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SCAR–based PCR primers to detect the hybrid pathogen *Phytophthora alni* and its subspecies causing alder disease in Europe

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Abstract

Since the 1990s, a new *Phytophthora* species hybrid has been jeopardizing the natural population of alders throughout Europe. This new *Phytophthora, P. alni*, has been suggested as a natural hybrid between two closely related species of *Phytophthora*. Little is known about the epidemiology of this pathogen, because its direct isolation is not always satisfactory. In this study we developed three pairs of Polymerase Chain Reaction (PCR) primers derived from Sequence Characterized Amplified Regions (SCAR) that allow discrimination among the three subspecies of *P. alni*: *P. alni* subsp. *alni, P. alni* subsp. *uniformis* and *P. alni* subsp. *multiformis*. These molecular tools were successfully used to detect *P. alni* directly in different substrates such as infested river water and soil, and necrotic alder bark, without the need for any prior baiting or isolation stages. An Internal Amplification Control (IAC) was included to help discriminate against false negative samples due to the potential presence of inhibitory compounds in DNA extracts. These molecular tools should be useful for epidemiological studies on this emerging disease.

Introduction

At the beginning of the 1990s, a new destructive and lethal disease of Alder (*Alnus* spp.) was described in Great Britain in riparian populations as well as horticultural shelterbelts (Gibbs et al., 1994). The disease exhibited characteristic symptoms: thinning of the crown, sometimes with abnormally small, sparse and yellowish leaves and tarry or rusty exudations on the stems. These external symptoms were consequences of the destruction of strips of inner bark and/or roots necrosis (Gibbs et al., 1994; Gibbs, 1995). The disease has since been described throughout Europe and has had a particularly destructive impact in Great Britain, but is also found in France, Belgium and Germany where it represents an increasing threat to natural riparian alder populations (Brasier et al., 1995; Gibbs, 1995; Streito et al., 2002a; Gibbs et al., 2003; Jung and Blaschke, 2004). The disease was initially shown to be caused by a previously unknown *Phytophthora* sp. resembling *P. cambivora* (Gibbs et al., 1994; Brasier et al., 1995). Further investigations led Brasier et al. (1999) to hypothesize that the *Phytophthora* involved was a natural hybrid between *P. cambivora* and another unknown taxon of *Phytophthora* close to *P. fragariae*. According to cultural features, cytological evidence, ITS sequences and genomic DNA fingerprinting, Brasier et al. (1999) also showed that the alder *Phytophthora* consisted of a range of heteroploid species hybrids. These can be divided into a 'standard' type and

several variants, all pathogenic to the different species of European alder. The standard type is nearly tetraploid (4n + 2, n = 18–22 chromosomes) and exhibits an unusual ITS polymorphism, i.e. dimorphic sites within ITS sequences for a single isolate. On the other hand, the respective ploidy for the different variants ranges from 2n + 2 for the Swedish variant to 2n + 7 for the German variant. In contrast to the standard type, the variants show a nearly homogenous ITS sequence. The ITS sequence for the Dutch, German and UK variants only differs from *P. fragariae* by a few bases whereas the ITS sequence for the Swedish variant is very close to the *P. cambivora* sequence (Brasier et al., 1999). The respective aggressiveness of the different types of alder *Phytophthora* are slightly different (Brasier and Kirk, 2001; Santini et al., 2003) but these hybrids are the only known *Phytophthora* species to be pathogenic to alder. In contrast, these hybrids are not pathogenic to other woody hosts such as *Quercus*, *Acer* or *Fagus* (Brasier and Kirk, 2001). Recently, Brasier et al. (2004) formally named these different types of alder *Phytophthora* as *P. alni*. Moreover, according to extensive morphological, cytological and genetic data, Brasier et al. (2004) have split *P. alni* into three subspecies: *P. alni* subsp. *alni* corresponding to the standard type, *P. alni* subsp. *uniformis* corresponding to the Swedish variant type and *P. alni* subsp. *multiformis* including the UK, German and Dutch variant types.

Recent studies have demonstrated that the pathogens were able to disseminate along rivers by producing large quantities of waterborne zoospores (Streito et al., 2002a, b) and could also be brought from infected areas to an initially healthy area by planting infected alder plants (Jung and Blaschke, 2004). However, the epidemiology as well as the aetiology of this new disease are still unclear, perhaps because direct isolation requires a certain level of technical skill and experience in correctly recognizing the symptoms and the hyphae produced by the pathogen. In addition, Streito et al. (2002b) and Streito (2003) reported that the efficiency of classical detection techniques such as direct isolation or baiting with this Oomycete could be poor. Moreover, hybrid fungi and hybrid Oomycetes are unlikely to be identified or detected by conventional methods which are mainly based on morphology, as their features are close or identical to those of the parental species (Brasier, 2001). During the last decade, molecular markers have proven to be useful for species-specific detection of plant pathogens. Generally, based on a Polymerase Chain Reaction (PCR), reliable and accurate diagnostic tests are now widely used, especially for economically important plant pathogens such as quarantine listed fungi (Bonants et al., 1997, 2003; Ioos and Frey, 2000). Nevertheless, Internal Transcribed Spacers (ITS)-based PCR techniques, despite being used very frequently for species discrimination, are not appropriate in the case of *P. alni*. Indeed, the ITS homology between the putative parental species and the variant types, on the one hand, and the running ITS sequence rearrangement for the standard type, on the other, make these regions inappropriate for diagnostic purposes.

Alternative strategies were developed for the design of species or strain-specific markers using randomly selected sequences (Wiglesworth et al., 1994; Boehm et al., 2001). Sequence Characterized Amplified Regions (SCAR) can be selected from RAPD (Random Amplified Polymorphic DNA) fingerprints and used to mark specific alleles (Paran and Michelmore, 1993) or generate species-specific PCR markers (Schilling et al., 1996; Schubert et al., 1999).

The aims of our study were first to generate SCARs from RAPDs carried out on a panel of different subspecies of *P. alni* and closely related species, i.e. *P. cambivora* and *P. fragariae*, in order to find markers that could be specific to the hybrids. Secondly, we designed sets of PCR primers that exhibit a complementary range of specificity, including a universal primer pair that enables the specific detection of the different subspecies of *P. alni* in various substrates. The distinction of the different subspecies might be useful for epidemiological purposes as, in contrast to *P. alni* subsp. *alni*, *P. alni* subsp. *multiformis* and *P. alni* subsp. *uniformis* were shown to be able to complete meiosis, despite the fact that no germination of the resulting oospores has ever been observed during *in vitro* studies (Delcan and Brasier, 2001).

