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PCR-based DNA Markers for Identifying Hybrids within *Phytophthora alni*

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Abstract

Two pairs of oligonucleotide primers were designed for the polymerase chain reaction (PCR)-based detection and differential identification of naturally occurring interspecific hybrid types (subspecies) of *Phytophthora alni*, all of which cause collar rot of alder trees. Primer pairs were derived from randomly amplified polymorphic DNA (RAPD) fragments that were unique to various subspecies of this alder pathogen. The primer pair set, SAP1/SAP2 (SAP), was derived from a 0.93-kb RAPD fragment amplified from *P. alni* ssp. *alni*. The primer pair set, SWAP1/SWAP2 (SWAP), was derived from a 1.13-kb fragment amplified from *P. alni* ssp. *uniformis*. Patterns of SAP and SWAP amplification enabled distinction among the three subspecies. No PCR products were amplified from isolates of 31 other *Phytophthora* spp. examined, including *P. cambivora* and *P. fragariae*, the suspected progenitors of *P. alni*. The SAP and SWAP primer sets were able to detect a minimum of 10 pg of DNA from pure cultures or DNA extracted from as few as 10 zoospores. Pathogen DNA could also be amplified directly from bark lesions of artificially inoculated and naturally infected common alders and from lesions developed on common cherry-laurel leaves used in baiting the pathogen from infested soil. Direct detection of pathogen DNA from alder tissue using SAP and SWAP primer sets should prove useful in developing measures for effective quarantine and management of *P. alni*.

Introduction

Phytophthora is an oomycetous genus of destructive, primarily soil-borne plant pathogens. The genus contains approximately 70 species that affect a wide range of crop plants, shrubs and trees throughout the world (Erwin and Ribeiro, 1996). In view of the economic importance of *Phytophthora*-related diseases, accurate and rapid diagnosis of the causal species is an indispensable element in international quarantine legislation, as well as in disease management and control.

Detection or identification of *Phytophthora* spp. by traditional, predominantly morphologically based approaches, is difficult. There are species, for instance, which do not produce asexual and/or sexual structures in culture media, or sometimes even in plants (Erwin and Ribeiro, 1996). In other species induction of reproductive structures requires subtle manipulations of nutritional and environmental factors. Diagnosis based upon phenotypic characteristics has also proven to be insufficient when intraspecific variations in reproductive and other traits are great. Furthermore, phenotypic similarities may occur among species coexisting in the same habitat. Traits that overlap species boundaries have been suggested to be the result of somatic or gametangial fusion between related species (Brasier, 1992). The resultant hybrid organisms are even less likely to be recognized by conventional diagnostic approaches (Brasier, 2000; Olson and Stenlid, 2002; Schardl and Craven, 2003).

Molecular tools may help overcome the shortcomings of traditional diagnostic methods (e.g. Lee et al., 1993; Bonants et al., 1997; Brasier et al., 1999; Judelson and Tooley, 2000). Rapid and reliable identification of several *Phytophthora* species has been successfully achieved using polymerase chain reaction (PCR) to directly produce diagnostic DNA sequences from internal transcribed spacer (ITS) regions of rRNA genes or total genomic and mitochondrial DNAs (e.g. Érsek et al., 1994; Tooley et al., 1997; Ristaino et al., 1998; Winton and Hansen, 2001; Grote et al., 2002; Hayden et al., 2004; Kroon et al., 2004; Martin et al., 2004). These types of molecular diagnostic tools have been particularly useful for proving conclusively the occurrence of interspecific hybridization among related *Phytophthora* species either in the laboratory or in nature (Goodwin and Fry, 1994; Érsek et al., 1995; Man in't Veld et al., 1998; Brasier et al., 1999).

Of special interest in the present study is an unusual organism that represents a previously unknown *Phytophthora* morphological type. The undefined organism caused widespread mortality of alder trees (*Alnus* spp.)

in southern Britain in the early 1990s (Brasier et al., 1995) and has since occurred widely across Europe (e.g. Gibbs, 2003; Jung and Blaschke, 2004). This new aggressive pathogen superficially resembles *P. cambivora*, which is not pathogenic to alder. However, unlike the heterothallic *P. cambivora*, the alder pathogen is homothallic, exhibits an unusually high level of zygotic abortion and has lower cardinal temperatures for growth (Brasier et al., 1999; Brasier and Kirk, 2001). Assessments of ITS sequences and genomic polymorphisms suggested that the unknown pathogen was a hybrid of two developmentally different species, *P. cambivora* and a homothallic *P. fragariae*-like species (Brasier et al., 1999).

The alder *Phytophthora*, formally designated by Brasier et al. (2004) as *P. alni*, comprise a range of heteroploid organisms. These include a commonly encountered near tetraploid hybrid, *P. alni* ssp. *alni* and two less common subspecies, *P. alni* ssp. *uniformis*, and *P. alni* ssp. *multiformis* with chromosome numbers intermediate between diploid and tetraploid. Isolates of *P. alni* ssp. *alni* and ssp. *uniformis* were formerly termed standard types and Swedish variants, respectively, and *P. alni* ssp. *multiformis* included divergent hybrid types that were formerly considered to be Dutch, German and the UK variants of the pathogen. Isolates of *P. alni* ssp. *alni* are generally more aggressive than those of the other two subspecies (Brasier and Kirk, 2001). Furthermore, they have ornamented oogonia and elongated two-celled antheridia, features typical of *P. cambivora*. Both *P. alni* ssp. *uniformis* and ssp. *multiformis* have unique reproductive structures. Isolates of *P. alni* ssp. *uniformis* uniformly form oogonia with a smooth surface. In contrast, *P. alni* ssp. *multiformis* produces oogonia that are typically ornamented, but antheridia and gametangial fusions may vary in morphology (Brasier et al., 1999).

The significant morphological diversity of *P. alni* has made precise identification by traditional methods difficult. In lieu, molecular markers have been developed that overcome the constraints of morphology-based assessments (Érsek et al., 2003; De Merlier et al., 2005). Based upon sequence analysis of a randomly amplified polymorphic DNA (RAPD) amplicon, De Merlier et al. (2005) generated a PCR primer set that is specific to *P. alni* ssp. *alni* and *P. alni* ssp. *uniformis* but unable to differentiate among subspecies of the pathogen.

