et al. (1998) demonstrated that a new unknown *Phytophthora* pathogen active on Primula and Spathiphyllum in hydroponic culture systems was actually a hybrid between *P. cactorum* and *P. nicotianae*. Another recently described *Phytophthora* hybrid, highly aggressive on alder trees (Alnus spp.), is currently spreading in natural ecosystems across Europe (Gibbs et al., 2003). Gibbs (1995) previously described this alder *Phytophthora* as a taxon similar to *P. cambivora*, which is a common pathogen on hardwood trees. Brasier et al. (1999) then demonstrated that this pathogen was actually an interspecific hybrid. Based on morphological, serological, and genotypic traits, Brasier et al. (2004) formally described this

new pathogen as *Phytophthora* alni. Because this new *Phytophthora* hybrid does not consist of a single entity but comprises a range of phenotypically diverse allopolyploid genotypes, *P. alni* was split into three subspecies: *P. alni* subsp*. alni* (*Paa*), *P. alni* subsp. *uniformis* (*Pau*), and *P. alni* subsp. *multiformis* (*Pam*) (Brasier et al., 2004).

In contrast with typical *Phytophthora* species, which are diploid organisms, *Paa* was shown to be near-tetraploid, consistent with possessing an allopolyploid genome (Brasier et al., 1999). *Paa* also displays an unusual internal transcribed spacer (ITS) polymorphism, i.e., numerous dimorphic sites within ITS sequences for a single isolate. On the other hand, the ploidy levels for the two other subspecies range from 2n + 2 to 2n + 7 for *Pau* and *Pam,* respectively. In contrast with *Paa,* nearly homogeneous ITS sequences were observed in both *Pam* and Pau: the ITS sequence for *Pam* differs from *P. fragariae* var. *fragariae* (*Pff*) or *P. fragariae* var. *rubi* (*Pfr*) by only a few bases, whereas the ITS sequence for *Pau* is very close to the *P. cambivora* (Pc) sequence (Brasier et al., 1999). According to chromosome karyotyping, ITS sequences, and amplified fragment length polymorphism (AFLP) fingerprinting, Brasier et al. (1999) hypothesized that *P. cambivora* was a parent of P. alni. On the other hand, recent isozyme analysis precludes the possibility that *P. fragariae* is involved *sensu stricto* in the hybridization process (Brasier, 2003; Nagy et al., 2003). Although *P. alni* was demonstrated to be a hybrid species, its origin remains unclear and additional data are required to trace back the ancestry to the parental species.

To address this problem, the use of molecular markers is particularly useful, and both nuclear and mitochondrial data may contribute to the understanding of the hybridization process. First, nuclear single-copy genes should be of particular interest for phylogeny purposes. In addition, the biparental inheritance and the absence of intergenomic concerted evolution in comparison to low copy or high copy nuclear genes make them particularly useful for studying the origin of hybrids and polyploidy lineages. Moreover, the use of genes containing introns, which are generally highly polymorphic regions, could potentially be of great interest for the discrimination of closely related *Phytophthora* species, such as the putative parents of *P. alni* (Brasier et al., 2004). Although the number of genes containing introns is much lower in *Phytophthora* sp. than in true Fungi (B. Tyler, personal communication), a few nuclear genes with introns have already been described for two *Phytophthora* species: P. parasitica (TRP1, Karlowsky and Prell, 1991) and *P. infestans* (RAS-Ypt; Chen and Roxby, 1996; GPA1, Laxalt et al., 2002). Besides, mitochondrial DNA analysis provides valuable information in the case of hybridization between two taxa; in the case of sexual mating for the *Phytophthora* genus, mitochondrial DNA is exclusively inherited from one of the parental lines (Whittaker et al., 1994).

In the present work, we studied the allelic distribution for orthologs of TRP1, RAS-Ypt, and GPA1 genes, along with another recently described single-copy gene containing one intron (ASF-like, Munakata et al., 2000). Mitochondrial DNA was studied through restriction pattern analysis and by the sequencing of two mitochondrial genes, cytochrome c oxidase subunit 1 (cox1) and NADH dehydrogenase subunit 1 (nadh1) (Kroon et al., 2004). This research, combining nuclear and mitochondrial data obtained from a large Europe-wide collection of *Paa, Pam, Pau,* Pc, *Pff,* and *Pfr* isolates, provides new insights into the hybrid status of P. alni.

2. Materials and methods

2.1. Source and culture of Oomycete isolates for DNA extraction

The Oomycete isolates used in this study are listed in Table 1. French isolates of *P. alni* or *Phytophthora* spp. were obtained by isolation from diseased plant material or biological baits carried out on soil sampled beneath the host, using the *Phytophthora*-selective PARBHY medium (Robin et al., 1998). Foreign isolates of *P. alni* and *Phytophthora* spp. were obtained from CBS (Centraalbureau voor Schimmelcultures, Utrecht, The

Table 1. List of the isolates of *Phytophthora* spp. and *Pythium* spp. used in this study

Species	Isolate	Isolator/supplier, reference	Host	Origin	Year isolated	mtDNA pattern
P. alni* subsp. *alni	PAA2	J.C. Streito (2N0685)	*Alnus glutinosa*	France (Yrieu lake, Landes)	2002	M
	PAA20	J.C. Streito (71T1)	*Alnus glutinosa*	France (Oise river, Aisne)	1997	*nd*
	PAA23	J.C. Streito (82T1A)	*Alnus glutinosa*	France (Oise river, Aisne)	1997	M
	PAA24	J.C. Streito (84T2)	*Alnus glutinosa*	France (Nied river, Bas-Rhin)	1997	*nd*
	PAA34	J.C. Streito (98-7-5)	*Alnus glutinosa*	France (La Combauté river, Haute-Saône)	1998	M
	PAA35	J.C. Streito (98-7-6)	*Alnus glutinosa*	France (La Combauté river, Haute-Saône)	1998	*nd*
	PAA38	J.C. Streito (2N0529)	*Alnus glutinosa*	France (Aa river, Pas-de-Calais)	2002	M
	PAA44	J.C. Streito (DSFO98172)	*Alnus glutinosa*	France (Thouet river, Maine-et-Loire)	1998	*nd*
	PAA47	J.C. Streito (AUL026/1)	*Alnus glutinosa*	France (Moselle river, Vosges)	1999	U
	PAA52	J.C. Streito (9900783.4)	*Alnus glutinosa*	France (Meurthe river, Meurthe-et-Moselle)	1999	*nd*
	PAA53	J.C. Streito (1R0152)	*Alnus glutinosa*	France (Nied Française river, Moselle)	2001	M
	PAA58	J.C. Streito (1N0201)	*Alnus glutinosa*	France (Rhône river, Savoie)	2001	M
	PAA100	R. Ioos (P1bisa)	*Alnus glutinosa*	France (Semouse river, Haute-Saône)	2003	M
	PAA102	R. Ioos (P3d)	*Alnus glutinosa*	France (Semouse river, Haute-Saône)	2003	M
	PAA107	R. Ioos (Priva)	*Alnus glutinosa*	France (Semouse river, Haute-Saône)	2003	M
	PAA111	C. Husson (Ainvelle Sol)	*Alnus glutinosa* soil	France (Ainvelle forest, Haute-Saône)	2003	M
	PAA112	C. Husson (2ALD03)	*Alnus glutinosa*	France (Moine river, Maine-et-Loire)	2003	M
	PAA113	C. Husson (102-1)	*Alnus glutinosa*	France (Sarre river, Moselle)	2003	M
	PAA114	C. Husson (Moselle)	*Alnus glutinosa*	France (Moselle river, Vosges)	2002	M
	PAA115	C. Husson (370-2)	*Alnus glutinosa*	France (Sarre river, Moselle)	2002	M
	PAA116	R. Ioos (3N10094-5a)	*Alnus glutinosa*	France (Ognon river, Haute-Saône)	2003	M
	PAA120	R. Ioos (3N10048-3a)	*Alnus glutinosa*	France (Ognon river, Haute Saône)	2003	*nd*
	PAA126	C. Husson (Ainvelle4-4)	*Alnus glutinosa*	France (Semouse river, Haute-Saône)	2003	M
	PAA129*	G. Capron (703)	*Alnus glutinosa*	France (Luy river, Landes)	2003	U
	PAA130*	R. Ioos (1429-6b)	*Alnus glutinosa*	France (Saône river, Haute-Saône)	2003	M
	PAA131	C. Husson (Sol A15)	*Alnus glutinosa* soil	France (Ainvelle forest, Haute Saône)	2003	M
	PAA133	C. Husson (Sol A7)	*Alnus glutinosa* soil	France (Ainvelle forest, Haute Saône)	2003	*nd*
	PAA147	B. Thoirain (478-4)	*Alnus glutinosa*	France (Sarre river, Moselle)	2004	M
	PAA149	B. Thoirain (19BT)	*Alnus glutinosa*	France (Vezouze river, Meurthe et Moselle)	2004	M
	PAA150	B. Thoirain (10BT)	*Alnus glutinosa*	France (Vezouze river, Meurthe et Moselle)	2004	M
	PAA151*	B. Thoirain (2051000-D12)	*Alnus glutinosa*	France (Moselle river, Vosges)	2004	U
	PAA153	B. Thoirain (C2.1-2070250)	*Alnus glutinosa*	France (Meurthe river, Meurthe et Moselle)	2004	M
	PAA160	B. Thoirain (C9)	*Alnus glutinosa*	France (Mortagne river, Meurthe et Moselle)	2004	M
	PAA161	B. Thoirain (C18)	*Alnus glutinosa*	France (Chiers river, Meurthe et Moselle)	2004	M
	PAA185	R. Ioos (4N1605)	*Alnus glutinosa*	France (Ill river, Bas-Rhin)	2004	M
	PAA195	R. Ioos (Jouy2)	*Alnus glutinosa*	France (Eure river, Eure)	2005	M
	PAA29	J.C. Streito (9900715.6)	*Alnus incana*	Belgium (Train river, Brabant)	1999	*nd*
	PAA86	D. De Merlier (2198[c])	*Alnus glutinosa*	Belgium (Salm river, Luxembourg)	1999	M
	PAA88	D. De Merlier (2295[c])	*Alnus glutinosa*	Belgium (Lesse river, Luxembourg)	2001	M
	PPA70	W. Man in't Veld (PD 20010933)	*Alnus* sp.	The Netherlands (Horst, Southern-Limburg)	Unknown	M
	PAA74	G. Mackaskill (P1275)	*Alnus glutinosa*	Great Britain (Scotland)	2000	U
	PAA75	J. Gibbs (P1272)	*Alnus viridis*	Great Britain (Scotland)	2000	M
	PAA76	J. Gibbs (P1271)	*Alnus glutinosa*	Great Britain (Scotland)	2000	M
	PAA77	J. Delcan (P1270)	*Alnus glutinosa*	Great Britain (Scotland)	2000	M
	PAA78	J. Delcan (P1960)	*Alnus glutinosa*	Great Britain (England)	1997	*nd*
	PAA79	J. Delcan (P957[a])	*Alnus glutinosa*	Great Britain (England)	1997	*nd*
	PAA80	J. Delcan (P950[a])	*Alnus glutinosa*	Great Britain (England)	1997	*nd*
	PAA81	J. Delcan (P937)	*Alnus glutinosa*	Great Britain (England)	1997	U
	PAA82	S. Gregory (P850)	*Alnus glutinosa*	Great Britain (England)	1996	M
	PAA85	C. Brasier (P834[c])	*Alnus glutinosa*	Great Britain (England)	Unknown	M
	PAA91	Z. Nagy (6[d])	*Alnus glutinosa*	Hungary (Hévíz)	2001	M′
	PAA92	Z. Nagy (8[d])	*Alnus glutinosa* soil	Hungary (Hévíz)	2001	M′
	PAA93	Z. Nagy (9[d])	*Alnus glutinosa* soil	Hungary (Hévíz)	2001	M′
	PAA94	Z. Nagy (1a[d])	*Alnus glutinosa* soil	Hungary (Hévíz)	2001	M′
	PAA95	Z. Nagy (4-2[d])	*Alnus glutinosa*	Hungary (Hévíz)	2001	M′
	PAA134	K. Kaminski (BBA 23/00)	*Alnus glutinosa*	Germany (Hessen)	2000	M
	PAA162*	R. Ioos (9a)	*Alnus glutinosa*	Germany (Bavaria)	2004	U
	PAA176	R. Ioos (1c)	*Alnus glutinosa*	Germany (Bavaria)	2004	U
	PAA141	T. Cech (Pucking B10)	*Alnus glutinosa*	Austria (Donau Valley)	Unknown	U′
	PAA143*	L. Orlikowski (PO 192)	*Alnus glutinosa*	Poland (Pilicia river, Bialobrzegi)	2002	M″
	PAA144	L. Orlikowski (PO 193)	*Alnus glutinosa*	Poland (Vistula river, Koszyce)	2003	M″
	PAA145	L. Orlikowski (PO 203)	*Alnus glutinosa*	Poland (Pisia river, Radziejowice)	2004	M″
	PAA146	L. Orlikowski (PO 205)	*Alnus glutinosa*	Poland (Vistula river, Pulawy)	2002	M″
	PAA178	L. Orlikowski (PO318)	*Alnus glutinosa*	Poland (Notec river, Naklo)	2003	M″
	PAA180	L. Orlikowski (PO355)	*Alnus glutinosa*	Poland (Rudawka river, Morawica)	2004	M″
	PAA181	L. Orlikowski (PO379)	*Alnus glutinosa*	Poland (Bug river, Wlodawa)	2004	M″
	PAA182	L. Orlikowski (PO385)	*Alnus glutinosa* soil	Poland (Bug river, Wlodawa)	2004	M″
	PAA183	L. Orlikowski (PO399)	*Alnus glutinosa* soil	Poland (nursery, Siedliska)	2004	M″
	PAA184	L. Orlikowski (PO400)	*Alnus glutinosa*	Poland (nursery, Kornie)	2004	M″
	PAA189	L. Orlikowski (*P. alni* soil)	*Alnus glutinosa* soil	Poland (nursery, Siedliska)	2004	M″
	PAA191	L. Orlikowski (121 AL/KO/03)	*Alnus glutinosa*	Poland (Vistula river, Koszyce)	2004	M″
	PAA192	L. Orlikowski (106 AL/PV)	*Alnus glutinosa*	Poland (Bug river, Wlodawa)	2004	M″
	PAA193	L. Orlikowski (107/AL/B/03)	*Alnus glutinosa*	Poland (Bug river, Wlodawa)	2004	M″