Materials and methods

Cultures of Oomycetes

French isolates of *P. alni* or *Phytophthora* spp. were obtained by isolation from naturally infected

tissues on PARPHY medium (Robin et al., 1998). Foreign isolates of *P. alni* and *Phytophthora* spp. were obtained from CBS (Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands) or from collaborative researchers (Table 1). Assignation 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. (1995) and restriction patterns of the ITS region using a series of enzymes, according to Brasier et al. (1999) and Cooke et al. (2000) (data not shown). All the cultures were kept at 10 °C in the dark on V8 agar slants (Miller, 1955) and as small V8 agar blocks flooded with sterile distilled water (SDW). Oomycete DNA was extracted from 5-day-old cultures grown in shaken liquid V8-juice medium (Miller, 1955) at 20 °C.

Zoospore production

The zoospores used in this study were produced by an isolate of *P. alni* subsp. *alni* (1429-6b) isolated from a bark necrosis on alder in France. Sporangia were produced by incubating 20 plugs of active margin culture of *P. alni* subsp. *alni* isolate 1429-6b for 48 h in the dark in 25 ml of pond water previously filtered through a 47 mm dia 5 μm pore Durapore® membrane (Millipore, Molsheim, France). Then, agar plugs bearing numerous sporangia were carefully rinsed with SDW and transferred into a sterile Petri dish. To release zoospores, 30 ml of pre-chilled pond water previously filtered through a 0.2 μm pore cellulose acetate filter were added and the plugs were incubated for 2 h under fluorescent light at 20 °C. The zoospore suspension was filtered through a 45 μm sieve to remove mycelium and agar plugs. One millilitre of the initial suspension was thoroughly vortexed for 2 min in a microcentrifuge tube to encyst zoospores in order to facilitate counting. The initial concentration of zoospores was determined by using a haemocytometer under a microscope at 250× magnification. First, 50 ml of river water were filtered through a 100 μm mesh sieve to eliminate large debris. The filtrate was subsequently filtered through a 47 mm dia Durapore® membrane with 5 μm pores. An initial suspension of 50×10^3 zoospores ml^{-1} was diluted by aliquots of 50 ml of filtered river water in order to obtain different quantities of zoospores. We tested six series of 50 ml of artificially contaminated river water containing from 1.5×10^6 to 30 zoospores.

DNA extraction from Oomycete and fungal cultures

DNA was extracted using a plant DNA extraction kit (DNeasy plant mini kit®, Qiagen, Courtaboeuf, France) following the manufacturer's instructions with slight modifications. For pure Oomycete culture, 200 mg of fresh mycelium was harvested and mixed in a 2 ml tube with 400 μl of lysis buffer and 4 μl of the RNase provided. The mixture was ground for 2 min with two 3 mm tungsten carbide beads at a frequency of 30 Hz, using a mixermill grinder (Tissuelyser® Qiagen, Courtaboeuf, France). The ground solution was subsequently centrifuged for 5 min at 14,000 rpm to compact the debris and the supernatant was treated following the manufacturer's instructions. DNA concentrations were estimated using a spectrophotometer (BioPhotometer®, Eppendorf, Le Pecq, France)

DNA extraction from lignified woody tissues, soil and water

For DNA extraction from woody tissues, thin wood shavings were taken from symptomatic tissues (bark necrosis) using a sterile scalpel blade. The shavings were transferred to a sterile 2 ml microcentrifuge tube with 500 μl of DNeasy® lysis buffer, 500 μl of powdered skimmed milk (0.2 g/25 ml distilled water) and 4 μl of the RNase provided by the manufacturer. The sample was ground with two 3 mm tungsten carbide beads and DNA was extracted as described above. For soil DNA extraction, about 1 g of sampled soil was transferred to a 2 ml centrifuge tube and DNA was extracted as described above for woody tissues. Water DNA was extracted following a protocol derived from Kong et al. (2003) with slight modifications. Fifty millilitres of river water artificially inoculated with *P. alni* subsp. *alni* zoospores was filtered through a 47 mm dia Durapore® membrane with 5 μm pores. The membrane was removed carefully from the filtering unit and cut into pieces of approximately 0.25 cm^2 using sterile forceps and scissors. All the pieces were transferred to a sterile 2 ml microcentrifuge

Table 1. Polymerase chain reaction amplification of DNA from isolates of different subspecies of *Phytophthora alni* recovered from different geographic locations, isolates of different species from the genus *Phytophthora* and *Pythium* and isolates of fungi commonly recovered from alder bark necrosis, using the three *P. alni* primer pairs designed in this study