In this report we describe the development of another RAPD-derived PCR marker system for specific detection of *P. alni*. The work was based on a preliminary RAPD analysis in which a 0.93-kb amplifiable RAPD fragment was detected consistently in isolates of *P. alni* ssp. *alni* but was absent in either ssp. *uniformis* or the presumed parental species (Nagy et al., 2003). We hypothesized that such a fragment would have utility for pathogen diagnosis and population analysis. The objective of the present study was to derive oligonucleotide primer pairs from prominent, unique RAPD fragments that can amplify single PCR products diagnostic of *P. alni* subspecies.

Materials and Methods

Pathogen isolates and plant materials

One hundred and five isolates of *Phytophthora*, *Pythium*, *Mortierella*, *Armillaria* and *Alternaria* spp. were examined in this study (Table 1). The collection included 14 Hungarian isolates that were recovered from bark tissues and the rhizosphere of alders and were either morphologically characterized in the present study or in previous work (Szabó et al., 2000; Nagy et al., 2003). Most of the isolations were carried out in mid-fall, from late-September to mid-October. The collections also included isolate P770, the type isolate of *P. alni* ssp. *multiformis*, and isolate P772, the type isolate of *P. alni* ssp. *alni*. Identity of other *Phytophthora* spp. baited from alder soil was confirmed by morphological features and analysis of the entire ITS sequences (White et al., 1990) followed by a BLAST search. All cultures were maintained on pea-broth (Erwin and Ribeiro, 1996) or carrot agar (Brasier, 1972) plates at 15–20°C.

Sporulation and zoospore release were initiated for one isolate each of *P. alni* ssp. *alni* and *P. alni* ssp. *uniformis*. Five 1-cm-diameter agar plugs were cut from the actively growing region of a culture and placed in a Petri plate containing 20 ml of pea broth. After 5 days incubation at 25°C, colonies were decanted and thoroughly washed with distilled water. Non-sterile garden soil extract was prepared by combining equal volumes of soil and tap water. After overnight incubation the soil extract was filtered and added to just cover the colonies (Erwin and Ribeiro, 1996). Zoospores were released abundantly after 3–5 days of additional incubation in the dark at 16°C. Serially diluted spores were used to assess the specificity and sensitivity of designed PCR primers.

Plant inoculation and pathogen isolation

Stems of 2-year-old common alder (*A. glutinosa*) were wound-inoculated 10 cm above the ground level. Using a cork borer, a 3-mm-diameter hole was punched through the bark to the wood surface of each of three replicate trees. A 3-mm-diameter carrot agar plug was cut from the margin of an actively growing colony of *P. alni* ssp. *alni* P772 or *P. alni* ssp. *uniformis* P876 and inserted into the wound of each tree. Equivalent control inoculations were made with sterile agar plugs.

After 10 days of incubation at 24–28°C in the greenhouse, necrotic and asymptomatic bark pieces were excised at points below and above the inoculation sites using a 10-mm-diameter cork borer. Excised tissues were surface sterilized in 10% solution of commercial sodium hypochlorite, rinsed in sterile deionized water and then processed for PCR. Inoculation tests were carried out twice.

In mid-fall of 2004, bark samples were collected from trunks of naturally infected alder trees that exhibited collar rot symptoms. Bark samples were also

Table 1
Isolates of *Phytophthora* spp. and other genera used to investigate specificity of primers SAP and SWAP in polymerase chain reaction (PCR) amplification from pure cultures

Isolate[a]	Host/origin	Product with primers[b] SAP	SWAP
P. alni ssp. *alni*			
4/2, 6	Alder root, Hungary	+[c]	+
1/a, 8, 9	Alder soil, Hungary	+	+
H-5/02, *H-88/04*, *H-89/04*, *H-95/04*, H-97/04	Alder bark, Hungary	+	+
P772^{CB} (type)	Alder bark, UK	+	+
P834^{DC}, AP101^{RI}, AP115^{RI}	Alder bark, France	+	+
2200^{AC}, 2303^{AC}	Alder bark, Belgium	+	+
P. alni ssp. *uniformis*			
155a^{ISZ}	Alder root, Hungary	–	+
155b^{ISZ}, 155c^{ISZ}	Alder soil, Hungary	–	+
H-105/04	Alder bark, Hungary	–	+
P876^{CB}, P887^{DC}	Alder bark, Sweden	–	+
2277^{AC}	Alder bark, Belgium	–	+
P. alni ssp. *multiformis*			
P770^{DC} (type)	Alder bark, the Netherlands	+	–
P889^{DC}	Alder bark, Germany	+	–
2274^{AC}	Alder bark, Belgium	+	–
P. cactorum			
H 1016 (DAR 37628)^{AH}	Unknown	–	–
ICMP 11853	Apple root, New Zealand	–	–
INRA 195^{FP}	Strawberry, France	–	–
P. cambivora			
P1010^{CB}, P1011^{CB}	Oak soil, England	–	–
P199^{DC}	Beech, UK	–	–
CAM1^{DC}	*Rubus* sp., Scotland	–	–
IMI 340633, P1996^{MC}	Almond, Australia	–	–
PCJC17^{RI}, PC428^{RI}	Chestnut soil, France	–	–
INRA 253^{FP}	Sycamore, France	–	–
BPIC 1173^{EK}	European chestnut, Greece	–	–
P0592^{MC}	Noble fir, Oregon	–	–
P3671^{MC}	Cherry, Michigan	–	–
P6360^{MC}, P7140^{MC}	Apple, Australia	–	–
P. capsici			
ATCC 15399^{JD}	Pepper, New Mexico	–	–
1794^{EB}	Soil, California	–	–
P. cinnamomi			
H 1000^{AH}	Soil, Victoria, Australia	–	–
CIN1^{RI}	Chestnut, France	–	–
IMI 022938	Cinnamon, Indonesia	–	–
P. citricola			
H-1/02	Alder soil, Hungary	–	–
BCIP 1178^{EK}	Lemon, Greece	–	–
P. citrophthora			
H 1018^{AH}	Sweet orange, NSW, Australia	–	–
BPIC 1133^{EK}	Almond, Greece	–	–
IMI 129906	Orange, Australia	–	–
P. colocasiae			
ICMP 11844	Taro, American Samoa	–	–
P. cryptogea			
H 1050^{AH}	*Melaleuca* sp., Australia	–	–
ICMP 9673	Asparagus, USA	–	–
BPIC 1184^{EK}	Eggplant, Greece	–	–
P. drechsleri			
ICMP 9771	USA	–	–
BPIC 1135^{EK}	Cucumber, Greece	–	–
P. erythroseptica			
H 1019^{AH}	Potato, Victoria, Australia	–	–

