A Rapid Diagnostic Test to Distinguish Between American and European Populations of *Phytophthora ramorum*

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ABSTRACT

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A new devastating disease in the United States, commonly known as Sudden Oak Death, is caused by *Phytophthora ramorum*. This pathogen, which previously was described attacking species of *Rhododendron* and *Viburnum* in Germany and the Netherlands, has established itself in forests on the central coast of California and is killing scores of native oak trees (*Lithocarpus densiflora*, *Quercus agrifolia*, *Q. kelloggii*, and *Q. parvula* var. *shrevei*). The phytosanitary authorities in the European Union consider non-European isolates of *P. ramorum* as a threat to forest trees in Europe. To date, almost all European isolates are mating type A1 while those from California and Oregon are type A2. The occurrence of both mating types in the same region could lead to a population capable of sexual recombination, which could generate a new source of diversity. To prevent contact between these two populations, a rapid, reliable, and discriminating diagnostic test was developed to easily distinguish the two populations. Based on a DNA sequence difference in the mitochondrial Cytochrome c oxidase subunit 1 (*Cox*1) gene, we developed a single-nucleotide polymorphism (SNP) protocol to distinguish between isolates of *P. ramorum* originating in Europe and those originating in the United States. A total of 83 isolates of *P. ramorum* from Europe and 51 isolates from the United States were screened and all isolates could be consistently and correctly allocated to either the European or the U.S. populations using the SNP protocol.

Additional keywords: detection, Oomycete, quarantine, species-specific.

The genus *Phytophthora* comprises over 60 described species, and new species are constantly emerging, either by hybridization of species already known (3,6,26) or by the discovery of previously undetected species (12,16,25,28). One of the newly described species is *P. ramorum*, a pathogen of woody plants, including *Rhododendron*, *Viburnum*, and *Quercus* spp. (31,34). Two distinct populations of *P. ramorum* from different regions have been characterized thus far. In the coastal forests of California and Oregon (U.S.), *P. ramorum* has been killing thousands of trees of native oak species (*Lithocarpus densiflora*, *Quercus agrifolia*, *Q. kelloggii*, and *Q. parvula* var. *shrevei*) (31) while surviving and causing damage on a wide range of other shrubs and trees, including bay laurel (*Umbellularia californica*), rhododendron (*Rhododendron* spp.), Douglas fir (*Pseudotsuga menziesii*), and coast redwood (*Sequoia sempervirens*). A second population of *Phytophthora ramorum* has been found in Europe (EU) on *Rhododendron* and *Viburnum* spp. used as ornamental plants in nurseries and landscapes, but so far has been found only rarely in forest ecosystems. These populations appear to be distinct based on amplified fragment length polymorphism (AFLP)-fingerprint patterns (2,19) and differ in mating type (35,36).

Until recently, only A1 mating type isolates have been found in Europe; whereas, in the United States, only A2 type isolates appeared to occur (15,33,35). The presence of two mating types indicates that the pathogen is heterothallic and requires interaction between isolates of opposite mating types for meiotic recombination. These mating types do not correspond to dimorphic forms of the pathogen but are distinguished by the production of specific hormones that induce the formation of gametangia in the opposite mating type. Fusion of male gametangia (antheridia) and female gametangia (oogonia) leads to formation of persistent diploid sexual spores, or oospores (5,21,30). The occurrence of both mating types on a common host has the potential to give rise to meiotic recombination during oospore formation, which could lead to the presence of a sexually reproducing population and greater genetic diversity in the species. The possibility of this occurring is especially poignant given the additional problems encountered with attempts to manage *P. infestans* after introduction of a sexually reproducing population in Europe (10, 17,32).

It is important that A1 type isolates of *P. ramorum* that occur in Europe at the present time can be differentiated from A2 type isolates that presently occur in the United States. Mating type determination tests for *P. ramorum* are carried out with tester strains of other heterothallic species of *Phytophthora* (e.g., *P. cryptogea*). These tester strains are more suitable for mating type assays, because the ability of *P. ramorum* to form oospores in intraspecific crosses often is impaired (35). The mating type of *P. ramorum* isolates is determined by pairing them with tester strains (either of the A1 or the A2 type) and observing the formation of oogonia and antheridia at the interaction zone between the tester strain and the *P. ramorum* isolate. Tester strains of the A2 mating type will induce formation of oogonia and antheridia in *P. ramorum* isolates with mating type A1 (and vice versa), whereas tester strains of the A1 mating type will induce formation of these structures in *P. ramorum* isolates with mating type A2 (and vice versa). However, mating type assays are time consuming and sometimes unsuccessful (35) and, therefore, are not suited for routine diagnostic procedures in a quarantine laboratory. Moreover, it is not yet clear that differences in mating type between

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EU and U.S. isolates are sufficiently consistent; only limited numbers of isolates have been tested so far. The underlying assumption for using mating type tests is that both the EU and U.S. populations are the clonal progeny of single introductions (of an A1 and an A2 isolate, respectively) from an unknown mother population. Recent findings of A2 type isolates in the European population (33) or A1 type isolates in the U.S. population (15) will require the use of more solid, preferentially molecular tests to confirm the genetic background and putative origin of these rare findings.