Table 1, continued

Species	Isolate	Isolator/supplier, reference	Host	Origin	Year isolated	mtDNA pattern
P. alni* subsp. *uniformis	PAU60*	J.C. Streito (AUL028)	*Alnus glutinosa*	France (Moselle river, Vosges)	1999	U″
	PAU84*	C. Olsson (P875[a,b,c,f])	*Alnus glutinosa*	Sweden (Gothenburg)	1997	U′
	PAU87	D. De Merlier (2271[c])	*Alnus glutinosa*	Belgium (Amblève river, Liège)	2001	U
	PAU187	D. De Merlier (2276[c])	*Alnus glutinosa*	Belgium (Canal du Centre, Hainaut)	2001	U
	PAU188	D. De Merlier (2277[c])	*Alnus incana*	Belgium (Rulles river, luxembourg)	2001	U
	PAU89*	P. Capretti (CBS109280[e])	*Alnus cordata*	Italy (Northern Tuscany)	2000	U
	PAU96	Z. Nagy (155-a[d])	*Alnus glutinosa*	Hungary (Hanság)	1999	U
	PAU97	Z. Nagy (155-b[d])	*Alnus glutinosa* soil	Hungary (Hanság)	1999	U
	PAU98	Z. Nagy (155-c[d])	*Alnus glutinosa* soil	Hungary (Hanság)	1999	U
	PAU142	A. Munda (Phy-A-Slo)	*Alnus glutinosa*	Slovenia (Lubljana)	2003	U
P. alni* subsp. *multiformis	PAM54*	J.C. Streito (DSFO/0125)	*Alnus glutinosa*	France (Aff river, Ille et Vilaine)	2000	M
	PAM71*	W. Man in't Veld (W1139)	*Alnus glutinosa* soil	The Netherlands (De Wieden, Overijssel)	Unknown	M
	PAM90	W. Man in't Veld (P972[a,c,f])	*Alnus glutinosa* soil	The Netherlands (De Wieden, Overijssel)	Unknown	M
	PAM73*	S. Gregory (P841[a,c,f])	*Alnus glutinosa*	Great Britain (England)	1996	M
	PAM186	D. De Merlier (2274[c])	*Alnus glutinosa*	Belgium (Sambre river, Namur)	2001	M′
P. cambivora	PC463	INRA Bordeaux	*Castanea sativa*	France (Lot et Garonne)	1994	C2
P. cambivora	PC643*	INRA Bordeaux	*Castanea sativa* soil	France (Aude)	2000	C1
P. cambivora	PCJC17*	C. Delatour	*Quercus* sp. soil	France (Haute Saône)	1999	C2
P. cambivora	PCGA1	C. Delatour	*Quercus* sp. soil	France (Haute Saône)	1999	C2
P. cambivora	PC99428	R. Ioos	*Castanea sativa*	France	1999	C2
P. cambivora	PCST3R1	C. Delatour	*Quercus petraea*	France (Gironde)	1999	C2
P. cambivora	PC627 (oecdc)	INRA Bordeaux	*Castanea sativa*	Italy	2000	C2
P. cambivora	PC1A21	INRA Bordeaux	*Quercus* sp. soil	France (Vienne)	1999	C2
P. cambivora	PC4N1425	LNPV-UMAF	*Castanea sativa*	France (Yvelines)	2004	C2
P. cambivora	PC4N444	LNPV-UMAF	*Castanea sativa*	France (Eure)	2004	C2
P. fragariae var. *fragariae*	PFF1	K. Hughes	*Fragaria x ananassa*	Unknown	Unknown	*nd*
P. fragariae var. *fragariae*	PFF209.46	CBS (CBS209.46)	*Fragaria x ananassa*	Great Britain (England)	1946	FF
P. fragariae var. *fragariae*	PFF309*	CBS (CBS 309.62)	*Fragaria x ananassa*	Great Britain (Scotland)	1962	FF
P. fragariae var. *rubi*	PFRVR 59	D. Cooke (FVR 59)	*Rubus* sp.	Great Britain	Unknown	FR
P. fragariae var. *rubi*	PFR163-2	A. Baudry (163-2)	*Rubus* sp.	France	Unknown	FR
P. fragariae var. *rubi*	PFR2	K. Hughes	*Rubus* sp.	Great Britain	Unknown	FR
P. fragariae var. *rubi*	PFR967.95	CBS (CBS967.95)	*Rubus* sp.	Great Britain (Scotland)	1985	FR
P. fragariae var. *rubi*	PFR109*	CBS (CBS109.892)	*Rubus* sp.	Great Britain (Scotland)	1991	FR
P. cactorum	CAC4810/TJ	C. Delatour	Unknown	France	Unknown	
P. cinnamomi	DSFO2N0964	J.C. Streito	*Castanea sativa*	France	2002	
P. cinnamomi	DSFA970060	J.C. Streito	*Quercus suber*	France	1997	
P. cinnamomi	DSFO990050	J.C. Streito	*Castanea sativa* soil	France	1999	
P. cinnamomi	P382	C. Brasier	*Nothofagus procera* soil	Great Britain	1980	
P. citricola	2N0750-171	J.C. Streito	Unknown	France	2002	
P. citricola	AUL 045 AP7	J.C. Streito	*Alnus glutinosa*	France	1999	
P. citricola	2AE5	C. Delatour	*Quercus* sp. soil	France	1998	
P. citricola	3N1345-17	R. Ioos	*Alnus glutinosa*	France	2003	
P. citrophthora	2N1021	J.C. Streito	*Rosa* sp.	France	2002	
P. cryptogea	990675	J.C. Streito	*Actinidia sinensis*	France	1999	
P. erythroseptica	960713	J.C. Streito	*Polygonum oberti*	France	1999	
P. europaea	AL5	C. Delatour	*Quercus* sp. soil	France	1998	
P. europaea	2AU2	C. Delatour	*Quercus* sp. soil	France	1999	
P. gonapodyides	Gonap 4	C. Delatour	*Quercus* sp. soil	France	1998	
P. gonapodyides	AB4	C. Delatour	*Quercus* sp. soil	France	1998	
P. humicola	3N1245-j	R. Ioos	*Alnus glutinosa* soil	France	2003	
P. ilicis	3N1245-l	R. Ioos	*Alnus glutinosa* soil	France	2003	
P. inundata	9500802	J.C. Streito	*Alnus glutinosa* soil	France	1998	
P. lateralis	98093.1-SPV	J.C. Streito	*Chamaecyparis* sp.	France	1998	
P. megasperma	3N1245-m	R. Ioos	*Alnus glutinosa* soil	France	2003	
P. megasperma	BK1	C. Delatour	*Quercus* sp. soil	France	1998	
P. megasperma	03-12	C. Delatour	water under *Quercus* sp.	France	1998	
P. megasperma	mega 1	T. Jung	Unknown	Germany	1998	
P. megasperma	8RPOC3	C. Delatour	*Quercus* sp. soil	France	1998	
P. nicotianae	960579	J.C. Streito	*Nicotiana tabacum*	France	1996	
P. taxon forestsoil	8CARPPOC1	C. Delatour	*Quercus* sp. soil	France	1998	
P. palmivora	970423	J.C. Streito	*Hedera* sp.	France	1997	
P. parasitica	970029	J.C. Streito	*Lycopersicon esculentum*	France	1997	

Table 1, continued

Species	Isolate	Isolator/supplier, reference	Host	Origin	Year isolated	mtDNA pattern
P. taxon Pgchlamydo	Haye,3,1	C. Delatour	*Quercus* sp. soil	France	1998	
P. pseudosyringae	EW5	C. Delatour	*Quercus* sp. soil	France	1998	
P. psychrophila	FF20	C. Delatour	*Quercus* sp. soil	France	1998	
P. quercina	FNA	C. Delatour	*Quercus* sp. soil	France	1999	
P. quercina	Mers2	C. Delatour	*Quercus* sp. soil	France	1999	
P. ramorum	2N0983	C. Saurat	*Rhododendron* sp.	France	2002	
P. ramorum	3N0003	C. Saurat	*Viburnum* sp.	France	2002	
P. sojae	443	F. Panabières	*Glycine max*	Unknown	Unknc	
P. syringae	2JZ2	C. Delatour	*Quercus* sp. soil	France	1999	
Pythium aphanidermatum	Ctsa	R. Ioos	Unknown	France	2003	
Pythium sylvaticum	0675/a	R. Ioos	Unknown	France	2003	
Pythium intermedium	02/84/1	S. Verger	Unknown	France	Unknown	
Pythium irregulare	02/57/1	S. Verger	Unknown	France	Unknown	
Pythium ultimum	433/3	S. Verger	Unknown	France	Unknown	
Pythium sp.	3N1345-11	R. Ioos	*Alnus glutinosa* soil	France	2003	

The different mitochondrial DNA patterns resolved in this study are indicated.
nd, not determined.
[a] Also studied by Delcan and Brasier (2001).
[b] Also studied by Brasier et al. (1999).
[c] Also studied by De Merlier et al. (2005).
[d] Also studied by Nagy et al. (2003).
[e] Also studied by Santini et al. (2003).
[f] Also studied by Brasier and Kirk (2001).
[*] Isolate used for sequencing in this study.