Table 1
Continued

Isolate[a]	Host/origin	Product with primers[b] SAP	SWAP
BPIC 1136^{EK}	Potato, Greece	–	–
P. fragariae var. *rubi*			
P823^{CB}	*Rubus* sp., Scotland	–	–
FVR11^{DC}	Raspberry, Scotland	–	–
PFR163^{RI}	*Rubus* sp., France	–	–
P. fragariae var. *fragariae*			
FVF7^{DC}	Unknown	–	–
FVF12^{DC}	Unknown	–	–
P. fragariae			
P3289^{MC}	Raspberry, New York	–	–
P3408^{MC}	Strawberry, Scotland	–	–
P3569^{MC}	Strawberry, Oregon	–	–
P3843^{MC}	Strawberry, Canada	–	–
P. glycinea			
ICMP 9056	Soybean, Australia	–	–
P. gonapodyides			
H-4/02	Alder soil, Hungary	–	–
ICMP 14157	Chestnut, New Zealand	–	–
BUKN1b^{JN}	Beech soil, Germany	–	–
P. taxon Salixsoil[d]			
H-15/02, H-16/02	Alder soil, Hungary	–	–
P. humicola			
P6701^{MC}	Citrus, Taiwan	–	–
P. idaei			
INRA 477^{FP}	Raspberry, UK	–	–
P. infestans			
H-3/1/93	Potato, Hungary	–	–
US930287^{WF}	Potato, USA	–	–
P. inundata			
IMI 390121	*Olea* sp., Spain	–	–
P. katsurae			
INRA 441^{FP}	Soil, Taiwan	–	–
P. macrochlamydospora			
INRA 511^{FP}	Soybean, Australia	–	–
P. meadii			
H 1031^{AH}	Unknown	–	–
P. megakarya			
INRA 293^{FP}	Cocoa, Togo	–	–
P. multivesiculata			
ICMP 14172	*Cymbidium* sp., New Zealand	–	–
P. nicotianae			
W1^{JD}	Tomato, California	–	–
C2-CL^{MM}	Citrus, Arizona	–	–
P. palmivora			
H 1104^{AH}	Pawpaw, Queensland, Australia	–	–
P. porri			
ICMP 14271	Broccoli, New Zealand	–	–
P. pseudosyringae			
L70^{JN}	Oak soil, Germany	–	–
P. pseudotsugae			
INRA 479^{FP}	Douglas fir, Oregon	–	–
P. quercina			
INRA 493^{FP}	Oak soil, France	–	–
P. sojae			
ICMP 9488	Asparagus soil, New Zealand	–	–
P. syringae			
H 1055^{AH}	*Cymbidium* sp., NSW, Australia	–	–
Pythium spp.			
H-3/03, H-4/03, H-5/03, P87/04	Alder soil, Hungary	–	–
Pythium undulatum			
P.1.Str.b^{JN}	Forest soil, Germany	–	–

Table 1
Continued

Isolate[a]	Host/origin	Product with primers[b] SAP	SWAP
Pythium dissotocum			
Myo 1[JN]	*Myosotis* soil, Germany	–	–
Mortierella sp.[LV]	Apple, Hungary	–	–
Armillaria gallica			
G53[GS]	Oak, Italy	–	–
Armillaria mellea			
M198[GS]	Chestnut, Italy	–	–
Alternaria sp.			
H-102/04	Alder bark, Hungary	–	–

[a]Isolate number from different laboratories that have worked with the same culture: CB, Clive Brasier; DC, David Cooke; RI, Renaud Ioos; AC, Anne Chandelier; ISZ, Ilona Szabó; AH, Adrienne Hardham; FP, Frank Panabières; MC, Michael Coffey; EK, Elena Kalomira; EB, Edward Butler; JN, Jan Nechwatal; WF, William Fry; JD, John Duniway; MM, Michael Matheron; LV, László Vajna; GS, Giovanni Sicoli. Isolates with no supplier indicated are directly from the type culture collections of IMI (CABI) and ICMP or from the authors' collection. Isolates in italics were also amplified directly from diseased plant tissues.
[b]SAP and SWAP represent SAP1/SAP2 and SWAP1/SWAP2 primer sets, respectively.
[c]+ indicates that the 0.93-kb and 1.13-kb fragments are produced by SAP and SWAP primers, respectively; – indicates that products are absent.
[d]This taxon was informally described by Brasier et al. (2003a).

collected from asymptomatic, healthy trees. Collection sites were located at randomly dispersed sites in a riparian woodland of northern Hungary. Bark sections (approximately 5 cm^2) were cut from four different points of the necrotic areas of each of four sampled trees. Bark samples from healthy trees were removed randomly at points from 30 to 60 cm above the ground level. Samples were surface-sterilized and stored either in sealed vials in a refrigerator (5°C) for 5 months or in sterile distilled water which was changed daily during storage at 10°C for 1–4 days.

After storage, a portion of each bark sample was plated on selective medium containing pimaricin, ampicillin, rifampicin, benomyl and pentachloronitrobenzene (PCNB) (Erwin and Ribeiro, 1996). Emerging colonies were identified to subspecies level based on morphological characteristics. DNA was extracted from 20 mg of the remaining bark tissue of each sample for subspecies identification by PCR.