Species	Code	Host	Geographical origin	Year	Isolator /supplier	PA-F/R	PAM-F/R	PAU-F/R	ITS1–ITS4 or ITS6–ITS4*
P. alni subsp.*alni*	2N0685	*Alnus glutinosa*	France	2002	J.C. Streito	+	+	+	+
	71T1	*Alnus glutinosa*	France	1997	J.C. Streito	+	+	+	+
	77T4	*Alnus glutinosa*	France	1997	J.C. Streito	+	+	+	+
	82T1A	*Alnus glutinosa*	France	1997	J.C. Streito	+	+	+	+
	84T2	*Alnus glutinosa*	France	1997	J.C. Streito	+	+	+	+
	9900715.6	*Alnus glutinosa*	Belgium	1999	J.C. Streito	+	+	+	+
	98-7-5	*Alnus glutinosa*	France	1998	J.C. Streito	+	+	+	+
	98-7-6	*Alnus glutinosa*	France	1998	J.C. Streito	+	+	+	+
	2N0529	*Alnus glutinosa*	France	2002	J.C. Streito	+	+	+	+
	DSFO98172	*Alnus glutinosa*	France	1998	J.C. Streito	+	+	+	+
	AUL026/1	*Alnus glutinosa*	France	1999	J.C. Streito	+	+	+	+
	9900783.4	*Alnus glutinosa*	France	1999	J.C. Streito	+	+	+	+
	1R0152	*Alnus glutinosa*	France	2001	J.C. Streito	+	+	+	+
	1N0201	*Alnus glutinosa*	France	2001	J.C. Streito	+	+	+	+
	9500802	*Alnus glutinosa*	France	1995	J.C. Streito	+	+	+	+
	PD2010953	*Alnus* sp.	The Netherlands	ND	W. Man in't Veld	+	+	+	+
	P1275	*Alnus glutinosa*	Scotland	2000	G. Mackaskill	+	+	+	+
	P1272	*Alnus viridis*	Scotland	2000	J. Gibbs	+	+	+	+
	P1271	*Alnus glutinosa*	Scotland	2000	J. Gibbs	+	+	+	+
	P1270	*Alnus glutinosa*	Scotland	2000	J. Delcan	+	+	+	+
	P1960	*Alnus glutinosa*	England	1997	J. Delcan	+	+	+	+
	P957[a]	*Alnus glutinosa*	England	1997	J. Delcan	+	+	+	+
	P950[a]	*Alnus glutinosa*	England	1997	J. Delcan	+	+	+	+
	P937	*Alnus glutinosa*	England	1997	J. Delcan	+	+	+	+
	P850	*Alnus glutinosa*	England	1996	S. Gregory	+	+	+	+
	P834[e]	*Alnus glutinosa*	England	ND	C. Brasier	+	+	+	+
	2198[c]	*Alnus glutinosa*	Belgium	1999	D. De Merlier	+	+	+	+
	2295[c]	*Alnus glutinosa*	Belgium	2001	D. De Merlier	+	+	+	+
	6[d]	*Alnus glutinosa*	Hungary	2001	Z. Nagy	+	+	+	+
	8[d]	*A. glutinosa* soil	Hungary	2001	Z. Nagy	+	+	+	+
	9[d]	*A. glutinosa* soil	Hungary	2001	Z. Nagy	+	+	+	+
	1a[d]	*A. glutinosa* soil	Hungary	2001	Z. Nagy	+	+	+	+
	4-2[d]	*Alnus glutinosa*	Hungary	2001	Z. Nagy	+	+	+	+
	P1bisa	*Alnus glutinosa*	France	2003	R. Ioos	+	+	+	+
	P3a	*Alnus glutinosa*	France	2003	R. Ioos	+	+	+	+
	Priva	*Alnus glutinosa*	France	2003	R. Ioos	+	+	+	+
	Privb	*Alnus glutinosa*	France	2003	R. Ioos	+	+	+	+
	P6-2	*Alnus glutinosa*	France	2003	R. Ioos	+	+	+	+
	P6-1	*Alnus glutinosa*	France	2003	R. Ioos	+	+	+	+
	Ainvelle Sol	*A. glutinosa* soil	France	2003	C. Husson	+	+	+	+
	2ALD03	*Alnus glutinosa*	France	2003	C. Husson	+	+	+	+
	102-1	*Alnus glutinosa*	France	2003	C. Husson	+	+	+	+
	Moselle	*Alnus glutinosa*	France	2002	C. Husson	+	+	+	+
	370-2	*Alnus glutinosa*	France	2002	C. Husson	+	+	+	+
	3N10094-5a	*Alnus glutinosa*	France	2003	R. Ioos	+	+	+	+
	3N10094-5c	*Alnus glutinosa*	France	2003	R. Ioos	+	+	+	+
	3N10048-3a	*Alnus glutinosa*	France	2003	R. Ioos	+	+	+	+
	3N10048-3b	*Alnus glutinosa*	France	2003	R. Ioos	+	+	+	+
	3N10048-3f	*Alnus glutinosa*	France	2003	R. Ioos	+	+	+	+
	Ainvelle4-4	*Alnus glutinosa*	France	2003	C. Husson	+	+	+	+
	Ainvelle1-2	*Alnus glutinosa*	France	2003	C. Husson	+	+	+	+
	Ainvelle1-1	*Alnus glutinosa*	France	2003	C. Husson	+	+	+	+
	703	*Alnus glutinosa*	France	2003	G. Capron	+	+	+	+
	1429-6b	*Alnus glutinosa*	France	2003	R. Ioos	+	+	+	+
	Sol A15	*A. glutinosa* soil	France	2003	C. Husson	+	+	+	+
	Sol A1	*A. glutinosa* soil	France	2003	C. Husson	+	+	+	+
	Sol A7	*A. glutinosa* soil	France	2003	C. Husson	+	+	+	+

Table 1. (Continued).