Table 2. List of the degenerate primers designed in this study

Gene	Original reference (GenBank Accession No.)	Additional sequences[a]	Primer (forward/reverse)	Sequence (5′–3′)	Size[b]	Intron(s)[c]
ASF-like	*Homo sapiens* (AB028628)	*P. sojae* (Scaffold_6 1068853-1069936)	ASF-E1-1F	ACCAACATCACCGTGCTGGAC	388–402	1
		P. ramorum (Scaffold_43 348071–349019)	ASF-E2-2R	CGTTGTTGACGTAGTAGCCCAC		
*GPA*1	*P. infestans* (AY050536), *P. palmivora* (AY050537)	*P. sojae* (Scaffold_34 298473–301143)	GPA-E1-1F	GGACTCTGTGCGTCCCAGATG	286–312	1
		P. ramorum (Scaffold_59 316212–318351)	GPA-E2-1R	ATAATTGGTGTGCAGTGCCGC		
RAS-Ypt	*P. infestans* (U30474)	*P. cinnamomi* (AF454368) *P. cryptogea* (AF454367) *P. citricola* (AF454369) *P. sojae* (Scaffold_30 367461–369077) *P. ramorum* (Scaffold_16 280386–282150)	RAS-E1-1F RAS-E5-1R	ATGAACCCCGAATAGTRCGTGC TGTTSACGTTCTCRCAGGCG	666–698	4
*TRP*1	*P. parasitica* (M64473)	*P. sojae* (Scaffold_25 46183–57099)	TRP-E1-1F	GAGGAGATCGCGGCGCAGCG	661–765	2
		P. ramorum (Scaffold_52 330699–333530)	TRP-E3-1R	GCGCACATRCCGAGVTTGTG		

[a] Refer to GenBank accession number for other species or localization of the respective similar sequence in the *P. sojae* and the *P. ramorum* JGI sequencing projects.
[b] Range of amplicon length yielded in silico using the designed primer pair with all available sequences.
[c] Number of introns that are located within the ampli W ed region using the designed primers.

Netherlands) or from several European colleagues. Assignment of the isolates to one of the three subspecies of *P. alni* was achieved by combining the examination of the morphological features of each isolate in pure culture according to Brasier et al. (2004), and analyzing restriction patterns of the ITS region using a series of enzymes, according to Brasier et al. (1999) and Cooke et al. (2000). The assignment was further confirmed using subspecific SCAR-based PCR primers (Ioos et al., 2005).

A panel of 15 isolates was selected from among *Paa, Pau, Pam, P. cambivora*, and *P. fragariae* and used for cloning and sequencing: five isolates of *P. alni* subsp. *alni* (PAA129, PAA130, PAA143, PAA151, and PAA162), three isolates of *P. alni* subsp. *uniformis* (PAU60, PAU84, and PAU89), three isolates of *P. alni* subsp. *multiformis* (PAM54, PAM71, and PAM73), two isolates of *P. cambivora* (PC643 and PCJC17), one isolate of *P. fragariae* var. *fragariae* (PFF309), and one isolate of *P. fragariae* var. *rubi* (PFR109), chosen from different geographical locations.

All the isolates were grown shaking in 6 ml of liquid V8 juice medium (Miller, 1955). After incubation at 22 °C for 4–7 days, the mycelium was harvested by filtration on a sterile Whatman N°1 paper (Maidstone, England) and stored in this condition at ¡20 °C until DNA extraction. DNA was extracted using commercial plant DNA extraction kits (DNeasy plant mini kit®, Qiagen, Courtaboeuf, France) and as previously described (Ioos et al., 2005).

2.2. RFLP of mitochondrial DNA

Total DNA was extracted using commercial plant DNA extraction kits (DNeasy plant mini kit®) as previously described (Ioos et al., 2005), except that the quantity of dried mycelium was increased from 200 to 600mg, and 10 l of proteinase K (20mg/ml) was added to the lysis buffer. Incubation time with the lysis buffer was also increased to 20min. Four to 8 g of total DNA was typically recovered. Mitochondrial DNA is present in multiple copies and can be separated from genomic DNA by digestion with restriction enzymes, which cut regions rich in G+C (Spitzer et al., 1989). Accordingly, 20 l of total DNA were digested twice for 12h with each time fIve units of HaeIII or HpaII and mtDNA patterns were resolved after a 15h electrophoresis (0.6V/cm) on a 0.8% agarose gel in TBE 0.5£ buffer. Gels were stained with ethidium bromide and images were recorded with a CCD camera and a GELDOC 2000® gel documentation system (Biorad, Marne-La-Coquette, France).

2.3. Design of nuclear gene-specific degenerate primers

For each of the four nuclear genes studied, namely ASF-like, GPA1, RAS-Ypt, and TRP1, the original sequence deposited in GenBank was retrieved and used as a basis for similarity research in other *Phytophthora* DNA resources (see accession numbers in Table 2). Nucleotide similarities were searched for in the GenBank database, and the P. ramorum and the *P. sojae* assembled sequences recently released in the public domain (http://genome.jgi-psf.org/), using the BLASTn algorithm and the *Phytophthora* Functional Genomics Database, which gathers sequences from *P. infestans* and *P. sojae*, and using the PFGD search filter (http://www.pfgd.org/pfgd/filter.html).

For each gene, all the available orthologous sequences from different *Phytophthora* species were then aligned using ClustalW (Thompson et al., 1994) and a series of degenerate primer pairs were manually designed in highly conserved regions located in exons. The location of the primers was chosen in order to enable PCR amplification of the largest part of the gene, including as many intronic regions as possible. The primer pairs that best amplified the target region in all species of the 15-isolate panel were retained and used for cloning. Table 2 lists the respective forward and reverse PCR primers chosen for each of the four nuclear genes.

2.4. Amplification and cloning of the nuclear and the mitochondrial genes

Each of the four nuclear genes was amplified by PCR for each of the 15 isolates of the panel. Amplification of the four nuclear genes was carried out in a 20-µl mixture containing 1× Taq polymerase buffer (Sigma–Aldrich, L'Isle d'Abeau, France), 1.8 mM $MgCl_2$ (1.5 mM for TRP1), 0.7 g/µl bovine serum albumin (Sigma), 0.45 µM (0.2 µM for TRP1) of each forward and reverse gene-specific primer, 180 mM dNTPs, 0.6 U of Taq DNA Polymerase (Sigma–Aldrich), 2 µl of template DNA (30– 80 ng), and molecular biology grade water was added to 20 µl. The cycling profile for PCR included an initial denaturation step at 95°C for 3 min, followed by 35 cycles of denaturation for 30 s at 94°C, annealing for 30 s at 62 °C (58°C for RAS-Ypt), and elongation for 1 min at 72°C, and a final extension step at 72°C for 7 min.

Amplification of the two mitochondrial genes, nadh1 and cox1, was performed for a limited panel of six isolates: *Paa* (PAA129 and PAA130), *Pam* (PAM54), *Pau* (PAU60), and Pc (PCJC17 and PC643). PCRs were carried out with the mitochondrial gene-specific primers designed by Kroon et al. (2004) using the same parameters as described above for the nuclear genes with slight modifications. The annealing temperatures were lowered to 58 and 53 °C for nadh1 and cox1 amplifications, respectively, and the concentration of $MgCl_2$ was raised to 3.5 mM for cox1, as suggested by Kroon et al. (2004).

Each of the gene-specific PCR products was cloned for each isolate of the panel, using a TOPO®-TA cloning kit (Invitrogen, Cergy Pontoise, France) and following the manufacturer's instructions.

Table 3. GenBank accession numbers of the sequences obtained for the six genes cloned in this study for the 15-isolate panel

Species	Isolate	Nuclear genes				Mitochondrial genes	
		ASF-like	*GPA*1	*RAS-Ypt*	*TRP*1	*cox*1	*nadh*1
P. alni subsp. *alni*	PAA 129	DQ092818	DQ179047	DQ093974	DQ093997	DQ202499	DQ202488
		DQ092819	DQ179048	DQ179054	DQ093998		DQ202489
		DQ092820	DQ179049				
	PAA 130	DQ092821	DQ179050	DQ093975	DQ202482	DQ202500	DQ202490
		DQ092822	DQ179051	DQ093976	DQ202483		DQ202491
		DQ092823		DQ093977	DQ212060		
	PAA 143	DQ092824	DQ092851	DQ093978	DQ093999		
		DQ092825	DQ179052	DQ093979	DQ094000		
		DQ092831		DQ093980			
	PAA 151	DQ092826	DQ092852	DQ093981	DQ094001		
		DQ092827	DQ092853	DQ093982	DQ094002		
			DQ092854				
	PAA 162	DQ092828	DQ092855	DQ093983	DQ094003		
		DQ092829	DQ092856	DQ093984	DQ094004		
		DQ092830					
P. alni subsp. *uniformis*	PAU 60	DQ092811	DQ092857	DQ093971	DQ202480	DQ202498	DQ202486
		DQ092812					DQ202487
		DQ092813					
	PAU 84	DQ092814	DQ092849	DQ093972	DQ093996		
		DQ092815					
		DQ092816					
	PAU 89	DQ092817	DQ092850	DQ093973	DQ202481		
P. alni subsp. *multiformis*	PAM 54	DQ092806	DQ092843	DQ093966	DQ093991	DQ202496	DQ202484
		DQ092807	DQ092844	DQ179053	DQ093992	DQ202497	DQ202485
	PAM 71	DQ092808	DQ092845	DQ093967	DQ093993		
		DQ179044	DQ179046	DQ093968	DQ202479		
		DQ179045					
	PAM 73	DQ092809	DQ092847	DQ093969	DQ093994		
		DQ092810	DQ092848	DQ093970	DQ093995		
P. cambivora	PC643	DQ092834	DQ092861	DQ093987	DQ094007	DQ202502	DQ202494
		DQ092835	DQ092862	DQ093988		DQ202503	DQ202495
		DQ092836					
		DQ092837					
		DQ092838					
	PCJC17	DQ092839	DQ092860	DQ093989	DQ094008,	DQ202501	DQ202492
		DQ092840		DQ093990	DQ094009		DQ202493
		DQ092841					
		DQ092842					
P. fragariae var. *fragariae*	PFF309	DQ092832	DQ092858	DQ093985	DQ094005		
P. fragariae var. *rubi*	PFR109	DQ092833	DQ092859	DQ093986	DQ094006		