Rhizosphere soil was also collected from each sampled tree in the field. Each soil sample was mixed with deionized water in 1 : 1 ratio to induce zoospore release by resident *Phytophthora* species. Leaves of common cherry-laurel (*Prunus laurocerasus*) were floated abaxial side down on the water surface for 6–10 days to capture released zoospores (Themann and Werres, 1998; Nagy et al., 2003). DNA was extracted from 7-mm-diameter lesions on individual leaf baits for pathogen detection by PCR. Small pieces from the same leaf lesions were plated, and emerging colonies were identified on the basis of morphological features.

DNA extraction

Cultures of *P. alni* and other oomycete or fungal species produced in pea broth at 20–25°C or stem tissues of artificially inoculated or control plants were thoroughly washed in sterile deionized water, freeze-dried and pulverized in liquid nitrogen. DNA was extracted from pulverized mycelia of each test culture using a phenol/chloroform procedure (Goodwin et al., 1992) and stored in 10 mM Tris-HCl (pH 8.5) at −20°C. In addition, DNA was extracted and purified from wound-inoculated symptomatic or healthy stem tissue using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) and stored in sterile Milli-Q water. Purity and concentration of DNAs were determined spectrophotometrically or by quantitation on agarose gels after staining with ethidium bromide and comparing with commercially available DNA standards. For field samples and zoospores DNA was extracted using the REDExtract-N-Amp Plant PCR Kit (Sigma-Aldrich Ltd, Budapest, Hungary) in accordance with the manufacturer's instructions.

Development of primers and PCR conditions

RAPD fragments amplified using various OPG and OPK primers (Operon Technologies Inc., Alameda, CA, USA) were screened for specificity to design diagnostic primer pairs using aliquots of DNA from cultures of *P. alni* and other *Phytophthora* species. About 30–50 ng of template DNA from each test isolate was combined with 50 mM KCl, 10 mM Tris-HCl (pH 8.8), 0.8% Nonidet P40, 2 mM $MgCl_2$, dNTPs (200 μM each), 0.4 μM random primer and 2 units of *Taq* polymerase in a total volume of 50 μl. All reaction components, except for the primers, were purchased from MBI Fermentas (Vilnius, Lithuania). Reactions were run using a PTC-150 Mini Cycler (Bio-Rad Hungary Ltd, Budapest, Hungary) programmed as follows: initial denaturation at 94°C for 3 min, 36 cycles of denaturation at 94°C for 1 min, annealing at 36°C for 1 min, extension at 72°C for 2 min and one cycle of final elongation at 72°C for 10 min. Amplification products were resolved by electrophoresis in 1.5% agarose gels (Gibco-BRL, Gaithersburg, MD, USA).

Putative diagnostic RAPD fragments were detected by ethidium bromide-staining and recovered using the QIAquick gel extraction kit (Qiagen). Each fragment was cloned into a pGEM-T vector (Promega Corporation, Mannheim, Germany) and then transformed into competent cells of TOP10 *Escherichia coli* (Invitrogen GmbH, Karlsruhe, Germany). Recombinant cells were selected on LB plates containing 100 μg/ml ampicillin, 40 μg/ml X-gal and 50 μg/ml IPTG (Sambrook et al., 1989). Recovered clones were selected and grown overnight in 5 ml LB broth at 37°C before extracting vector DNA by alkali lysis (Sambrook et al., 1989) and purifying with Miniprep Express Matrix (Q-BIOgene, Carlsbad, CA, USA). Insert DNA was as sequenced by MWG Biotech AG (Ebersberg, Germany).

Two sets of *P. alni*-specific oligonucleotide primers were constructed (MWG Biotech AG) by elongating the RAPD primers, OPG-02 and OPK-12, to complement additional DNA sequences of *P. alni* ssp. *alni* or *P. alni* ssp. *uniformis*, respectively. Primer pair SAP1/SAP2, 5′-GGCACTGAGGGTTCCTC-3′/5′-GGCACTGAGGTCTAGATT-3′ was designed on the basis of terminal nucleotide sequences of the 0.93-kb fragment derived from the type isolate P772 of *P. alni* ssp. *alni* and contained the sequence of the random primer OPG-02 (underlined). Primer pair SWAP1/SWAP2, 5′-TGGCCCTCACATTAAAACTGCTGC-3′/5′-GGCCCTCACCAAATGCGAAATGA-3′ was similarly derived from the 1.13-kb fragment amplified from isolate P876 of *P. alni* ssp. *uniformis* and contained the sequence of the random primer OPK-12 (underlined).

Specificities of the primer pairs were tested by PCR using total genomic DNAs extracted from numerous oomycete and fungal cultures. Template DNA at concentrations ranging from 5 pg to 100 ng was combined with 50 mM KCl, 10 mM Tris-HCl (pH 8.8), 0.8% Nonidet P40, 1.5 mM $MgCl_2$, 200 μM each of dNTPs, 0.4 μM of each primer, and 1.5 units *Taq* polymerase in a total volume of 25 μl. In routine tests, 25–50 pg or 100 ng DNA was used from *P. alni* or from other micro-organisms, respectively. PCR cycling conditions corresponding to those of the aforementioned RAPD–PCR were tested over a range of annealing temperatures.

The sensitivity of pathogen detection was evaluated over a range of concentrations of DNA extracted from mycelia or zoospores of one isolate each of *P. alni* ssp. *alni* and *P. alni* ssp. *uniformis*. To test the influence of plant substances on the sensitivity of detection, dilutions of mycelial DNA were mixed with 4 μl of a onefold diluted DNA extract obtained from 20 mg of bark tissue of healthy alder using the REDExtract-N-Amp Plant PCR procedure. Corresponding control dilutions were spiked only with 2 μl each of REDExtract-N-Amp extraction and dilution buffers. The amplification was conducted using the PCR Ready Mix of the kit under the same cycling parameters as mentioned above.