In recent years, molecular techniques have been used for detection and identification of plant-pathogenic Oomycetes (4,8). In-

TABLE 1. Isolates of *Phytophthora* used in this study: designations, host plant, and country from which isolates were recovered initially, year of isolation, and source of isolates

	Isolate codes[a]		Isolate origin			
Phytophthora spp.	PD, IMI, ATCC	Other	Host plant[b]	Country[c]	Year	Isolate source[d]
P. ramorum	PD 93/56	CBS101327	*Rhododendron* 'Catawbiense'	The Netherlands	1993	Hans de Gruyter PD
P. ramorum	PD 98/5233	CBS101330	*Viburnum* sp.	The Netherlands	1998	Hans de Gruyter PD
P. ramorum	PD 99/2855	…	*Rhododendron* sp.	The Netherlands	1999	Hans de Gruyter PD
P. ramorum	PD 20011060	…	*Viburnum bodnantense* 'Dawn'	The Netherlands	2001	Hans de Gruyter PD
P. ramorum	PD 20011880-2	…	*Rhododendron* sp.	The Netherlands	2001	Hans de Gruyter PD
P. ramorum	PD 20018722	…	*Rhododendron* sp.	The Netherlands	2001	Hans de Gruyter PD
P. ramorum	PD 20019200	…	*Rhododendron* sp.	The Netherlands	2001	Hans de Gruyter PD
P. ramorum	PD 20019355	…	*Rhododendron* 'Cunningham's White'	The Netherlands	2001	Hans de Gruyter PD
P. ramorum	PD 20019414	…	*Rhododendron* sp.	The Netherlands	2001	Hans de Gruyter PD
P. ramorum	PD 20019958	…	*Rhododendron* 'Catawbiense'	The Netherlands	2001	Hans de Gruyter PD
P. ramorum	PD 20019535	BBA 16/99, CBS109278	*Viburnum bodnantense* 'Dawn'	Germany	1999	Sabine Werres BBA
P. ramorum	PD 20019538	BBA 2/4, CBS101554	*Rhododendron* sp.	Germany	…	Sabine Werres BBA
P. ramorum	PD 20019539	BBA 9/95, CBS101553	*Rhododendron* 'Catawbiense'	Germany	1995	Sabine Werres BBA
P. ramorum	PD 200110053	RH/122/98	*Rhododendron* sp.	Poland	…	Leszek Orlikowski RIPF
P. ramorum	PD 200110054	RH/2/00	*Rhododendron* sp.	Poland	…	Leszek Orlikowski RIPF
P. ramorum	PD 200110055	RH/6/00	*Rhododendron* sp.	Poland	…	Leszek Orlikowski RIPF
P. ramorum	PD 20017607	Pr-13	*Quercus agrifolia*	CA, United States	…	Matteo Garbelotto UCB
P. ramorum	PD 20017608	Pr-01	*Q. agrifolia*	CA, United States	…	Matteo Garbelotto UCB
P. ramorum	PD 21008928	Pr-05	*Lithocarpus densiflora*	CA, United States	…	Matteo Garbelotto UCB
P. ramorum	PD 21008929	Pr-06	*Q. agrifolia*	CA, United States	…	Matteo Garbelotto UCB
P. ramorum	PD 21008931	Pr-58	*Vaccinium* sp.	CA, United States	…	Matteo Garbelotto UCB
P. ramorum	PD 21008932	Pr-87	*Arbutus menziessi*	CA, United States	…	Matteo Garbelotto UCB
P. ramorum	PD 21008933	Pr-88	*Umbellularia californica*	Unites States	…	Matteo Garbelotto UCB
P. ramorum	…	Pr-52	*Rhododendron* sp.	CA, United States	…	Matteo Garbelotto UCB
P. ramorum	…	Pr-62	*Q. agrifolia*	CA, United States	…	Matteo Garbelotto UCB
P. ramorum	…	Pr-65	*Q. parvula* var. *shrevei*	CA, United States	…	Matteo Garbelotto UCB
P. ramorum	…	Pr-67	Soil	CA, United States	…	Matteo Garbelotto UCB
P. ramorum	…	Pr-70	*Vaccinium ovatum*	CA, United States	…	Matteo Garbelotto UCB
P. ramorum	…	Pr-102	*Q. agrifolia*	CA, United States	…	Matteo Garbelotto UCB
P. brassicae	…	CBS179.87	*Brassica oleracea*	The Netherlands	1987	CBS
P. cactorum	…	P6183	*Rubus idaeus*	United States	…	Hans de Gruyter PD
P. cinnamomi	…	10A6	Avocado	Italy	…	Querico Migheli
P. citricola[e]	…	P1817	*Medicago sativa*	South Africa	…	Hans de Gruyter PD
P. citrophthora[e]	PD94/353	CBS274.33	*Citrus limonium*	Cyprus	…	Hans de Gruyter PD
P. cryptogea	…	HR1/ss/pp/99	*Lycopersicon esculentum*	United Kingdom	…	Joeke Postma PRI
P. drechsleri	…	CBS292.35	*Beta vulgaris* var. *altissima*	CA, United States	1935	CBS
P. gonapodyides	…	P245	*Salix matsudana*	United Kingdom	1972	David Cooke SCRI
P. heveae[e]	IMI180616	CBS296.29	*Hevea brasiliensis*	Malaysia	1929	David Cooke SCRI
P. hibernalis	ATCC64708	CBS522.77	*Aquilegia vulgaris*	New Zealand	…	CBS
P. hybrid alder	PD92/1471	…	*Alnus cordata*	The Netherlands	1992	Hans de Gruyter PD
P. ilicis[e]	PD91/595	…	*Ilex aquifolium*	The Netherlands	1991	Hans de Gruyter PD
P. infestans	…	Pic99186	*Solanum stoloniferum*	Mexico	1999	Wilbert Flier PRI
P. lateralis	IMI040503	CBS168.42	*Chamaecyparis lawsoniana*	OR, United States	1942	David Cooke SCRI
P. megasperma	IMI133317	MEG23	*Malus sylvestris*	Australia	1968	David Cooke SCRI
P. nemorosa[f]	ATCC MYA-2948	P7	*L. densiflora*	CA, United States	…	Matteo Garbelotto UCB
P. nemorosa[f]	…	P13	*L. densiflora*	CA, United States	…	Matteo Garbelotto UCB
P. nicotianae[e]	…	P582	*Nicotiana tabacum*	United States	…	Hans de Gruyter PD
P. palmivora	…	CBS236.30	*Cocos nucifera*	India	…	CBS
P. pseudosyringae[f]	…	P8	*Q. agrifolia*	CA, United States	…	Matteo Garbelotto UCB
P. pseudosyringae[f]	…	IFB PSEU16	*Fagus sylvatica*	Germany	…	Thomas Jung
P. pseudotsugae	IMI331662	PSE1	*Pseudotsuga menziesii*	United States	…	David Cooke SCRI
P. syringae	IMI045169	CBS364.52	*Prunus armeniaca*	New Zealand	…	CBS