2.5. Selection of the clones and sequencing

For each isolate, hundreds of positive clones were typically recovered for each of the cloned genes. For each isolate x gene combination, 10 positive clones were randomly selected and tested by heteroduplex analysis in order to detect sequence polymorphisms among the 10 inserts, following a protocol adapted from Pinar et al. (1997). Heteroduplex analysis is a conformational technique that makes it possible to detect mutations in PCR-

amplified products. The migration of heteroduplex DNA in agarose gel electrophoresis is different from that of homoduplex DNA because of an altered tri-dimensional structure. Brie X y, for each group of 10 clones derived from the same gene, the insert was amplified by PCR using M13 F/R primers, following the PCR conditions recommended by the cloning kit's manufacturer. The ten PCR products were tested against each other by mixing directly 4 l of each PCR products in a PCR tube, corresponding to a total of 45 pairwise combinations. An extra 8-µl sample of a unique PCR product was also prepared to be used as a control for homoduplex patterns. All the mixtures were then placed in a Genamp PCR system 9700 (Applied Biosystem, Foster City, California). DNA strands were denatured at 96°C for 3 min and slowly cooled to 20°C at a rate of 0.25°C/s. The entire 8-µl mixture was then loaded onto a 1% agarose gel and separated by electrophoresis for 90 min at 3.6 V/cm. Heteroduplex banding patterns were distinguished from among all of the 45 combinations and, eventually, the 10 clones could be discriminated and clustered based on the occurrence of detectable sequence polymorphisms. For both nuclear and mitochondrial genes and for each isolate, all the polymorphic inserts were selected and double-strand DNA sequencing was performed by the di-deoxy-chain termination method using a T3-T7 sequencing kit on a CEQ 2000 XL DNA sequencer (Beckman, Fullerton, California). Forward and reverse sequences were assembled in Sequencher 4.2 (Genecodes, Ann Arbor, MI) and aligned using an on-line version of ClustalW software (http://www.genebee.msu.su/clustal/ basic. html). The sequence obtained for the four nuclear and the two mitochondrial genes was deposited in the GenBank database (Table 3).

Table 4. List of the allele-specific primers designed in this study

Gene	Allele-specific primer (F = forward, R = reverse)	Sequence (5′–3′)	Annealing temperature (°C)
ASF-like	ASF-PAM1-F[a]	GGT GCT GGA GGA AGT GCT T	64
	ASF-PAM2-F[a]	ACC GCC ATC ACC ACC ATA	60
	ASF-PAU-F[a]	CAC CGC CAC AAC ATC CAC TC	58
	ASF-PCJC17a6-F[a]	ACT ATC TCC TAT GAT ACA CT	54
	ASF-PC643e5-F[a]	TCC TAT GCT ACA CAC TAA C	60
	ASF-PCJC17c6-F[a]	GAT ACA GTG TAG CAC CAT C	58
*GPA*1	GPA-PAM1-R[b]	CTC GCA GCT CCC ACT GCG AG	60
	GPA-PAM2-R[b]	ACA GCA CCA AAC AAA GTA CC	60
	GPA-PAU-R[b]	TCC CAC TAT AAA CAT GTC A	54
RAS-Ypt	RAS-PAM1-F	AGA GGG ATA TAT TTG AGG TT	60
	RAS-PAM1-R	GTT GGA CCC GGG ACG GTC TTC	60
	RAS-PAM2-F	AGA GGG ATA TAT TTG CGG CT	60
	RAS-PAM2-R	TCA GCA ATC GGA GAG CAA GCT	60
	RAS-PAU-F	A TTT ACT TGC AGC CGC AGG CT	58
	RAS-PAU-R	ACC TAG GGC AGA CAA GCT AGT C	58
	RAS-PC643g1-F	AGC GGT AGA CTG ACC ACA CCG	60
	RAS-PC643g1-R	GCC TGG AGG TCA AAA CTT AG	60
	RAS-PC643a2-F	GCT GCT AAC AGA CAG CAG AC	60
	RAS-PC643a2-R	ATG AAG CAC TCC GAA CCG GT	60
	RAS-PFR109h1-F	TGT CGA GAG TGA TTT ATT	60
	RAS-PFR109h1-R	AA TGG CAA GGC TAG TTA CTA	60
*TRP*1	TRP-PAM1-R[c]	CCT GTA GCA ACA GAG CAA TG	60
	TRP-PAM2-F	CCC GTT GCT GCG GCT GGC	62
	TRP-PAM2-R	GGT CGC CTA CAC CGC GTG	62
	TRP-PAU-F	GTG CGT CGC TAG CCC ATC A	60
	TRP-PAU-R	CGC CTA CAG AGC ATC ATA G	60
	TRP-PCJC17c3-F	TGG ACG TAG AAG CCG CCA AG	60
	TRP-PCJC17c3-R	CAG GCA TAT ACC GTT TCC AC	60
	TRP-PFF309a9-F	CTA CCT CCC TAA GCT TAT CA	60
	TRP-PFF309a9-R	ACG CAG CAT CAT AGA AAA T	60

[a] Used in combination with gene-speci W c ASF-E2-2R as reverse primer.
[b] Used in combination with gene-speci W c GPA-E1-1F as forward primer.
[c] Used in combination with gene-speci W c TRP-E1-1F as forward primer.

2.6. Phylogenetic analyses

Phylogenetic analyses were performed with PAUP version 4.0 (Swofford, 2002), using the maximum parsimony method. Individual phylogenetic analyses were conducted for each mitochondrial and nuclear gene. Heuristic searches were performed using “tree-bisection–reconnection” (TBR) branch swapping algorithm, zero-length branches were collapsed and all characters equally weighted. Subsequent parsimony bootstrap analyses used 1000 replicates with TBR branch swapping.

2.7. Design and testing of allele-specific PCR primers within the nuclear genes

All the sequences obtained for each nuclear gene and for all of the 15 isolates were aligned using ClustalW. Based on different clusters of sequences, a series of allele-specific primers were manually designed from polymorphic regions or around insertion/deletion (indel) loci mainly located within introns. Some of the sequences could not be subjected to primer design due to an insufficient polymorphism level or scattered substitutions. To test the absence or the presence of the different alleles within the genome of the different *Phytophthora* isolates, all the primer pairs were tested by PCR, first with the 15-isolate panel to check their reliability and specificity, and then with all the isolates of the P. alni/*Phytophthora* spp. collection listed in Table 1. Allele-specific PCRs were carried out as described above for gene-specific amplification except that the $MgCl_2$ concentration was increased to 2.2 mM and that no BSA was used. The allele-specific annealing temperatures used are indicated in Table 4.

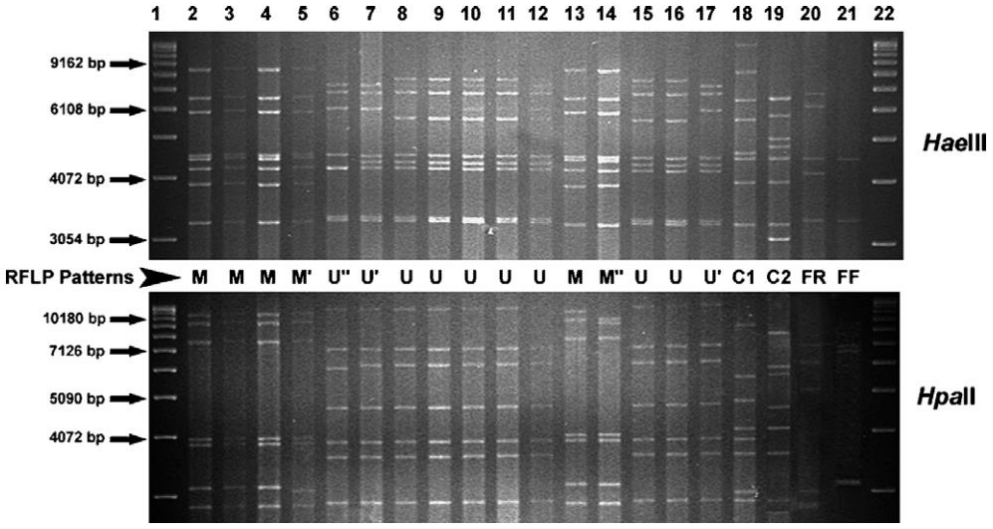

Fig. 1. Combination of the restriction patterns obtained by independent HaeIII and HpaII digestions of total DNA from a series of *P. alni* subsp. *multiformis* (*Pam), P. alni* subsp. *uniformis* (*Pau*), *P. alni* subsp. *alni* (*Paa*), *P. cambivora (Pc), P. fragariae* var. *rubi* (*Pfr*), and *P. fragariae* var. *fragariae* (Pff) isolates. Six different combinations were resolved for *P. alni* and named M, M’, M’’, U, U’, and U’’. Two different combinations were resolved for Pc, i.e., C1 and C2, respectively, whereas a single combination was resolved for *Pfr* (FR) and for *Pff* (FF). Lanes 1 and 22, molecular standard 1 kb DNA Ladder (Invitrogen, Cergy Pontoise, France); lanes 2–5, *Pam* isolates PAM54, PAM71, PAM73, and PAM186; lanes 6–11, *Pau* isolates PAU60, PAU84, PAU87, PAU89, PAU142, and PAU188; lanes 12–17, *Paa* isolates PAA129, PAA130, PAA143, PAA151, PAA162, and PAA141; lanes 18 and 19, *Pc* isolates PC643 and PCJC17; lane 20, *Pfr* isolate PFR109; lane 21, *Pff* isolate PFF309.

3. Results

3.1. Mitochondrial DNA patterns

Digestion of total DNA with the two endonucleases generated patterns with several discrete bands. Both HaeIII and HpaII revealed polymorphism in the mtDNA pattern of the isolates we tested. Four and five distinct patterns were revealed with HaeIII and HpaII restrictions, respectively, among the studied isolates from the three subspecies of P. alni, respectively (Fig. 1). Combining the patterns of the two enzymes, isolates of the three *P. alni* subspecies could be placed in six groups: M, M', and M'' groups encompassing only *Paa* and *Pam* isolates, and U, U', and U'' groups, which included only *Paa* and *Pau* isolates. Most of the *Pam* isolates displayed the M pattern, whereas most of the *Pau* isolates displayed the U pattern. The pattern M' was only observed for a single *Pam* isolate originating from Belgium (PAM186). Likewise, the two patterns U' and U'' were both displayed by a single *Pau* isolate. Pattern U' was encountered in PAU84 from Sweden, whereas pattern U'' was encountered in PAU60 from France.

Five out of the six patterns, M, M', M'', U, and U', were observed among the different *Paa* isolates we examined. No strong relationship could be established between the pattern observed and isolate origin or sampling date (Table 1). M was the most frequent pattern encountered in the *Paa* isolates. By contrast, the M' pattern was characteristic of *Paa* isolates from Hungary, whereas M'' was only found in Polish isolates. Despite being less frequent than M, the U pattern was also identified in *Paa* from locations throughout Europe, whereas the closely related U' pattern was only detected for one isolate originating in Austria (PAA141).

Additional patterns were resolved for *P. cambivora* isolates (C1 and C2) and for *P. fragariae* for which var. *fragariae* and var. *rubi* could be separated into FF and FR patterns, respectively (Fig. 1).

3.2. Cloning and sequencing of the four nuclear and the two mitochondrial genes

Each of the four nuclear genes (ASF-like, GPA1, RAS-Ypt, and TRP1) could be efficiently amplified using the designed exon-based primer pairs. Table 2 lists the series of primer pairs that best enabled the amplification of each gene. The two mitochondrial genes, cox1 and nadh1, were also successfully amplified using the gene-specific primers designed by Kroon et al. (2004).