Results

RAPD analysis

Screening of arbitrary 10-mer OPG and OPK primers revealed two reproducible RAPD fragments whose amplification patterns distinguished among isolates of *P. alni* ssp. *alni*, *P. alni* ssp. *uniformis* and *P. alni* ssp. *multiformis*. Primer OPG-02 amplified a 0.93-kb product in isolates of *P. alni* ssp. *alni* and isolates P889 and P770 of *P. alni* ssp. *multiformis* (Fig. 1). Primer OPK-12 amplified a 1.13-kb product in isolates of both *P. alni* ssp. *uniformis* and *P. alni* ssp. *alni* (Fig. 1). Neither amplicon was detected in the putative parental species, *P. cambivora* and *P. fragariae* var. *rubi*, or in any *Phytophthora* species that may coexist with alder *Phytophthora* in the same soil habitat (data not shown).

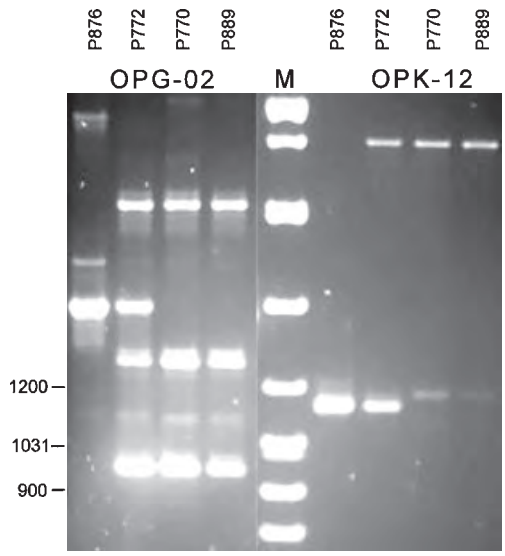

Fig. 1 Randomly amplified polymorphic DNA (RAPD) patterns derived from isolates of *Phytophthora alni* ssp. *uniformis* (P876), ssp. *alni* (P772) and ssp. *multiformis* (P770 and P889). Polymerase chain reaction (PCR) was performed using random primers OPG-02 and OPK-12. Lane M includes molecular size markers (GeneRuler DNA Ladder Mix; MBI Fermentas; fragment sizes indicated are as bp)

Specificity of *P. alni*-specific DNA markers

Under optimal conditions, candidate diagnostic primers SAP1/SAP2 (SAP) amplified a single 0.93-kb DNA product from tested *P. alni* ssp. *alni* isolates and from isolates P770 and P889 of *P. alni* ssp. *multiformis* (Table 1; Fig. 2). In contrast, candidate primers SWAP1/SWAP2 (SWAP) amplified a single 1.13-kb

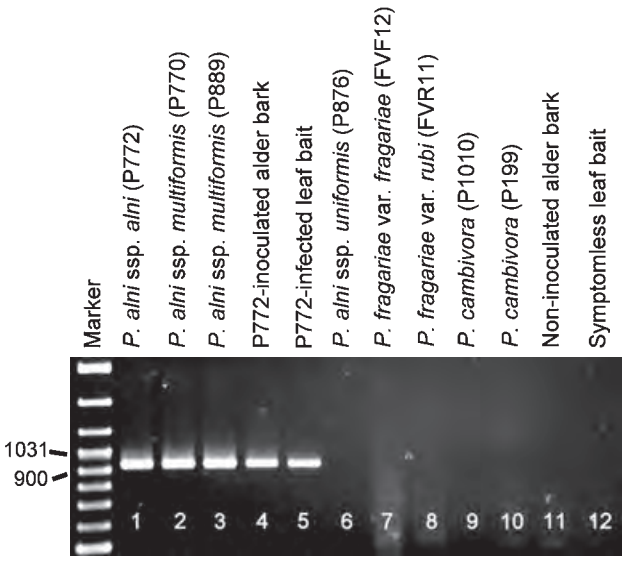

Fig. 2 Detection of a 0.93-kb DNA fragment after polymerase chain reaction (PCR) using oligonucleotide primer pair SAP1/SAP2 specific to *Phytophthora alni* ssp. *alni* and ssp. *multiformis*. DNA was extracted from pure mycelial cultures or plant tissues. About 50 pg (lanes 1–3) or 100 ng (lanes 4 and 6–10) of extracted DNA was used as template for PCR. DNA from one plant tissue sample (lane 5) was not quantified. GeneRuler DNA Ladder Mix (MBI Fermentas) is included in marker lane (fragment sizes indicated are as bp)

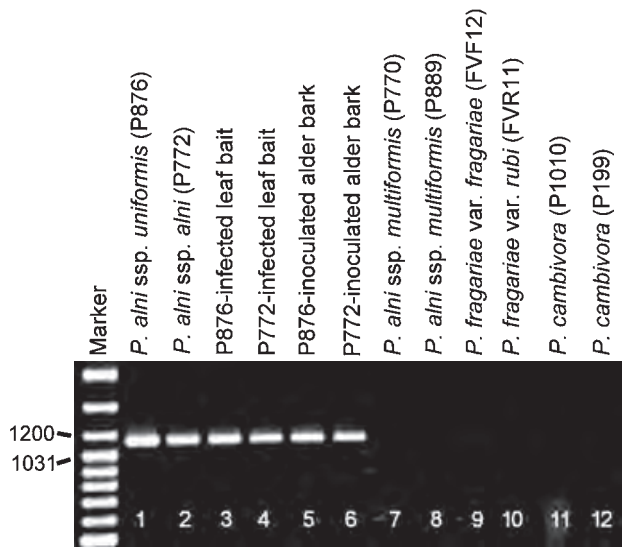

Fig. 3 Detection of a 1.13-kb DNA fragment with oligonucleotide primer pair SWAP1/SWAP2 specific to *Phytophthora alni* ssp. *alni* and ssp. *uniformis*. DNA was obtained from pure mycelial cultures or plant tissues. About 50 pg (lanes 1 and 2) or 100 ng (lanes 5–12) of extracted DNA was used as template for polymerase chain reaction (PCR). DNA from some plant tissue samples was not quantified (lanes 3 and 4). GeneRuler DNA Ladder Mix (MBI Fermentas) is included in marker lane (fragment sizes indicated are as bp)