[a] PD = isolate code used by the Plant Protection Service, The Netherlands; IMI = isolate code used by the Commonwealth Agricultural Bureaux; ATCC = isolate code used by the American Type Culture Collection; CBS = isolate code used by the Centraal Bureau voor Schimmelcultures, The Netherlands; BBA = isolate code used by the Biologische Bundesanstalt, Germany.

[b] Plant species from which the isolate was recovered.

[c] Country from which the isolate was initially recovered; CA = California and OR = Oregon.

[d] RIPF = Research Institute for Pomology and Floriculture, Poland; UCB = University of California, Berkeley, United States; PRI = Plant Research International, The Netherlands; SCRI = Scottish Crop Research Institute, UK.

[e] These species of *Phytophthora* are only included in the sequence analysis, not in the restriction fragment length polymorphism (RFLP) assay.

[f] These species of *Phytophthora* are only included in the RFLP assay, not in the sequence analysis.

ternal transcribed spacer (ITS) sequences of rDNA and single-strand-conformation polymorphisms have proven useful in the identification of *Phytophthora* spp. (7,23). Based upon ITS sequence analysis, a species-specific polymerase chain reaction (PCR) detection method for *P. ramorum* was developed and validated using stem and leaf material from plants infected with *P. ramorum* (13). Real-time detection tests for *P. ramorum* also have been developed using molecular beacons and Taqman probes (1,18,24). However, these tests do not differentiate between A1 and A2 isolates of the pathogen. AFLP DNA fingerprinting showed significant differences between EU and U.S. populations (2,19), but this technique is laborious and results sometimes are hard to analyze consistently.