Thanks to the heteroduplex analysis carried out for each individual isolate of the panel, several clones containing polymorphic inserts for each of the six genes could be selected.

The sequencing of the selected clones potentially containing different insert sequences showed that each isolate possessed one to four different alleles, depending on the nuclear gene, and either one or two slightly different sequences for the mitochondrial genes. For each of the four nuclear genes, all the coding regions of the sequences obtained were first translated and compared by sequence alignment to the published reference sequence(s) in order to check the identity of the cloned sequences (data not shown). The sequences obtained from the mitochondrial genes were compared using BLASTn software with the series of sequences published by Kroon et al. (2004) and shown to represent orthologs of cox1 and nadh1, as expected (data not shown).

3.3. Sequence analysis for the nuclear genes

Separate phylogenetic analyses were conducted for individual nuclear genes using all of the sequences obtained from the 15-isolate panel, and the retrieved ortholog sequences from P. ramorum and *P. sojae* were considered as outgroups.

For each of the four nuclear genes, a single allele was found in all of the *Pau* isolates. By contrast, two different alleles were systematically observed for all the *Pam* isolates on our panel, whereas at least two, and sometimes three different alleles, were present for all the *Paa* isolates. All four phylogenetic trees showed the same clustering pattern regarding *P. alni*

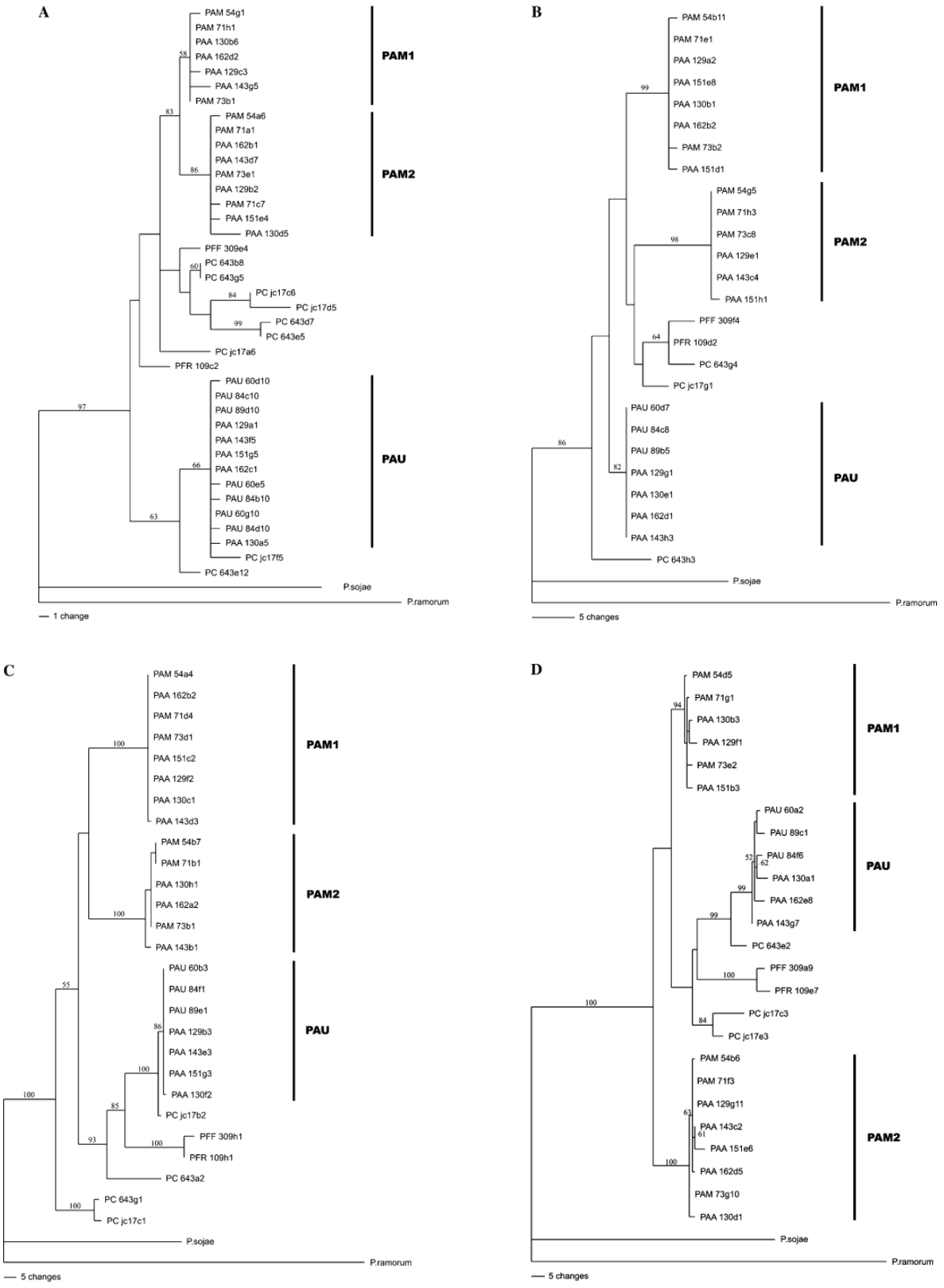

Fig. 2. Phylogenetic trees constructed using the parsimony method for each individual nuclear gene: ASF-like (A), GPA1 (B), RAS-Ypt (C), and TRP1 (D). Values given above the branches represent the bootstrap values from 1000 replicates; only values greater than 50% are shown. Clusters of similar *P. alni* sequences are defined on the right side of each tree.

sequences (Fig. 2). Indeed, regardless of the gene considered, the different *P. alni* alleles could be split into three clusters, respectively, designated here as PAM1, PAM2, and PAU. For each of the four genes, PAM1 and PAM2 clusters only contained sequences originating from *Paa* and *Pam* isolates, whereas *Pau* clusters contained sequences originating from *Paa* and *Pau* isolates.

Single alleles were observed for the four studied genes for *P. fragariae* var. *fragariae* and *P. fragariae* var. *rubi* isolates. By contrast, one or two different alleles of GPA1, TRP1, and RAS-Ypt genes were identified for each *P. cambivora* isolate, whereas up to four different alleles of the ASF-like gene could be observed (Fig. 2A).

Some *P. cambivora* alleles were closely related to the *Pau* cluster but, except for ASF-like, were not included in this *Pau* cluster. The alleles identified for the two different varieties of *P. fragariae* clustered together in another group, different from those containing *P. alni* or *P. cambivora* sequences. Nevertheless, for the ASF-like gene, the respective alleles for the two varieties were separated (Fig. 2A).

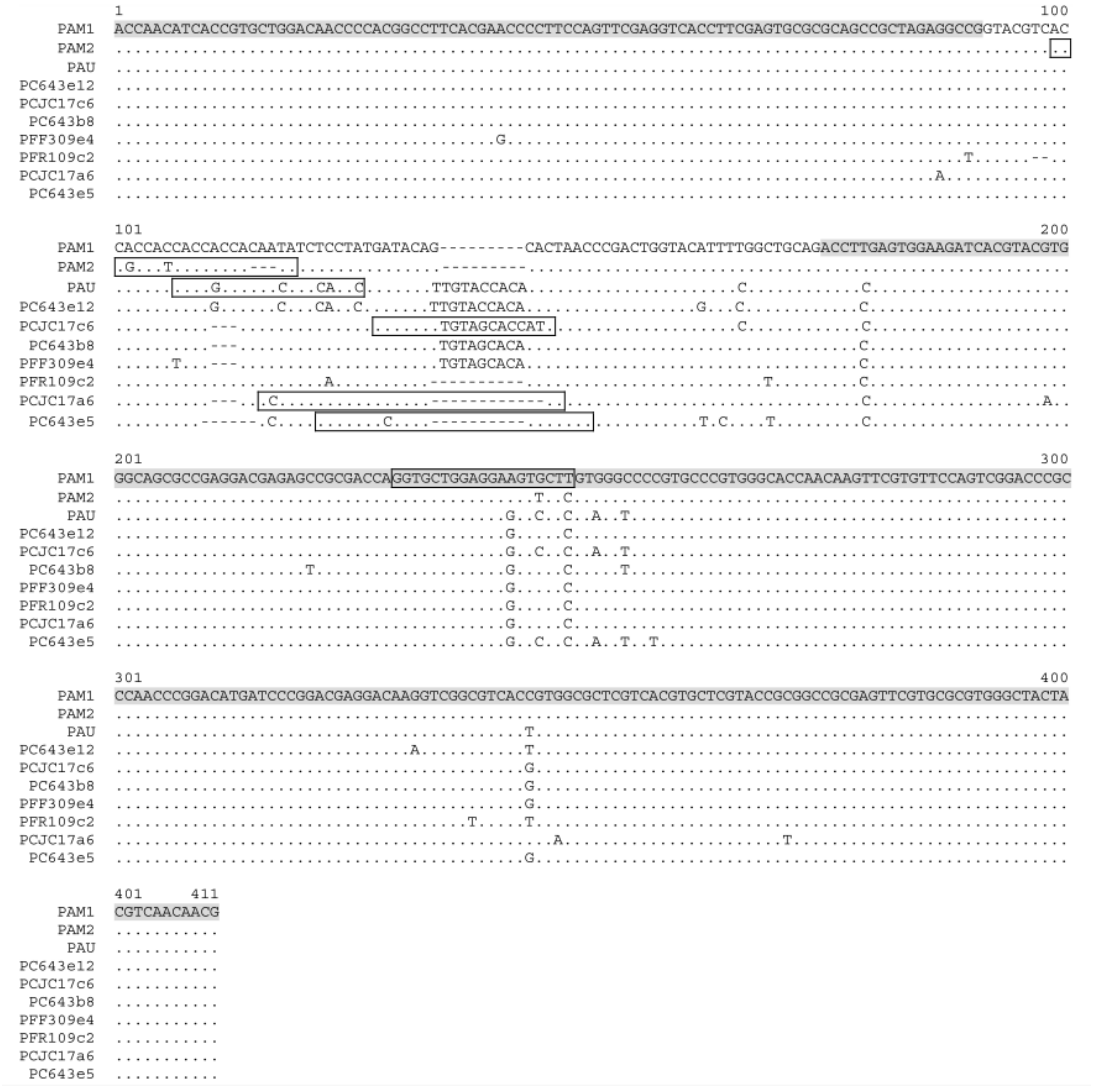

Fig. 3. Sequence alignment using the different groups of sequences collected from the 15-isolate panel for the ASF-like gene. Sequences are designated according to the clusters designed from the phylogenetic trees or the clone from which they are derived (see Fig. 2A). Boxed characters indicate the regions from which allele-specific primers could be designed. Shaded regions correspond to exons.