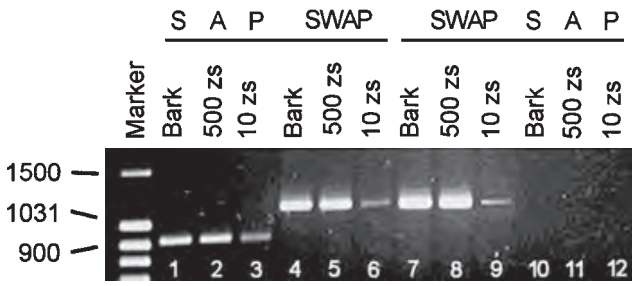

Fig. 4 Polymerase chain reaction (PCR)-based detection of *Phytophthora alni* DNA from naturally infected alder bark and 10 or 500 zoospores (zs) produced by isolates cultured from bark tissues. PCR was performed using SAP and SWAP primer sets. Amplification products derived from isolate H-88/04 of *P. alni* ssp. *alni* (lanes 1–6) and from isolate H-105/04 of *P. alni* ssp. *uniformis* (lanes 7–12). MassRuler DNA Ladder Mix (MBI Fermentas) is included in marker lane (fragment sizes indicated are as bp)

fragment from isolates of *P. alni* ssp. *uniformis* and *P. alni* ssp. *alni* (Table 1; Fig. 3). Neither the 0.93- or 1.13-kb fragment nor any other discernible sequences were amplified from up to 100 ng of mycelial DNA of *P. cambivora* and *P. fragariae*, the parental species, or any other *Phytophthora* sp. or *Pythium*, *Mortierella*, *Armillaria* and *Alternaria* species tested (Table 1). DNA from zoospores liberated from pure cultures of a *P. alni* ssp. *alni* isolate (H-88/04) and a *P. alni* ssp. *uniformis* isolate (H-105/04) in non-sterile soil extract also produced the diagnostic fragments with these primer pairs (Fig. 4). Ten zoospores provided sufficient target DNA for resolvable amplification.

The annealing temperature, a critical factor for optimizing product formation, differed with the template–primer system. Highest yields of hybrid-specific products and the clearest agarose gel images based on SAP and SWAP primers were obtained at annealing temperatures of 60°C and 68°C, respectively. These empirically derived values differed from the respective theoretically derived optimal temperatures of 54 and 59.2°C, at which faint non-specific bands were detected (data not shown).

Detection of *P. alni* in diseased plant tissues

The SAP and SWAP primers were able to amplify DNA of *P. alni* subspecies in symptomatic alder tissue. For example, each primer pair was tested with two samples per inoculation and reproducibly amplified its respective diagnostic product from 100 ng DNA extracted from necrotic tissue after wound inoculation with the *P. alni* ssp. *alni* type isolate P772 or the *P. alni* ssp. *uniformis* isolate P876 (Figs 2 and 3).

In field isolation trials, identification of *P. alni* subspecies determined by PCR, also agreed with identifications based on morphological assessments. For example, nine of 12 symptomatic bark samples (three samples from each of three trees) stored for 1–4 days in water produced amplicons with both primer pairs, suggesting that the pathogen in each tissue section represented *P. alni* ssp. *alni* (Table 2; Fig. 4). Three representative isolates recovered from these bark samples, including isolates H88/04, H89/04 and H95–04, also exhibited morphological characteristics typical of *P. alni* ssp. *alni*. Three bark samples from the remaining tree amplified with the SWAP primers only and thus, they appeared to contain isolates of *P. alni* ssp. *uniformis* (Table 2; Fig. 4). One representative isolate, H105/04, recovered from these bark samples, was also morphologically identical to *P. alni* ssp. *uniformis*. Identity of each of these isolates was further confirmed with PCR using DNA from pure cultures derived from the bark samples. No amplicon diagnostic of *P. alni* ssp. *multiformis* was produced in field samples tested. Background amplification was not observed with DNA from healthy bark tissues. The bark samples of the same trees that were refrigerated for 5 months, produced the expected PCR amplicons, but all failed to develop colonies when plated on selective medium (Table 2).

Cherry-laurel leaves that were used to bait *Phytophthora* from rhizosphere soil associated with the four sampled alder trees developed water-soaked lesions after infection. Only *P. alni* ssp. *alni* or *P. alni* ssp. *uniformis* were detected in 11 of 14 leaf lesions sampled by PCR using the SAP and SWAP primers. All lesions caused by *P. alni* ssp. *alni* or *P. alni* ssp. *uniformis* came from rhizosphere soil of three and one of the trees with bark colonized by the respective subspecies (Table 2; Figs 2 and 3). The remaining three leaf lesions were found to be colonized by *Phytophthora* species other than *P. alni* (Table 2). Analyses of morphological features and ITS sequences showed that one of the lesions was colonized by *P. citricola*, whereas the two other lesions were colonized by

Table 2
Molecular and/or morphological identification of *Phytophthora* spp. in field samples collected from bark sections and soil habitat of symptomatic alder trees

Source of samples[a]	Number of samples[b]	*P. alni*-specific amplification[c]	Isolation per attempt[d]	*Phytophthora* identified[e]		
				Paa	*Pau*	Others
Bark, fresh	12	12	4/4	9 (3)	3 (1)	0
Bark, stored[f]	4	4	0/4	3	1	0
Soil	14	11	3/3	8	3	3

[a]Samples were collected from the trunk and the soil of four trees exhibiting collar rot symptoms.
[b]Number of bark tissue sections examined (four samples per tree) and lesions formed on cherry-laurel leaves used to capture the pathogen from soil. All samples were processed for PCR.
[c]Samples amplifying with *P. alni*-specific SAP or SWAP primer sets.
[d]To validate PCR-based diagnosis, comparable sections of a bark sample from each tree was plated on selective medium for pathogen isolation and morphological identification. Of leaf bait lesions tested, only those not amplifying with the *P. alni*-specific primers were plated on medium for pathogen isolation and identification.
[e]Number of samples identified as *P. alni* ssp. *alni* (*Paa*) or *P. alni* ssp. *uniformis* (*Pau*) or other than *P. alni*, i.e. *P. citricola*, *P. gonapodyides* and *P. taxon* Salixsoil. Of all the samples identified as *P. alni* by PCR those confirmed by culturing are in parentheses.
[f]Bark tissue sections were stored in sealed vials in a refrigerator at 5°C for 5 months.