Species	Code	Host	Geographical origin	Year	Isolator /supplier	PA-F/R	PAM-F/R	PAU-F/R	ITS1–ITS4 or ITS6–ITS4*
	BBA 23/00	*Alnus glutinosa*	Germany	2000	K. Kaminski	+	+	+	+
	PO 192	*Alnus glutinosa*	Poland	ND	G. Skuta	+	+	+	+
	PO 193	*Alnus glutinosa*	Poland	ND	G. Skuta	+	+	+	+
	PO 203	*Alnus glutinosa*	Poland	ND	G. Skuta	+	+	+	+
	PO 205	*Alnus glutinosa*	Poland	ND	G. Skuta	+	+	+	+
	Pucking B10	*Alnus glutinosa*	Austria	ND	T. Cech	+	+	+	+
P. alni subsp. *uniformis*	AUL028	*Alnus glutinosa*	France	1999	J.C. Streito	+	–	+	+
	155-a[d]	*Alnus glutinosa*	Hungary	1999	Z. Nagy	+	–	+	+
	155-b[d]	*A. glutinosa* soil	Hungary	1999	Z. Nagy	+	–	+	+
	155-c[d]	*A. glutinosa* soil	Hungary	1999	Z. Nagy	+	–	+	+
	CBS109280[e]	*Alnus cordata*	Italy	ND	P. Capretti	+	–	+	+
	P875[a,b,c,f]	*Alnus glutinosa*	Sweden	ND	C. Olsson	+	–	+	+
	2271[c]	*Alnus glutinosa*	Belgium	2001	D. De Merlier	+	–	+	+
	Phy-A-Slo	*Alnus glutinosa*	Slovenia	2003	A. Munda	+	–	+	+
P. alni subsp. *multiformis*	W1139	*Alnus* sp.	The Netherlands	ND	W. Man in't Veld	+	+	–	+
	P972[a,c,f]	*Alnus* sp.	The Netherlands	ND	W. Man in't Veld	+	+	–	+
	P841[a,c,f]	*Alnus glutinosa*	UK	1996	S. Gregory	+	+	–	+
	DSFO/0125	*Alnus glutinosa*	France	2000	J.C. Streito	+	+	–	+
P. cambivora	463	*Castanea sativa*	France	ND	INRA Bordeaux	–	–	–	+
P. cambivora	643	*C. sativa* soil	France	ND	INRA Bordeaux	–	–	–	+
P. cambivora	JC17	*Quercus* sp. soil	France	ND	C. Delatour	–	–	–	+
P. cambivora	GA1	*Quercus* sp. soil	France	ND	C. Delatour	–	–	–	+
P. cambivora	99428	*Castanea sativa*	France	ND	R. Ioos	–	–	–	+
P. cambivora	ST3R1	*Quercus petraea*	France	ND	C. Delatour	–	–	–	+
P. cambivora	627	ND	France	ND	INRA Bordeaux	–	–	–	+
P. cambivora	1A21	*Quercus* sp. soil	France	ND	INRA Bordeaux	–	–	–	+
P. fragariae var. *fragariae*	1	*Fragaria x ananassa*	ND	ND	K. Hughes	–	–	–	+
P. fragariae var. *fragariae*	209.46	*Fragaria x ananassa*	ND	ND	CBS	–	–	–	+
P. fragariae var. *fragariae*	309.62	*Fragaria x ananassa*	ND	ND	CBS	–	–	–	+
P. fragariae var. *rubi*	FVR 59	*Rubus* sp.	UK	ND	D. Cooke	–	–	–	+
P. fragariae var. *rubi*	163-2	*Rubus* sp.	France	ND	A. Baudry	–	–	–	+
P. fragariae var. *rubi*	2	*Rubus* sp.	UK	ND	K. Hughes	–	–	–	+
P. fragariae var. *rubi*	967.95	*Rubus* sp.	UK	ND	CBS	–	–	–	+
P. fragariae var. *rubi*	109.892	*Rubus* sp.	UK	ND	CBS	–	–	–	+
P. cactorum	CAC4810/TJ	ND	France	ND	C. Delatour	–	–	–	+
P. cinnamomi	DSFO2N0964	*Castanea sativa*	France	ND	J.C. Streito	–	–	–	+
P. cinnamomi	DSFA970060	*Quercus suber*	France	ND	J.C. Streito	–	–	–	+
P. cinnamomi	DSFO990050	*C. sativa* soil	France	ND	J.C. Streito	–	–	–	+
P. cinnamomi	P382	*Nothofagus procera* soil	UK	ND	C. Brasier	–	–	–	+
P. citricola	2N0750-171	ND	France	ND	J.C. Streito	–	–	–	+
P. citricola	AUL 045 AP7	*Alnus glutinosa*	France	ND	J.C. Streito	–	–	–	+
P. citricola	2AE5	*Quercus* sp. soil	France	ND	C. Delatour	–	–	–	+
P. citricola	3N1345-17	*Alnus glutinosa*	France	ND	R. Ioos	–	–	–	+
P. citrophthora	2N1021	*Rosa* sp.	France	ND	J.C. Streito	–	–	–	+
P. cryptogea	990675	*Actinidia chinensis*	France	ND	J.C. Streito	–	–	–	+
P. erythroseptica	960713	*Polygonum oberti*	France	ND	J.C. Streito	–	–	–	+
P. europaea	AL5	*Quercus* sp. soil	France	ND	C. Delatour	–	–	–	+
P. europaea	2AU2	*Quercus* sp. soil	France	ND	C. Delatour	–	–	–	+
P. gonapodyides	Gonap 4	*Quercus* sp. soil	France	ND	C. Delatour	–	–	–	+
P. gonapodyides	AB4	*Quercus* sp. soil	France	ND	C. Delatour	–	–	–	+
P. humicola	3N1245-j	*A. glutinosa* soil	France	ND	R. Ioos	–	–	–	+
P. ilicis	3N1245-l	*A. glutinosa* soil	France	ND	R. Ioos	–	–	–	+
P. inundata	9500802	*A. glutinosa* soil	France	ND	J.C. Streito	–	–	–	+
P. lateralis	98093.1-SPV	*Chamaecyparis* sp.	France	ND	J.C. Streito	–	–	–	+
P. megasperma	3N1245-m	*A. glutinosa* soil	France	ND	R. Ioos	–	–	–	+
P. megasperma	BK1	*Quercus* sp. soil	France	ND	C. Delatour	–	–	–	+
P. megasperma	03-12	water under *Quercus* sp.	France	ND	C. Delatour	–	–	–	+
P. megasperma	mega 1	ND	Germany	ND	T. Jung	–	–	–	+

Table 1. (Continued).

Species	Code	Host	Geographical origin	Year	Isolator /supplier	PA-F/R	PAM-F/R	PAU-F/R	ITS1–ITS4 or ITS6–ITS4*
P. megasperma	8RPOC3	*Quercus* sp. soil	France	ND	C. Delatour	–	–	–	+
P. nicotianae	960579	*Nicotiana tabacum*	France	ND	J.C. Streito	–	–	–	+
P. taxon forestsoil	8CARPPOC1	*Quercus* sp. soil	France	ND	C. Delatour	–	–	–	+
P. palmivora	970423	*Hedera* sp.	France	ND	J.C. Streito	–	–	–	+
P. parasitica	970029	*Lycopersicon esculentum*	France	ND	J.C. Streito	–	–	–	+
P. taxonPgchlamydo	Haye,3,1	*Quercus* sp. soil	France	ND	C. Delatour	–	–	–	+
P. pseudosyringae	EW5	*Quercus* sp. soil	France	ND	C. Delatour	–	–	–	+
P. psychrophila	FF20	*Quercus* sp. soil	France	ND	C. Delatour	–	–	–	+
P. quercina	FNA	*Quercus* sp. soil	France	ND	C. Delatour	–	–	–	+
P. quercina	Mers2	*Quercus* sp. soil	France	ND	C. Delatour	–	–	–	+
P. ramorum	2N0983	*Rhododendron* sp.	France	ND	C. Saurat	–	–	–	+
P. ramorum	3N0003	*Viburnum* sp.	France	ND	C. Saurat	–	–	–	+
P. sojae	443	*Glycine max*	No	ND	F. Panabières	–	–	–	+
P. syringae	2JZ2	*Quercus* sp. soil	France	ND	C. Delatour	–	–	–	+
Pythium aphanidermatum	Ctsa	*A. glutinosa* soil	France	ND	R. Ioos	–	–	–	+
Pythium sylvaticum	0675/a	*A. glutinosa* soil	France	ND	R. Ioos	–	–	–	+
Pythium intermedium	02/84/1	ND	France	ND	S. Verger	–	–	–	+
Pythium irregulare	02/57/1	ND	France	ND	S. Verger	–	–	–	+
Pythium ultimum	433/3	ND	France	ND	S. Verger	–	–	–	+
Pythium sp.	3N1345-11	*A. glutinosa* soil	France	ND	R. Ioos	–	–	–	+
Botryosphaeria obtusa	467a	*Alnus glutinosa*	France	2003	R. Ioos	–	–	–	+
Trichoderma harzanium	1790a	*Alnus glutinosa*	France	2003	R. Ioos	–	–	–	+
Fusarium avenaceum	1790b	*Alnus glutinosa*	France	2003	R. Ioos	–	–	–	+
Microsphaerosis olivaceae	467b	*Alnus glutinosa*	France	2003	R. Ioos	–	–	–	+
Phoma sp.	1790c	*Alnus glutinosa*	France	2003	R. Ioos	–	–	–	+
Epicoccum nigrum	1790	*Alnus glutinosa*	France	2003	R. Ioos	–	–	–	+
Fusarium sporotrichioides	P1bis1	*Alnus glutinosa*	France	2003	R. Ioos	–	–	–	+
Aspergillus sp.	Priv1	*Alnus glutinosa*	France	2003	R. Ioos	–	–	–	+
Alternaria sp.	A6b	*Alnus glutinosa*	France	2003	R. Ioos	–	–	–	+
Graphium sp.	P3-3	*Alnus glutinosa*	France	2003	R. Ioos	–	–	–	+

ND, not determined.
* ITS6 and ITS4 primers were used for *Phytophthora* and *Pythium* spp. whereas ITS1 and ITS4 primers were used for other fungi (White et al., 1990).
[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).

tube and DNA was extracted following the same protocol described above for woody tissues.