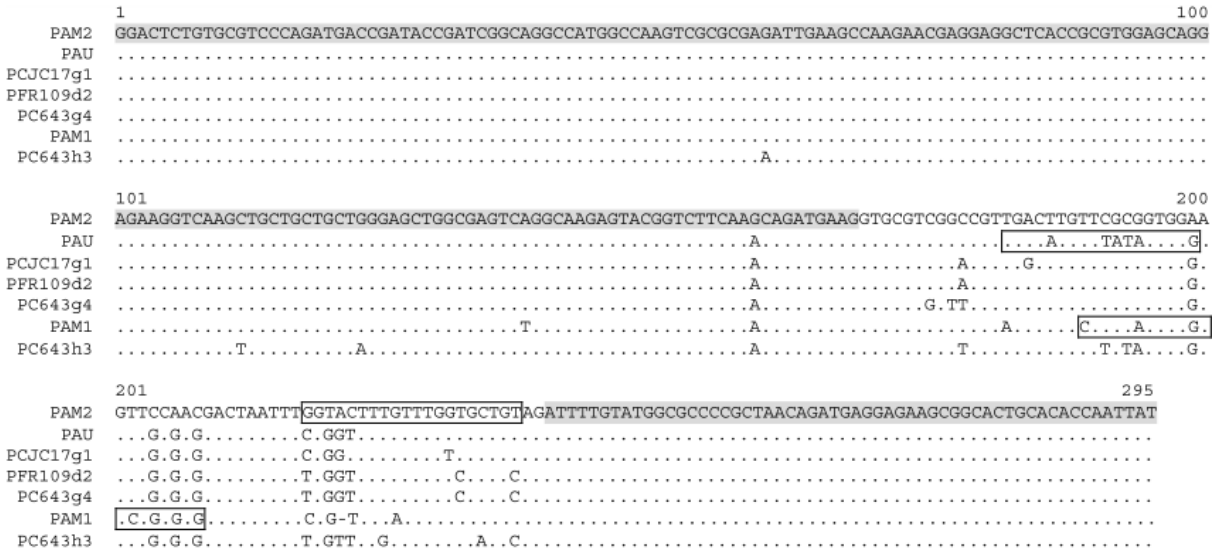

Fig. 4. Sequence alignment using the different groups of sequences collected from the 15-isolate panel for the GPA1 gene. Sequences are designated according to the clusters designed from the phylogenetic trees or the clone from which they are derived (see Fig. 2B). Boxed characters indicate the regions from which allele-specific primers could be designed. Shaded regions correspond to exons.

3.4. Allele-specific PCRs for the nuclear genes

Sequence alignments using all the data collected for each nuclear gene and for each isolate showed that polymorphism mainly occurred in the intronic regions. These polymorphisms were used to design a series of allele-specific PCR primers for each nuclear gene (Figs. 3–6). For each nuclear gene, it was possible to design pairs of primers specific to each of the three clusters of *P. alni* alleles, i.e., PAM1, PAM2, and *Pau,* respectively (Table 4). Additionally, for the two related taxa, *P. cambivora* and P. *fragariae*, a series of PCR primers targeted regions that were specific to sequences obtained from at least one isolate and were named according to the clone they were designed from (Table 4). Unfortunately, it was sometimes impossible to design PCR primers from some of the sequences obtained from *P. cambivora* or *P. fragariae* because of possible cross-reactions with other sequences. Such sequences are indicated in italics in Table 5. Likewise, it was not possible to design primers that only targeted sequences present in one or the other variety of *P. fragariae* because of the very strong similarity between the sequences of the genes for the two varieties.

All the allele-specific primer pairs successfully yielded an amplicon of the expected size when tested with the DNA of the isolate the primers were designed from, confirming the reliability of the sequences. The testing of our Europe-wide collection of *P. alni* with the series of allele-specific PCR primers showed that, for each of the four nuclear genes, all the *Paa* isolates possessed three alleles, referred to as PAM1, PAM2, and *Pau,* whereas all the *Pam* possessed two alleles, PAM1 and PAM2 (Table 5). By contrast, all the *Pau* isolates possessed only single alleles (PAU). These results confirm the previous results obtained by sequencing with the 15-isolate panel.

Based on these specific PCRs, single isolates of *P. cambivora* were shown to possess more alleles for each gene than previously derived from sequencing and displayed a more complex allelic pattern than expected for this species (Table 5). The occurrence of two different alleles of both TRP1 and RAS-Ypt genes, of three alleles of the GPA1 gene, and of two to four alleles for the ASF-like gene, could be derived from allele-specific PCRs.

All the isolates of *P. fragariae* var. *rubi* and *P. fragariae* var. *fragariae* yielded only a positive signal with the primers derived from *Pff* or *Pfr* sequences, showing that these two varieties possessed single alleles of each of the four nuclear genes as well.

Finally, the series of allele-specific primers was tested with the collection of *Phytophthora* spp. and *Pythium* spp. listed in Table 1. None of the isolates yielded any PCR product with the allele-specific PCR primers, showing that the series of primers designed was specific to some of the three species studied here, *P. alni, P. cambivora,* and *P. fragariae*.

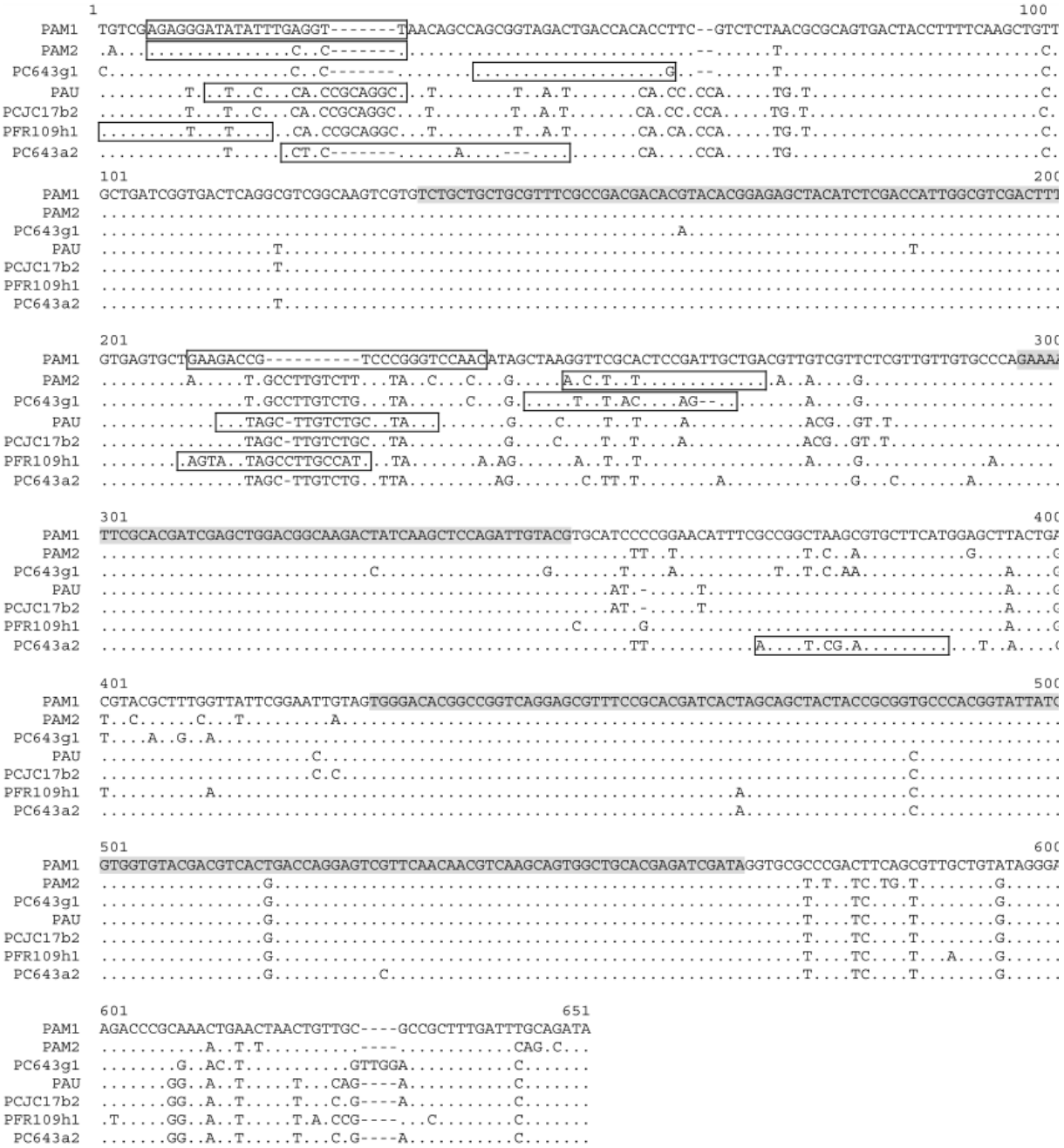

Fig. 5. Sequence alignment using the different groups of sequences collected from the 15-isolate panel for the RAS-Ypt gene. Sequences are designated according to the clusters designed from the phylogenetic trees or the clone from which they are derived (see Fig. 2C). Boxed characters indicate the regions from which allele-specific primers could be designed. Shaded regions correspond to exons.

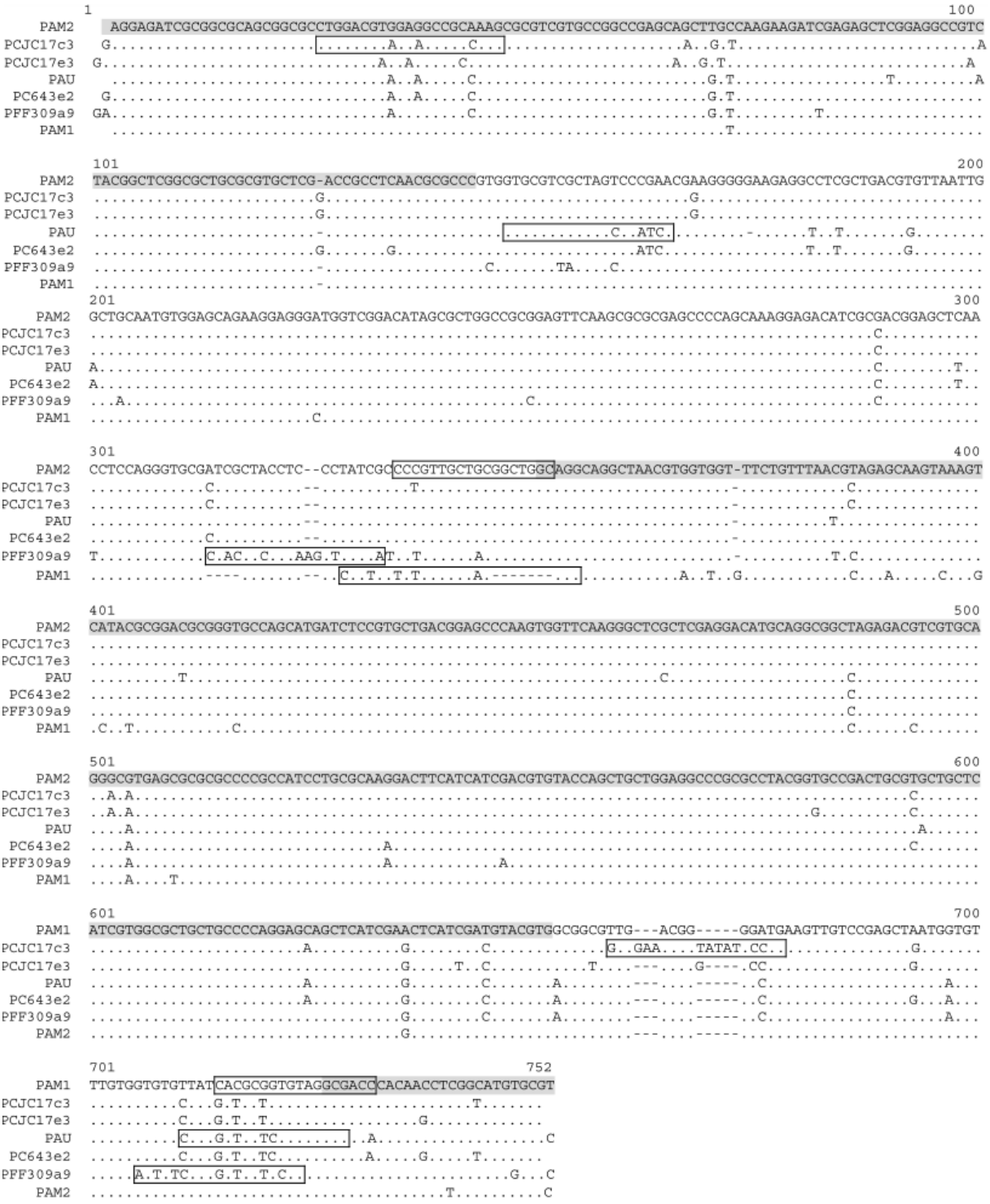

Fig. 6. Sequence alignment using the different groups of sequences collected from the 15-isolate panel for the TRP1 gene. Sequences are designated according to the clusters designed from the phylogenetic trees or the clone from which they are derived (see Fig. 2D). Boxed characters indicate the regions from which allele-specific primers could be designed. Shaded regions correspond to exons.