P. gonapodyides or the informally designated *P. taxon* Salixsoil (Brasier et al., 2003a). Neither primer pair amplified discernible DNA fragments from asymptomatic leaf tissues.

Sensitivity of detection

At 60°C, SAP primers optimally produced a diagnostic amplicon from 25 pg (but not from 12.5 pg) mycelial DNA derived from a culture of *P. alni* ssp. *alni* or *P. alni* ssp. *multiformis* (data not shown). At an annealing temperature of 68°C, SWAP primers required 25 and 50 pg of mycelial DNA to amplify a product diagnostic for *P. alni* ssp. *uniformis* and *P. alni* ssp. *alni*, respectively (data not shown).

To test the effect of plant DNA extracts on the sensitivity of pathogen detection, healthy plant samples were processed with the REDExtract-N-Amp PCR Ready Mix and added to purified mycelial DNA. With mycelial DNA in control samples, the pathogen detection limit was 10 pg (Fig. 5). The sensitivity of detection declined over a range of test concentrations of mycelial DNA when combined with constant concentrations of plant DNA extracts. For example, when 100 pg of mycelial DNA from a culture of *P. alni* ssp. *alni* type isolate P772 was mixed with 4 μl of a 1 : 1 dilution of DNA extract (the same volume that would be added if doing the assay with symptomatic tissue) from healthy alder bark tissue, the intensity of product amplification was equivalent to that of 10 pg of mycelial DNA in extraction and dilution buffers alone. Similar 10-fold reductions in amplification intensity were observed for all mycelial DNA concentrations tested (Fig. 5). Comparable reductions in sensitivity were also obtained with DNA dilutions from *P. alni* ssp. *uniformis* P876 using the SWAP primer pair (data not shown). The electrophoretic resolution of hybrid-specific products obtained either from 100 ng total DNA from an artificially inoculated stem, DNAs obtained with the REDExtract-N-Amp Plant PCR procedure from 20 mg naturally infected bark tissue, or from 7-mm-diameter disc of leaf bait and from 500 zoospores compared favourably with the resolution achieved with approximately 50 pg DNA from pure hybrid cultures (cf. Figs 2–4).

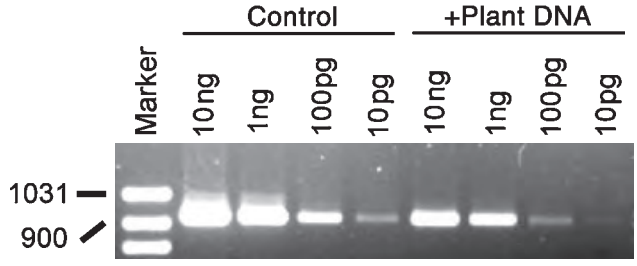

Fig. 5 Sensitivity of polymerase chain reaction (PCR)-based detection of isolate P772 of *Phytophthora alni* ssp. *alni*. PCR was performed using primer set SAP1/SAP2 over a range of DNA quantities from 10 pg to 10 ng. DNA was extracted from mycelial cultures in phenol/chloroform and amplified using the reaction mixture of the REDExtract-N-Amp Plant PCR Kit. In control treatments, reaction mixtures were spiked with ingredients used for processing plant DNA. In +plant DNA treatments, reaction mixtures were spiked with DNA from symptomless alder bark tissue. The MassRuler DNA Ladder Mix (MBI Fermentas) is included in marker lane (fragment sizes indicated are as bp)

Discussion

Phytophthora alni killed approximately 10% of the alders in southern Britain within a few years of its initial discovery in that country (Brasier et al., 1995; Gibbs et al., 1999). Recent surveys indicate that this disease is even more severe in riparian ecosystems in Bavaria (Jung and Blaschke, 2004) and in north-eastern France (Streito et al., 2002). The rapid spread of *P. alni* through Europe has probably been caused by the dissemination of infested nursery stock (Brasier and Jung, 2003; Jung and Blaschke, 2004). There is great concern about the potential impact of *P. alni* should it be dispersed to susceptible alder populations in other temperate regions of the world. To prevent dispersal, it is important to devise methods to rapidly detect and identify these pathogens to enable effective implementation of quarantines and control measures.

Phytophthora alni includes a range of heteroploid subspecies, including *P. alni* ssp. *alni*, ssp. *uniformis* and ssp. *multiformis* (Brasier et al., 1999, 2004). Distinguishing among subspecies by morphological traits, or by analysis of isozymes, amplified fragment length polymorphism (AFLP) or RAPD polymorphisms, is time-consuming or unsuitable for diagnostic purposes (Brasier et al., 1995, 1999, 2004; Nagy et al., 2003; J. Bakonyi, unpublished data). The DNA marker system developed in this study provides a means to rapidly detect and identify *P. alni* and its subspecies.

High sequence similarity between the ITS regions of rDNA of *P. alni* and progenitors (Brasier et al., 1999) previously hindered diagnostic marker development using this region. RAPD polymorphisms provided a means of circumventing this limitation (Érsek et al., 2003; De Merlier et al., 2005). In this study, two putative hybrid-specific RAPD products, a 0.93-kb fragment and a 1.13-kb fragment amplified with random primers OPG-02 and OPK-12, respectively, were selected for diagnostic marker development. The 0.93-kb fragment occurred in isolates of both *P. alni* ssp. *alni* and ssp. *multiformis*, and the 1.13-kb product was characteristic of both *P. alni* ssp. *uniformis* and *P. alni* ssp. *alni*. Neither product was amplified in the putative parental species nor other species of the genus that may coexist with *P. alni* in the same soil habitat (Brasier, 1999, 2003; Bakonyi et al., 2003; Brasier et al., 2003a,b; Jung and Blaschke, 2004).