RAPD and PCR amplification conditions

The amplification reactions were carried out on a Genamp 9700 thermocycler (Applied Biosystems, Foster City, California). The cycling profile for RAPD included an initial denaturation step at 95 °C for 3 min followed by 40 cycles of denaturation, annealing and elongation for respectively 30 s at 94 °C, 30 s at 36 °C and 1 min at 72 °C, and a final extension step at 72 °C for 7 min. RAPDs were carried out in a 20 μl mixture containing 1× *Taq* DNA polymerase buffer (Sigma-Aldrich, Lyon, France), 2 mM $MgCl_2$, 0.5 μM of 10-mer RAPD primer (kit OPE, OPF, OPG and OPH, Operon Technologies, Alameda, California), 150 μM dNTPs, 0.8 μg μl^{-1} Bovine Serum Albumin (BSA), 1 unit of *Taq* DNA Polymerase (Sigma-Aldrich), 2 μl of template DNA and molecular biology grade water was added to 20 μl. The cycling profile for PCR was the same as that described above except that the annealing tem-

perature was raised to 58 °C and only 35 amplification cycles were necessary to obtain a significantly positive signal. PCRs were carried out in a 20 μl mixture containing 1× polymerase buffer (Sigma-Aldrich), 1.8 M $MgCl_2$, 0.45 μM of each primer, 180 μM dNTPs, 0.7 μg μl^{-1} BSA, 0.6 unit of *Taq* DNA Polymerase (Sigma-Aldrich), 2 μl of template DNA and molecular biology grade water was added to 20 μl. RAPD and PCR fragments were separated, together with a 100 bp DNA ladder (Invitrogen, Cergy Pontoise, France), by a 4 h and a 1 h electrophoresis, respectively, on a 1% agarose gel at 4 V cm^{-1}. Gels were stained with ethidium bromide and images were recorded with a CCD camera and a GELDOC 2000® system (Biorad, Marne-La-Coquette, France).

Cloning and sequencing of RAPD fragments

All the RAPD fragments were cloned from products generated with DNA from *P. alni* subsp. *alni* isolate 703 with the pCR® 4-TOPO® – TA cloning kit (Invitrogen, Cergy Pontoise, France). Ten microlitres of the bulk RAPD products containing the band(s) of interest were subjected to a 30 min elongation step at 72 °C with 0.5 μl of 4 × 25 mM dNTPs mix and 0.3 U *Taq* DNA Polymerase in order to ensure the addition of an adenosyl base at each 3′ end of the amplicons, as recommended by the manufacturer. Five microlitres were then transferred to a sterile 1.5 ml microcentrifuge tube and the amplicons were ligated to a TOPO® vector (Invitrogen) as recommended by the manufacturer in the presence of 1 μl of the salt solution provided. Ligated plasmids were used to transform TOP 10® competent cells (Invitrogen) according to the manufacturer's instructions. Positive clones were selected by PCR amplifications of inserts with M13 sequencing primers. The PCRs were carried out directly with a suspension of transformed bacteria in ultrapure water. Clones containing the RAPD band of interest were selected according to the expected PCR product size. PCR products were then purified using Millipore purification microplates (Millipore, Molsheim, France) on a vacuum manifold (Millipore). 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).

Construction of an Internal Amplification Control (IAC)

A heterologous DNA template with 5′ and 3′ ending sequences identical to the primer pair PA-F and PA-R was constructed according to the protocol described by Langrell (2002), with slight modifications. Briefly, DNA extracted from leaves of *Populus trichocarpa* × *P. deltoides* 'Beaupré' was subjected to RAPD following the protocol described above, except that 0.45 mM of 10-mer primer was replaced by 0.45 mM of each of the 20-mer primers PA-F and PA-R. A typical RAPD pattern was revealed by electrophoresis on a 1% agarose gel and an 850-bp fragment was chosen as IAC for PA-F/R specific PCR. The entire RAPD product was cloned with the pCR® 4-TOPO® – TA cloning kit (Invitrogen), using the protocol described above. The clone containing the selected fragment was screened by PCR with M13 primers. Clones containing the selected fragment were screened according to the expected PCR product size. These clones were also tested in three different PCRs: one with the primer PA-F, one with the primer PA-R and the last one with both primers to ensure that the selected clones exhibit both recognition sites in 5′ and in 3′. Ready-to-use IAC templates were stored as a suspension of transformed bacteria in ultra pure water at −20 °C until used for PCR. A series of concentrations of IAC copies were mixed with a 200 ng to 0.5 pg range of Oomycete DNA and tested by PCR with PA-F/R primers. Six hundred copies of IAC in each PCR tube proved to be adequate to allow the amplification of both targets in the presence of a wide range of *P. alni* DNA concentrations and this method was therefore chosen to be used in routine analysis.

Primer design

Forward and reverse sequences were edited with Sequencher software (Gene Codes, Ann Arbor, Michigan). The presence of the RAPD primer was checked at both ends of the sequences and generally two sets of primers were designed with the help of Primer 3 software (Rozen and Skaletsky, 2000). Commonly, the first primer pair consisted mainly of the 10 bases of the RAPD primer completed by the following 3′ base sequence to design a 20–23 mer primer. In addition, a set of internal primers was designed within the SCAR. These

internal primers were chosen to have GC contents between 50 and 60% with a GC clamp at the 3′ end. Primers were custom synthesized by Invitrogen (Cergy Pontoise, France).