3.5. Sequence analysis for the mitochondrial genes

The mitochondrial gene sequences obtained with the limited six-isolate panel were aligned with the sequences of cox1 and nadh1 previously published by Kroon et al. (2004)

for *P. alni* subsp. *multiformis* (isolate PD92/1471, formerly designated as P. hybrid-Dutch variant) (GenBank Accession Nos. AY564168 and AY563995, respectively), *P. fragariae* var. *fragariae* (GenBank Accession Nos. AY564177 and AY564178, and AY564004 and AY564005, respectively), and *P. fragariae* var. *rubi* (GenBank Accession Nos. AY564179 and AY564180, and AY564006 and AY564007, respectively).

Table 5. Occurrence and distribution of the different alleles for the four nuclear genes in the genome of the different subspecies of *P. alni, P. cambivora,* and the two varieties *of P. fragariae*, as inferred from allele-specific PCR tests

Species	*ASF*-like	*GPA*1	*RAS-Ypt*	*TRP*1
P. alni subsp. *alni*	ASF-PAM1 ASF-PAM2 ASF-PAU	GPA-PAM1 GPA-PAM2 GPA-PAU	RAS-PAM1 RAS-PAM2 RAS-PAU	TRP-PAM1 TRP-PAM2 TRP-PAU
P. alni subsp. *multiformis*	ASF-PAM1 ASF-PAM2	GPA-PAM1 GPA-PAM2	RAS-PAM1 RAS-PAM2	TRP-PAM1 TRP-PAM2
P. alni subsp. *uniformis*	ASF-PAU	GPA-PAU	RAS-PAU	TRP-PAU
P. cambivora	ASF-PAU ASF-PCJC17a6[a] ASF-PC643e5[b] ASF-PCJC17c6[a] *ASF-PC643b8*[c]	*GPA-PCJC17g1* *GPA-PC643h3* *GPA-PC643g4*	RAS-PAU[a] RAS-PC643g1 RAS-PC643a2[b]	TRP-PAU[b] TRP-PCJC17c3[a] *TRP-PCJC17e3*
P. fragariae var. *fragariae*	*ASF-PFF309e4*	*GPA-PFF309g4*	RAS-PFR109h1	TRP-PFF309a9
P. fragariae var. *rubi*	*ASF-PFR109c2*	*GPA-PFR109d2*	RAS-PFR109h1	TRP-PFF309a9
Phytophthora spp.	n.p.s.	n.p.s.	n.p.s.	n.p.s.
Pythium spp.	n.p.s.	n.p.s.	n.p.s.	n.p.s.

n.p.s., no positive signal when tested with all the available allele-speci W c primer pairs
[a] Except isolate PC643.
[b] Positive only for isolates PC643 and PC463.
[c] Italics indicate alleles of genes obtained by cloning and sequencing, and for which PCR primers could not be designed.

Separate phylogenetic analyses were conducted for each mitochondrial gene using all the sequences obtained with *Paa, Pam, Pau,* and Pc and the retrieved sequences from *Pam, Pff* , and Pfr. For the two mitochondrial genes, either a single or two nearly identical (1–3 substitutions) sequences were obtained for each individual isolate. For the two mitochondrial genes, the clusters regarding *P. alni* sequences were the same (Figs. 7A and B). Sequences from PAM54 and PAA130, if not identical, clustered together in the mtPAM cluster, along with sequences of *Pam* isolate PD92/ 1471 (Kroon et al., 2004), whereas another cluster called mtPAU gathered the sequences from PAU60 and PAA129. Sequences obtained from *P. cambivora* isolates significantly differed from those forming the two *P. alni* clusters. Moreover, sequences of isolates PC643 and PCJC17 were clearly separated in both phylogenetic trees. Sequences of *P. fragariae* retrieved from GenBank (Kroon et al., 2004) did not cluster with either *P. alni* or *P. cambivora*'s sequences.

4. Discussion

4.1. Allelic diversity within P. alni

This study performed on a large Europe-wide collection of *P. alni* isolates demonstrated that three different alleles of each of the four studied nuclear genes were present in the *P. alni* subsp*. alni* (Paa) genome. Two of these alleles, PAM1 and PAM2, were the same as those found in all the studied *P. alni* subsp. *multiformis* (Pam) isolates, whereas the third allele of each gene, i.e., *Pau,* corresponded to the single alleles found in all of the *P. alni* subsp. *uniformis* (Pau) isolates.

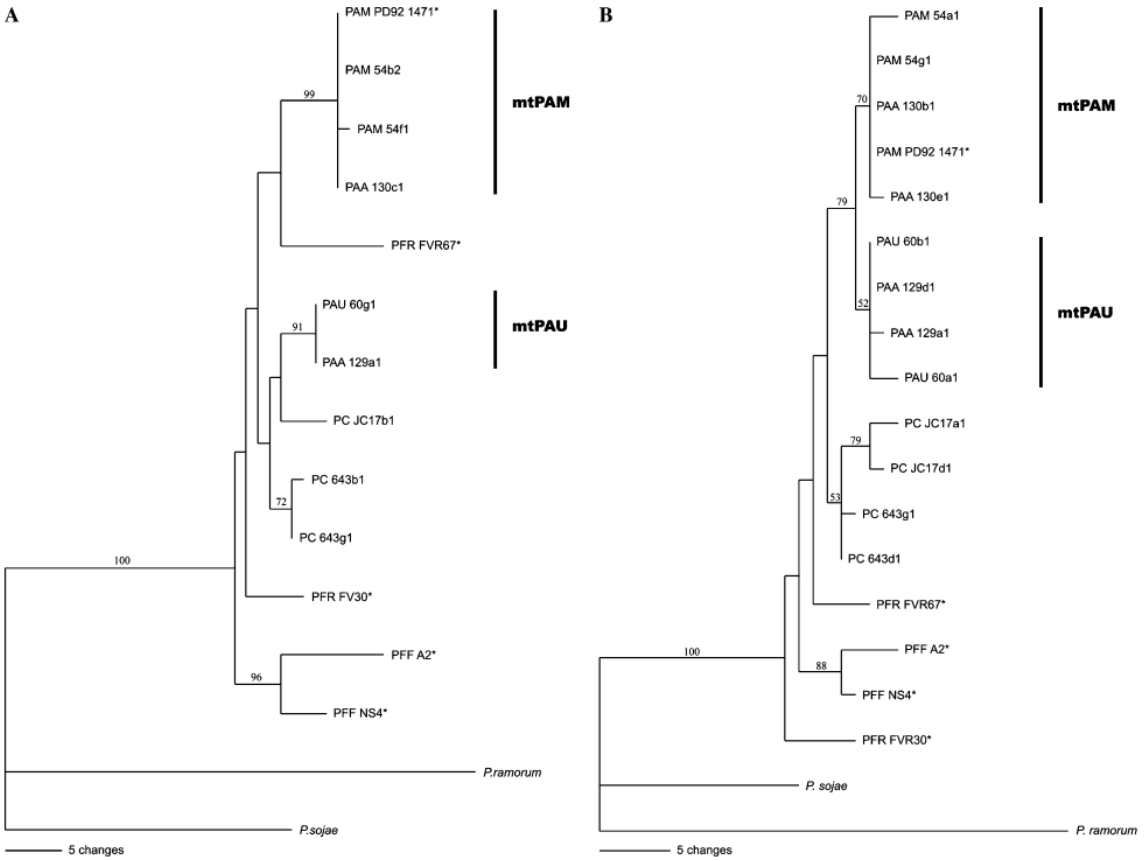

Fig. 7. Phylogenetic trees constructed using the parsimony method for each individual mitochondrial gene: cox1 (A) and nadh1 (B). Values given above the branches represent the bootstrap values from 1000 replicates; only values greater than 50% are shown. Sequences labeled with an asterisk are retrieved from the work of Kroon et al. (2004) and correspond to cox1 sequences for Pam isolate PD92/1471 (AY564168), Pff isolate A2 (AY564177), Pff isolate NS4 (AY564178), Pfr isolate FVR67 (AY564179), and Pfr isolate FVR30 (AY564180) (A), and to nadh1 sequences for Pam isolate PD92/1471 (AY563995), Pff isolate A2 (AY564004), Pff isolate NS4 (AY564005), Pfr isolate FVR67 (AY564006), and Pfr isolate FVR30 (AY564007) (B).

The presence of single alleles in all the *Pau* isolates and the low level of polymorphism observed suggest that this subspecies is probably close to homozygosity for the nuclear genes we studied. These data are consistent with this subspecies being near-diploid and with its high phenotypic and genetic uniformity (Brasier et al., 1999).

By contrast, two significantly divergent alleles (PAM1 and PAM2) of the four nuclear genes were observed in all the *Pam* isolates we studied. If, by extension, two alleles of most of the nuclear genes are present in *Pam,* then this subspecies should normally be near-tetraploid, which would be in disagreement with the ploidy levels (2n + 4 to 2n + 7) determined with the acetoorcein method by Brasier et al. (1999). A first explanation is that *Pam* is homoploid, i.e., near-diploid for the genus *Phytophthora*, but heterozygous with two alleles, PAM1 and PAM2, as diploid orthologs, probably on homolog chromosomes. As a result, *Pam* could be a cryptic polyploid hybrid species that regained a level of ploidy close to diploidy. However, *Pam* is homothallic and constant selfing should normally drive homothallic species to a homozygous state (Goodwin, 1997). Nevertheless, despite the homothallic nature of *Pam,* germinating oospores were not observed for any of the *P. alni* subspecies during an in vitro study (Delcan and Brasier, 2001) and, therefore, these species could be reproducing only asexually, which would maintain a high level of heterozygosity. A second

hypothesis would be that the occurrence of two alleles of the studied nuclear genes in *Pam* has been generated by autopolyploidization. Indeed, the studied nuclear genes are not physically linked since they are located on different scaffolds in the *P. sojae* or P. ramorum genomes (Table 2). Therefore, the occurrence of multiple polymorphic sequences might not have arisen by gene duplication, generating paralogs, because the simultaneous duplication of four physically unlinked genes seems very unlikely. Finally, it cannot be inferred from our data whether or not the presence of two alleles of the four studied nuclear genes arose from an ancient autopolyploidization generating series of homologous alleles in a *Pam* ancestor, followed by divergent evolution between the two respective genes, or was generated by an ancient reticulation event such as interspecific hybridization.