Oligonucleotide primer pairs, SAP1/SAP2 (SAP) and SWAP1/SWAP2 (SWAP), were designed from a 0.93- and 1.13-kb RAPD fragment, respectively, to amplify single hybrid-specific DNAs. The size of each amplicon matched that of the particular RAPD fragment used for primer construction. In addition, high levels of sequence similarity between these amplicons and the corresponding RAPD products were revealed by Southern hybridisation (Z. Á. Nagy, unpublished data). The SAP and SWAP primer sets successfully amplified *P. alni* DNAs but not DNA from any of the other 31 *Phytophthora* species and several other oomycete and fungal species examined. Remarkably, these primer sets did not cross-react with isolates of *P. fragariae* and *P. cambivora*, notwithstanding the high level of sequence homology between hybrids and the suspected parental species, particularly *P. cambivora* (Brasier et al., 1999; Nagy et al., 2003). Conversely, we found that an oligonucleotide primer pair specifically designed for *P. cambivora* (Schubert et al., 1999) amplified the corresponding DNA fragment in *P. alni* isolates (Z. Á. Nagy, unpublished data).

The occurrence of a single 0.93-kb amplicon in both *P. alni* ssp. *alni* and *P. alni* ssp. *multiformis* was in accordance with similarities of AFLP fingerprints of total genomic DNA or ITS sequences (Brasier et al., 1999) indicating that these subspecies are closely related. Detection of the 1.13-kb SWAP product in *P. alni* ssp. *alni*, in addition to *P. alni* ssp. *uniformis* also suggests high sequence similarity within the subgenomic target DNAs of these subspecies, notwithstanding that they were found to be more distantly related (Brasier et al., 1999).

Primers developed recently by De Merlier et al. (2005) produced a 366-bp amplicon after PCR with DNA extracted from cultures of *P. alni* ssp. *alni* or *P. alni* ssp. *uniformis* but *P. alni* ssp. *multiformis*, and stem tissues of wound-inoculated alder. Using the two primer pairs developed in this study allowed us to clearly distinguish among *P. alni* ssp. *alni*, *P. alni* ssp. *uniformis* and *P. alni* ssp. *multiformis*.

Different DNA preparation methods were used in the course of this study. While phenol/chloroform extraction and general PCR reagents worked well for mycelium, due to the need for rapid sample processing kits that provide a time effective procedure and do not generate organic solvent waste were tried for plant samples. We used the DNeasy Plant Mini Kit for PCR detection of the pathogen from artificially inoculated alder bark tissues and the REDExtract-N-Amp Plant PCR Kit for field samples and zoospores. All DNA extraction, purification and PCR methods were effective in detecting and distinguishing among *P. alni* subspecies. Because of its ease, the REDExtract kit was particularly useful for pathogen detection in field samples. Using this kit, we successfully amplified diagnostic markers from bark samples that had been stored for 5 months, even when the pathogen could no longer be isolated from these tissues. A similar situation, i.e. failure in isolation rather than molecular detection, may occur in association with seasonality as reported on *P. ramorum* by Hayden et al. (2004). Due to our successful isolations of *P. alni* in mid-fall, we have not studied the effect of season. Reportedly, the greatest opportunity for sampling may be when alder bark lesions are most obvious, from May through October in temperate regions (Brasier and Kirk, 2001; Streito et al., 2002; Jung and Blaschke, 2004). Isolation frequencies, however, are likely to be lower when temperatures in hot summer days rise above the maximum temperature (30°C) for growth of most *P. alni* isolates.

Detection of *P. alni* was obviously affected by the quality of DNA and/or the PCR mix. For instance, a minimum of 25–50 pg of DNA from pathogen mycelia was required for PCR detection when using the phenol/chloroform extraction and the general PCR protocol. This level of detection sensitivity was similar to levels (20 pg) described in studies by De Merlier et al. (2005). Using reagents including the special reaction mix from the REDExtract-N-Amp PCR kit, only 10 pg of phenol-extracted mycelial DNA was required for detectable amplification of diagnostic markers. Thus, differences in sensitivity are likely to be more of a reflection of the performance of the reaction mix rather than that of the extraction method.

Recent sequencing studies have provided insight into the genome size of various *Phytophthora* species. For instance, analyses have estimated the haploid genome size of *P. ramorum* and *P. sojae* to be 65 Mbp and 95 Mbp, respectively (Joint Genome Institute, California, USA). On the basis of these size estimates, the

average nucleus of each diploid species would contain 0.13 pg and 0.19 pg DNA, respectively. Accordingly, if these species and the near tetraploid *P. alni* are comparable in haploid genome size, the PCR detection threshold of 10 zoospores of *P. alni* would correspond to 2.6–3.8 pg nuclear DNA; this amount is approximately one-third of the detection limit of mycelial DNA.

Several studies have suggested that greater detection sensitivity can be achieved by using of nested-PCR to amplify ITS or mitochondrial sequences (Hayden et al., 2004; Kroon et al., 2004; Martin et al., 2004). For instance, Kroon et al. (2004) found that nested-PCR enabled the detection of 10 fg of mtDNA of *P. ramorum* in comparison with 100 pg of mtDNA that was detectable by standard PCR.

Nevertheless, our PCR marker system is sensitive enough to enable differential detection of *P. alni* subspecies from pure cultures or zoospores of the pathogen, and from infected alder trees. Furthermore, the use of the REDExtract-N-Amp PCR protocol for field samples and zoospores in this study, appears to be the first application of the kit to processing DNA of a micro-organism rather than plant tissue *per se*. As such, bypassing the need for pathogen culturing it (or any comparable kit) is applicable as a sensitive, ready-to-use assay to symptomatic alder bark tissue or leaf baits that diagnostic laboratories could utilize. The use of baits is likely to overcome difficulties regarding the direct detection of the pathogen from infested soil that is short of detectable propagules. An early diagnosis of the pathogen in nurseries might be useful to prevent transplanting of infected plants to reforestation plantings. The described marker system will also assist in evaluating population structure and monitoring geographical spread of the different subspecies of the pathogen.

Note added in proof

In a most recent study, Ioos et al. (2005) have developed three PCR primer pairs based on sequence characterized amplified regions of RAPDs. One of these molecular markers detected each subspecies whereas the other two allowed discrimination among the three subspecies of *P. alni*, at a level of detection sensitivity of 0.5–50 pg mycelial DNA. The authors, moreover, successfully attempted the detection of the pathogen directly from naturally infested soil or river water, in addition to symptomatic alder bark or zoospores.

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