Results

Sixty-eight RAPD primers were tested with a panel representing two isolates of *P. alni* subsp. *alni* (703 and 1429-6b), one isolate of *P. alni* subsp. *multiformis* (DSFO/0125), one isolate of *P. alni* subsp. *uniformis* (AUL028), and isolates of two closely related species: *Phytophthora cambivora* (PC463, PC643) and *P. fragariae* var. *rubi* (FVR 59, 163-2) (Table 1). For each primer, RAPDs were carried out twice to confirm reproducibility of the patterns, with low stringency conditions. Sixty-eight primers were tested and 41 bands were selected on the different patterns. Those bands seemed to be specific for either, *P. alni* subsp. *alni* and one of the other subspecies of *P. alni*, or specific for *P. alni* subsp. *alni* and the two other subspecies of *P. alni*. Thirty-nine out of these 41 bands could be cloned and sequenced. Firstly, the sequences obtained were investigated using the blastn and blastx programme (http://www.ncbi.nlm.nih.gov/BLAST/) to check for any similarity with known sequences in the Genbank database. Except for one SCAR showing 18% identity with a retrotransposon sequence in *P. infestans* and another in which translation showed partial identity with an ABC transporter protein (data not shown), no other significant similarity was found. Secondly, the SCAR sequences were compared to sequences retrieved from the *Phytophthora sojae* genome sequencing project (http://genome.jgi-psf.org/physo/). *Phytophthora sojae* occurs in a different ecological niche from *P. alni*, *P. cambivora* and *P. fragariae* but lies in the same phylogenetic clade (Cooke et al., 2000). Eighteen out of 41 SCARs showed partial or complete identity to *P. sojae* sequences. These regions were subsequently not used to design PCR primers within them.

Finally, 122 primers were designed from the SCAR sequences. Sixty-seven primer pairs were tested by PCR with a panel of 18 representative isolates of *P. alni* from different geographical origins and different subspecies, two isolates of *P. cambivora*, one isolate of *P. fragariae* var. *fragariae*, one isolate of *P. fragariae* var. *rubi*, and three species of *Phytophthora* frequently isolated from riparian ecosystems: *P. inundata*, *P. megasperma* and *P. gonapodyides* (Brasier et al., 2003a, b). Primer pairs producing a unique PCR product with the alder *Phytophthora* isolates but yielding no amplification with the other *Phytophthora* species described above were selected to be tested by a more exhaustive PCR assay, including all the isolates of *P. alni*, *Phytophthora* spp. and the other Oomycetes and fungi listed in Table 1.

Finally, 34 out of 67 primer pairs showed cross-reactions with at least one of *Phytophthora cambivora*, *P. fragariae* var. *fragariae*, *P. fragariae* var. *rubi*, *P. inundata*, and were not used in subsequent experiments for detection purposes. Interestingly, several PCR primer pairs showed different specificity patterns with *Phytophthora alni*.

One primer pair designed from RAPD with OPF4 primer produced a unique PCR amplicon of approximately 450 bp with all the isolates of different subspecies of *P. alni* (Figure 1A.) but yielded no amplicon with the other species of *Phytophthora* or with the other Oomycete or fungal species (Table 1). Nevertheless, very faint bands were visible when DNA extracts from *P. cambivora* isolates were tested, but their weakness and their much larger size (> 700 bp), allowed easy distinction from the *P. alni* isolates. Moreover, increasing the annealing temperature up to 62 °C overcame this problem. These forward and reverse primers were designated 'PA-F' and 'PA-R' respectively (Table 2).

Eight primer pairs designed from RAPD with OPG3, OPG8, OPG10 and OPH19 primers were shown to be specific to both *P. alni* subsp. *alni* and *P. alni* subsp. *multiformis* but did not cross-react with *P. alni* subsp. *uniformis*, or with the other species tested. One primer pair producing an amplicon of approximately 590 bp (Figure 1B.) was selected and designated as 'PAM-F/R' (Table 2). In addition, one primer pair designed from RAPD with OPF2 primer, was shown to be specific to both *P. alni* subsp. *alni* and to *P. alni* subsp. *uniformis* and did not cross-react with *P. alni* subsp. *multiformis*, or with the other species tested. This primer pair produced a unique PCR amplicon of approximately 750 bp (Figure 1C.) and was designated as 'PAU-F/R' (Table 2). Nevertheless, PAU-F/R amplification produced a

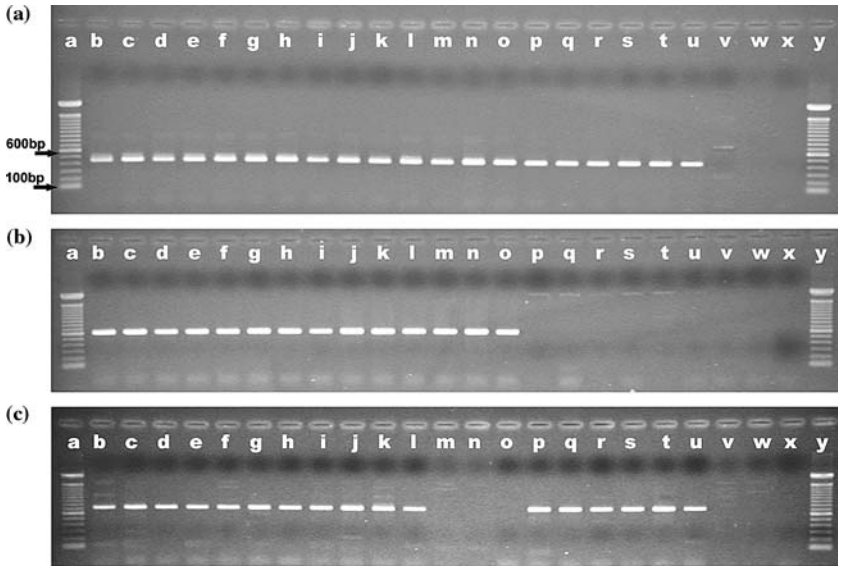

Figure 1. PCR products obtained with the primer pair PA-F/R (a), the primer pair PAM-F/R (b) and the primer pair PAU-F/R (c). Lanes b to l: *Phytophthora alni* subsp. *alni* 2N0685, 71T1, DSFO98172, PD2010953, P1272, P950, 2198, 155-a, BBA23/00, Pucking B10, PO192, Lanes m to o: *P. alni* subsp. *multiformis* isolates DSFO/0125, W1139, P841. Lanes p to u: *P. alni* subsp. *uniformis* isolates P875, CBS109280, 6, AUL028, Phy-A-SLO, 2271. Lane v: *P. cambivora* isolate PC643. Lane w: *P. fragariae* var. *rubi* isolate 163-2. Lane x: negative control with sterile ultra pure water. Lanes a and y: 100-bp DNA ladder.

very faint band of >1500 bp with *P. alni* subsp. *multiformis* DNA extracts. Although the size of this amplicon did not affect the interpretation of the results, this problem was overcome by increasing the annealing temperature to 62 °C.