The presence of three alleles in *Paa* and the level of divergence between their respective sequences, as high as the level of divergence between the ortholog sequences for each of the studied genes in *P. cambivora* and *P. fragariae*, are consistent with *Paa* being allopolyploid, as demonstrated by Brasier et al. (1999). Indeed, intraspecific polyploidization might not have generated three such divergent alleles and, therefore, the occurrence of multiple alleles in *Paa* would be the result of at least one reticulation. The three alleles are probably located on homoelogue chromosomes, following a reticulation event. Overall, the data from the nuclear gene are in full agreement with previous research using isozyme analysis. Nagy et al. (2003), as well as Brasier et al. (2004), revealed a trisomic state at the glucose-6-phosphate isomerase (Gpi) locus for *Paa,* which is consistent with the co-occurrence of three different alleles of the four nuclear genes. Likewise, heterozygosity at the Gpi locus for *Pam* and homozygosity at three different enzyme loci, including Gpi, for *Pau* (Brasier et al., 2004), are consistent with our findings of two and single alleles for *Pam* and *Pau,* respectively, for the four nuclear genes.

This multilocus analysis conducted on a Europe-wide collection of hybrids showed that the genome of the parental species has been conserved to a high degree in *Paa* and Pam. Thus, the co-existence of two and three alleles of most of the nuclear genes, probably located on respective homoelogue chromosomes, may be hypothesized. This co-existence does not mean that each allele is actually expressed, but may be an explanation why *Pam* and *Paa* are reported to be phenotypically more unstable than *Pau* (Delcan and Brasier, 2001; De Merlier et al., 2005), in which only single alleles were found. *Paa* is the most frequent subspecies in Europe at this time (Brasier, 2003; Ioos, unpublished results) and seems to propagate essentially by vegetative reproduction through the dissemination of zoospores. This subspecies cannot diversify by outcrossing, nor can it use meiosis to mitigate the accumulation of deleterious mutations known as Muller's ratchet (Muller, 1964). However, genetic redundancy in this hybrid and in *Pam,* to a lesser extent, might mask some deleterious mutations and may also enable the hybrid to be more fit in its ecological niche, i.e., to maintain itself as an aggressive pathogen on alder. For example, this type of selective advantage of hybrid species was demonstrated for a fungal endophyte species, *Neotyphodium coenophialum*, which also has three ancestors involved in two separate hybridization events (Moon et al., 2004). Based on the allelic distribution of the studied nuclear genes, it appears that (i) either the *Paa* genome contributed to the genomes of *Pam* and *Pau* by descent, or (ii) conversely, that *Pam* and *Pau* contributed to the genome of *Paa* by hybridization.

4.2. Dichotomous mitochondrial pattern within *P. alni* and relationship between the three subspecies

The mitochondrial DNA features combined with the different nuclear allelic patterns found indicate that *Pau* and *Pam* might not have arisen from the genetic breakdown of *Paa,* as previously hypothesized by Delcan and Brasier (2001). Indeed, *Paa* isolates have all three different alleles of the nuclear genes we studied, and all display either an M/M'/M'' or a U/U' mtDNA pattern. On the other hand, all the *Pam* isolates possess two of these alleles (PAM1 and PAM2) and display two closely related mtDNA patterns, M or M', whereas all the *Pau* isolates possess single alleles (PAU), and display three closely related mtDNA patterns, U,

U', or U''. If *Pam* and *Pau* had originated from *Paa* through segregation, then other combinations of nuclear and mitochondrial DNA would be expected, which has not been the case so far.

The mitochondrial DNA analysis showed that all the *Paa* isolates tested displayed either a pattern identical to *Pam* isolates (M/M'/M'') or to *Pau* isolates (U/U'). The two mitotypes are present throughout Europe with no obvious geographical or sampling time pattern (Table 1). Moreover, further analyses carried out on additional French *Paa* isolates showed that the mitotypes M' and U' were also present in France (Ioos, unpublished data). Furthermore, the sequencing of the cox1 and nadh1 mitochondrial genes demonstrated the occurrence of a unique mitotype within *P. alni* isolates. Moreover, the sequences of individual *Paa* isolates, i.e., PAA129 or PAA130, cluster either with those of *Pau* (PAU60) or with those of *Pam* (PAM54), respectively, which is therefore fully consistent with the dichotomous distribution of the mtDNA patterns resolved by RFLP (see Table 1).

Some of the mtDNA RFLP patterns obtained with HaeIII were identical to those obtained by Nagy et al. (2003) on the same subspecies; however, we obtained more patterns than previously described because we screened a broader collection of isolates. The occurrence of several closely related mtDNA patterns within each subspecies of P. alni, i.e., M, M', and M'', on the one hand, and U, U', and U'', on the other hand, is not surprising. Indeed, these patterns only differed by the size of one or two restriction fragments, which could be explained by insertion–deletion events and seems consistent with the level of intraspecific polymorphism already observed in other species such as *P. infestans* (Goodwin, 1991) or P. parasitica (Lacourt et al., 1994). By contrast, the two mitotype groups, M/M'/M'' and U/U'/U'', differed from each other by at least six restriction fragments for each enzyme, indicating a large divergence between the two mitotype groups.

Finally, the co-segregation of mtDNA along with nuclear patterns could also be explained either by linkage between mtDNA and the nuclear genes studied or by a poorer viability of the "missing" hybrids combining U/U'/U'' mtDNA and PAM1/PAM2 nuclear DNA or M/M'/M'' mtDNA and *Pau* nuclear DNA. However, linkage between mtDNA and four physically unrelated nuclear genes seems very unlikely, whereas poorer viability of the "missing" hybrids remains possible but difficult to demonstrate experimentally. Whittaker et al. (1994) proved that mtDNA was uniparentally inherited from A1 x A2 matings in *P. infestans* and that there was no evidence for biparental inheritance, recombination or segregation of mitotypes, regardless of the ages of the crossings. Later, Man in't Veld et al. (1998) demonstrated that only mtDNA from *P. nicotianae* was present in the natural hybrid *P. nicotianae* x *P. cactorum* isolates. Accordingly, we favor the most parsimonious hypothesis that *Paa* may have arisen via hybridization of two taxa close to *Pau* and *Pam,* if not *Pau* and *Pam* themselves. This hypothesis is well supported by other investigations conducted on a series of elicitin genes, which belong to a multigenic family encoding polypeptides specific to the genus *Phytophthora* and a few Pythium species (Ioos et al., in preparation). Moreover, since the mitochondrial DNA is only uniparentally inherited following crossing, this hypothesis might explain why *Paa* isolates display different mitochondrial DNA patterns, whereas they all possess the same three alleles for each of the four nuclear genes. From this point of view, sexual, rather than somatic, hybridization is more likely to have occurred.

Furthermore, the occurrence of significantly different mtDNA patterns among *Paa* isolates and the fact that these patterns are shared either with *Pau* or *Pam* also implies that different hybridization events might have occurred. Moreover, the uniparental inheritance of mtDNA and the occurrence of two groups of mitotypes in *Paa* suggest that the hybridization events may have occurred in both directions. Furthermore, different mtDNA patterns combined with intraspecific variation were observed among the *Paa* isolates that we gathered from different isolation dates. This suggests that the spread of this subspecies, within different countries and throughout Europe, might not be attributable to a single clone, as hypothesized by Brasier et al. (1995) and Nagy et al. (2003). The occurrence of multiple

hybridization events has already been demonstrated for other natural *Phytophthora* hybrids between *P. nicotianae* and *P. cactorum*, probably generated in hydroponic systems of greenhouses in the Netherlands (Bonants et al., 2000).

Whether or not *Pau* and *Pam* are the direct progenitors of *Paa* cannot be inferred from our nuclear and mitochondrial investigations. *Pam* and *Pau* are far less frequently isolated from alder lesions than *Paa,* regardless of their geographical origins (Brasier et al., 2004) and have proved to be significantly less aggressive on alder bark than *Paa* (Brasier and Kirk, 2001). One of the first *Pam* isolates sampled in the Netherlands was found in 1995 in soil in a natural alder stand where none of the alders showed any symptoms of *Phytophthora* disease (Streito, 2003), while Santini et al. (2003) reported the isolation of *Pau* from asymptomatic alder seedlings. Therefore, *Pam* and *Pau,* or *Pam*- and *Pau*-like species, might have existed for a long time on—or in the vicinity of— alder trees before the recent emergence of large-scale decay in the European alder population. The occurrence of these species in the past might not have been noticed because of the lack of conspicuous symptoms or declines of whole trees.

Considering the wide extant hybrid zone where the different subspecies of *P. alni* disseminate, the introgression may presumably continue through further reticulation events or backcrossings. Despite the fact that the *Paa, Pam,* and *Pau* isolates we tested only displayed a limited level of intraspecific nuclear polymorphism, the occurrence of new genotypes cannot be ruled out as a possibility. For instance, Jung and Blaschke (2004) and Brasier et al. (2004) reported the occurrence of additional major variants of *Pam* and minor variants of *Pau,* presumed to have been generated either by backcrossing or reassortments of the hybrid genome.

4.3. Relationship between the closely related *P. alni*, *P. cambivora*, and *P. fragariae*

Since no allele present in the two varieties of *P. fragariae* for the four nuclear and the two mitochondrial genes we studied was observed in any *P. alni* subspecies, our results confirm that this species is not among the parental species of P. alni, as previously suggested by Brasier (2003). Suggested to be one of the progenitors of *P. alni* by Brasier et al. (1999, 2004), *P. cambivora* was included in this study in order to unravel the relationship between the three subspecies of P. alni, only pathogenic on alder, and *P. cambivora*, pathogenic on numerous deciduous trees, but not on alder (Brasier and Kirk, 2001; Santini et al., 2003). Our results do not support the hypothesis that *P. cambivora* is directly involved as a parental species for P. alni. Although *P. cambivora* is undoubtedly close to *Pau* and *Paa,* with at least one allele of *P. cambivora* close to the allele shared by *Paa* and *Pau* for the four nuclear genes, there is never complete identity. Since the hybrid is believed to be in its nascent state (Brasier et al., 1999), one might expect to find alleles of the parental species for each nuclear gene in the hybrid genome. The series of *Pau* allele-specific primers designed in this study yielded a PCR product when tested with several isolates of *P. cambivora*. Nevertheless, sequencing of genes showed that within the *Pau* cluster, polymorphism occurred between the target regions of the two primers. In particular, *P. cambivora* sequences could be differentiated from *P. alni* sequences by several substitutions but, at the same time, could not be discriminated by *Pau*-specific PCRs. Therefore, our sequence data suggest that *P. cambivora* may not be directly involved as a parental species but it could still be one parental species of an ancient *Pau*-like species that has evolved. Nevertheless, considering the unexpected genetic variability among French isolates of *P. cambivora*, the involvement of another particular genotype of *P. cambivora*, either exotic or endemic to Europe, cannot be ruled out as a possibility. Further sequencing studies using the allele-specific primers designed in this study with a broader collection of *P. cambivora* isolates could be of great interest in order to validate such a hypothesis. *P. cambivora* isolates exhibited more alleles than expected for a typical diploid species, for example, with up to four different alleles of the ASF-like gene and two significantly different alleles of the RAS-Ypt gene. This is in agreement with previous observations based on

ribosomal DNA where ITS polymorphisms were revealed within individual isolates (Brasier et al., 1999; Cooke and Duncan, 1997) and suggests that independent gene duplications generating paralogs might have occurred for that species and/or, that *P. cambivora* might have been involved in reticulation events in the past.

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