The three selected primer pairs were tested with dilution series of purified DNA from several isolates of different subspecies of *P. alni* (Table 2). Primer pairs PA-F/R and PAM-F/R yielded the expected PCR product down to 0.5 pg of target DNA, whereas PAU-F/R could detect *P. alni* subsp. *alni* down to 5 pg and *P. alni* subsp. *uniformis* down to 50 pg (Table 2).

PCR tests using primer pairs PA-F/R were successfully carried out directly on total DNA extracted from inoculated or naturally infested plant samples and soil. In artificially contaminated river water, PCR with the PA-F/R primers yielded positive results for all five zoospore quantities from 1.5×10^6 down to 190 zoospores (Figure 2).

To help discriminate against false negatives due to the presence of inhibitory compounds in DNA extracts from naturally infected samples, an 850 bp heterologous fragment with identical primer recognition sites at both ends was constructed from *Populus* DNA using both primers PA-F/R under low stringency PCR conditions. The amplicon size chosen was larger than the *P. alni* target in order not to outcompete the efficient amplification of the Oomycete target DNA in routine PCR analysis. Two PCR products of the expected sizes were obtained when the IAC was

Table 2. Sequence, selectivity and sensitivity of the three *Phytophthora alni* primer pairs developed in this study

Primer pair	Sequence (5′–3′)	Amplicon size	Specificity	Sensitivity
PA-F	GGT GAT CAG GGG AAT ATG TG	450 bp	*P. alni* subsp. *alni*	<0.5 pg
PA-R	ATG TCC GAG TGT TTC CCA AG		*P. alni* subsp. *multiformis*	<0.5 pg
			P. alni subsp. *uniformis*	<0.5 pg
PAM-F	CTG ACC AGC CCC TTA TTG GC	590 bp	*P. alni* subsp. *alni*	<0.5 pg
PAM-R	CTG ACC AGC CAT CCC ACA TG		*P. alni* subsp. *multiformis*	<0.5 pg
PAU-F	GAG GAT CCC TAA CAC TGA ATG G	750 bp	*P. alni* subsp. *alni*	<5 pg
PAU-R	GAT CCC TGG TTG AAG CTG AG		*P. alni* subsp. *uniformis*	<50 pg

added to the DNA extracted from naturally infected alder tissues, from naturally infected alder soil or from *P. alni* zoospores trapped on the Durapore® membrane (Figure 3). In contrast, no amplification of the IAC was obtained when a large amount of target *P. alni* DNA was added as a template in the PCR tube, e.g. genomic DNA extracted from pure culture of the Oomycete (Figure 3, lane 7). However, since the target DNA could be amplified, the absence of the IAC amplification product only meant that the large amount of target DNA prevented the IAC amplification by outcompeting and that no inhibitory compound was present. In the case where only the IAC band was produced while no *P. alni* target was amplified, we concluded that the DNA extract did not contain a detectable amount of *P. alni* DNA. This was the case with DNA extracted from a hornbeam forest soil (Figure 3, lane 5).

Discussion

No generalized dieback of alders was reported in France before 1990. *Phytophthora alni* was isolated for the first time in France in 1996, but was supposed to have caused damage since the beginning of the 1990s (Streito et al., 2002a). Streito et al. (2002a) demonstrated that the disease is now widespread in France with particularly high damage in north-eastern and western France. The spread and severity of the disease in France are comparable with those recently observed in Bavaria, Germany (Jung and Blaschke, 2004). The disease has also been reported with a lower impact in most of the European countries (Streito, 2003). However, several European countries, as well as countries on other continents, are so far officially free of this destructive pathogen. Recently, the Canadian Food Inspection Agency added *P. alni* to its quarantine list (Anonymous, 2003). Once introduced into a river system, no efficient means of control exists to prevent downstream contamination of alder trees. Therefore, plants entering disease-free countries should be strictly checked with a reliable detection tool to limit the pathogen spread.

Up to the present time, direct isolation of *P. alni* using a non selective medium (Streito, 2003), a selective medium (Streito et al., 2002a; De Merlier et al., 2005) or a biological baiting (Jung and Blaschke, 2004) were the unique means of detecting the Oomycete in alder necrosis, infected soil or contaminated water. Unfortunately, the efficiency of these methods remains often poor and requires an active form of the Oomycete for successful isolation. Streito (2003) also reported that the onset of activity by the pathogen could also vary from season to season and from year to year. This

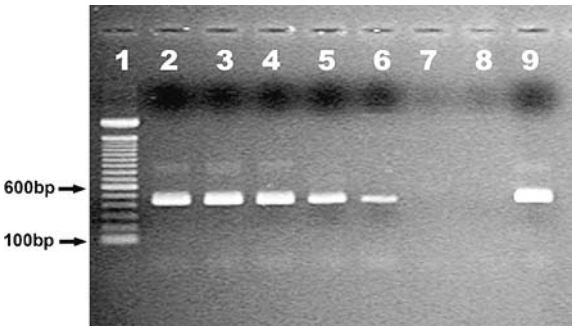

Figure 2. PCR products obtained with the PA-F/R primer pair and DNA extracted from different quantities of *Phytophthora alni* subsp. *alni* zoospores introduced into 50 ml of zoospore-free river water. Lane 1: 100-bp DNA ladder. Lanes 2 to 7: 1.5×10^6, 4.1×10^5, 7×10^4, 1.1×10^3, 190 and 30 zoospores, respectively, in 50 ml of river water. Lane 8: river water without introduced *P. alni* subsp. *alni* zoospore. Lane 9: positive control (genomic DNA from *P. alni* subsp. *alni* isolate 1429-6b).

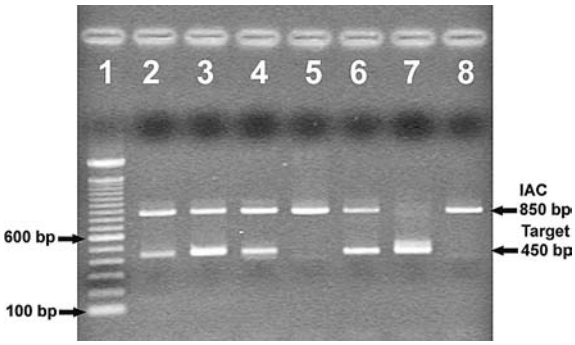

Figure 3. PCR products obtained with the primer pair PA-F/R and different DNA extracts. The internal amplification control was added directly in the PCR mixture to a final number of 600 IAC copies per PCR tube. Lane 1: 100-bp DNA ladder. Lanes 2 and 3: DNA extracted from inner bark of alder naturally infected by *Phytophthora alni* subsp. *alni*. Lane 4: DNA extracted from 50 ml of river water artificially contaminated with 1200 zoospores of *P. alni* subsp. *alni*. Lane 5: DNA extracted from an aliquot of *Carpinus betulus* forest soil. Lane 6: DNA extracted from an aliquot of river bank soil sampled in the vicinity of a *P. alni* subsp. *alni* infected alder. Lane 7: DNA extracted from pure culture of *P. alni* subsp. *alni* isolate 1429-6b. Lane 8: negative control (ultra pure water).

may therefore have a direct influence on the likelihood of successful isolation of *P. alni*. Moreover, competition between several microorganisms may occur during the isolation stage. Recently, De Merlier et al. (2005) successfully developed a SCAR-based PCR primer pair that is specific to *P. alni* subsp. *alni* and *P. alni* subsp. *uniformis*. However, *P. alni* subsp. *multiformis* isolates remained undetected with this primer pair. Moreover, this PCR test does not allow discrimination among *P. alni* subsp. *alni* and *P. alni* subsp. *uniformis* isolates and was only developed to detect these two subspecies in bark necrosis. The PCR primer pairs we developed enabled the specific detection of any subspecies of *P. alni* in different substrates like soil, water or woody tissues.

PCR tests with the PA-F/R primers were able to detect down to 0.5 pg of *P. alni* DNA. Theoretically, this amount represents less than the DNA content of one nucleus of the diploid *P. infestans* (Tooley and Therrien, 1987). Nevertheless, we could not achieve positive detection of *P. alni* subsp. *alni* with less than 190 zoospores trapped on a filtration membrane. There are probably several reasons why we could not get a positive signal below this threshold, such as DNA shearing during the DNA extraction process or suboptimal PCR conditions. However, we successfully detected *P. alni* in naturally infected soil as well as in fresh or old bark necrosis on alder trees from which we failed to isolate the Oomycete (data not shown). This probably means that there is often enough DNA of the pathogen to be detected by the PCR test we developed, even in cases where active forms of the Oomycete were absent. These molecular tools could therefore be useful for diagnosis purposes to confirm the presence of *P. alni* when symptoms of alder disease are observed, and when isolation proves to be difficult. These tools are currently under assessment in a large-scale epidemiological study of alder disease, requiring the routine analysis of hundreds of soil, water, and plant samples (Husson et al., 2004). This PCR test could also be of great interest to check plant stocks, in nurseries for example, as a means of preventing the spread of the pathogen into disease-free areas. With this in mind, the Internal Amplification Control we have developed in association to the PA-F/R primers will help to eliminate the 'false-negative' samples. Indeed, the presence of inhibiting compounds in DNA extracts from soil or woody tissues might be frequent and might lead to false negative PCR results if such an IAC is not used (Langrell, 2002).

Brasier et al. (1999) stressed that many hybrids or introgressants are unlikely to be detected by the conventional diagnosis methods used in international quarantine surveys. Even diagnoses using ITS-based PCR would not be appropriate if homogenization of the rDNA had occurred, thus returning to the parental species sequences. In the case of *P. alni* subsp. *alni*, which is the most frequent subspecies in Europe, ITS arrays are not yet stabilized and are still in the process of fixation (Brasier et al. 1999). We successfully found *P. alni*-specific markers in other regions of the genome through a large screening of RAPD profiles. The SCAR-based primers we designed did not cross-react with the closely related species *P. cambivora* and *P. fragariae*, even with low stringency PCR conditions. An initial explanation for this was to consider that the target sequences belonged to another unknown parental *Phytophthora* species. None of the *Phytophthora* species tested in this study appeared to be this unknown parent. Consequently, the SCAR primers we described here might also be used to identify the *Phytophthora* species involved in this particular case of natural hybrid. Another hypothesis is that these hybrid-specific sequences were generated during or after the hybridization process and did not exist in the genome of the parental species. This recombination phenomenon has already been described within the ITS of *P. alni* subsp. *alni* by Brasier et al. (1999).

In this study, we developed three primer pairs with distinct sub-specificity. The first primer pair PA-F/R allowed the specific detection of all the subspecies of *P. alni* we had in collection. However, the primer pair PAM-F/R gave a positive PCR signal with all the *P. alni* subsp. *alni* and all the *P. alni* subsp. *multiformis* isolates, whereas the primer pair PAU-F/R gave a positive PCR signal with all the *P. alni* subsp. *alni* and all the *P. alni* subsp. *uniformis* isolates. As we selected SCARs from RAPD profiles obtained with a *P. alni* subsp. *alni* isolate, we could not expect to find sequences exclusive to *P. alni* subsp. *multiformis* or subsp. *uniformis*. Nevertheless, these results confirm the close relationship between *P. alni* subsp. *alni* (i.e. the 'Standard' type) and the two other subspecies (i.e. the 'Variant' types) already demonstrated by Brasier et al. (1999) from AFLP data.

Interestingly, despite their different geographical origins, all the isolates belonging to a subspecies gave a positive signal with their respective subspecific primer pairs, as well as with other pairs of subspecific primers derived from the SCARs we had screened (Ioos, unpublished data). This might indicate that the different European isolates of *P. alni* have conserved markers characteristic of each subspecies within their genome. However, the origin of these subspecies of *P. alni* remains unclear and further investigations should be made to understand the hybridization process as well as the occurrence of the three subspecies throughout Europe.

Finally, although the primer sets we described in this paper were successfully checked with a large collection of *P. alni*, we can not totally exclude the possibility that their reliability might be challenged in the future, as this pathogen still seems to be evolving, especially for *P. alni* subsp. *alni* (Brasier et al., 1999). In addition, we could only investigate a few representative isolates of *P. alni* subsp. *uniformis* and *P. alni* subsp. *multiformis*. As hypothesized by Brasier et al. (2004), we cannot anticipate the existence of other types or the outbreak of new types of alder *Phytophthora*. Therefore, the specificity of our primers should be checked continuously in the future, even though they were successfully used with all of the recently collected European *P. alni* isolates that we have tested so far. Even though there is so far no 'non-pathogenic' isolates of *P. alni* described in the literature, we cannot exclude their occurrence. Therefore, any positive PCR detection of *P. alni*, directly in soil or in water, for example, would not discriminate between a pathogenic and a non-pathogenic isolate and should be interpreted carefully. Continuous checking of the pathogenicity of the different isolates of *P. alni* is necessary and, in this view, isolation of the pathogen by classical means remains therefore very useful.

Acknowledgements

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