A phylogenetic study on species of *Phytophthora*, using a multigene sequencing approach (L. P. N. M. Kroon, *unpublished data*), revealed sequence differences between EU and U.S. isolates of *P. ramorum* in the mitochondrial Cytochrome c oxidase subunit 1 (*Cox*1) gene. These differences were exploited to develop a diagnostic assay for *P. ramorum* isolates. The objectives of this study were to (i) test whether EU and U.S. populations of *P. ramorum* can be distinguished consistently by their mitochondrial *Cox*1 gene sequence; (ii) if so, develop a reproducible single-nucleotide polymorphism (SNP) assay to detect and differentiate between EU and U.S. populations of *P. ramorum*; and (iii) compare sequence-based restriction fragment length polymorphism (RFLP) profiles of isolates of *P. ramorum* with those of closely related species and species that occur on common hosts.

MATERIALS AND METHODS

Isolates and cultures. Stems and leaves from plants of *Rhododendron* and *Viburnum* spp. colonized by *P. ramorum* were collected during a national survey of nurseries and gardens in the Netherlands in 2001. Infected leaf and stem material, taken from the periphery of lesions, was surface sterilized in 50% ethanol and rinsed in water. Plant pieces were placed on selective P_5ARPH medium (per liter: 17 g of cornmeal agar [Difco Laboratories, Detroit], 5 mg of pimaricin, 250 mg of sodium ampicillin, 10 mg of rifampicin, 100 mg of pentachloronitrobenzene [75% a.i.], and 50 mg of hymexazol [70% a.i.]) (20) and incubated in the dark at 22°C. Individual isolates of *P. ramorum* were obtained by transferring agar pieces containing growing mycelium from colonies on P_5ARPH plates to plates of cherry agar (per liter: 100 ml of cherry pulp and 15 g of agar [Oxoid, Basingstoke, UK]) and incubated in the dark at 22°C for 1 to 2 weeks. Isolates were stored on V8 slants (per liter: 200 ml of V8 juice, 2 g of $CaCO_3$, 0.05 g of β-sitosterol, and 15 g of agar) at 4°C in the dark. Researchers from Europe and the United States provided us with a diverse range of additional samples, covering the known genetic and geographic diversity in *P. ramorum*. In total, 83 isolates of *P. ramorum* from Europe and 51 isolates from the United States were included in this study (Tables 1 and 2). In addition, isolates of 21 other species of *Phytophthora* were included in the analysis to evaluate the species specificity of the assay (Table 1).

DNA extraction. Isolates were grown in the dark for 10 to 14 days at 20°C in pea broth. This medium was prepared by autoclaving 120 g of frozen peas in 1 liter of tap water. The broth was filtered through cheesecloth to remove the peas and then was autoclaved again (11). Mycelium was harvested and lyophilized, and total DNA was extracted using the Puregene kit (Gentra/Biozyme, Landgraaf, The Netherlands) according to the manufacturer's instructions. The DNA pellet was dissolved in 100 µl of TE (10 mM Tris-HCl, pH 8.0, and 1 mM EDTA), and the solution was stored at –20°C. For infected plant material, DNA was extracted directly from tissue on the periphery of lesions on stems and leaves. From infected host plants found in the Netherlands, 20 samples of infected *Rhododendron* spp. and 4 samples of infected *Viburnum* spp. were analyzed. In addition, infected leaf material (12 samples) of plants inoculated with either an EU- or U.S.-type isolate in greenhouse experiments (9) was analyzed. Small pieces (100 to 200 mg) of surface-sterilized (15 s in a 1% sodium hypochlorite solution) diseased plant tissue were homogenized in 300 µl of extraction buffer (0.02 M phosphate-buffered saline, 0.05% Tween T25, 2% polyvinylpyrrolidone, 0.2% bovine serum albumine) using a cell disrupter (RiboLyzer, Hybaid Thermo, Ashford, UK). The resulting suspension was centrifuged at 13,200 rpm (16,100 × *g*) in a microcentrifuge (Eppendorf, Hamburg, Germany) for 5 s, and 75 µl of the supernatant was transferred to a new 1.5-ml microcentrifuge tube. Total DNA was extracted using the DNeasy Plant Mini Kit (Qiagen, Helden, Germany) according to the manufacturer's instructions. The DNA was eluted with 50 µl of AE buffer, further purified on a Micro Bio-Spin chromatography column filled with polyvinylpolypyrrolidone (Bio-Rad, Hercules, CA), and stored at –20°C.

PCR amplification. For amplification of the *Cox*1 gene, the complete mitochondrial DNA-sequence U17009 of *P. infestans* (29) was used as a template to develop primers. CoxF4N 5′ GTATTTCTTCTTTATTAGGTGC 3′ (base pairs 9,126 to 9,147) and CoxR4N 5′ CGTGAACTAATGTTACATATAC 3′ (base pairs 10,076 to 10,097) were chosen to amplify a fragment of 972 bp. DNA from 49 isolates of *P. ramorum* (42 EU and 7 U.S.) was used as template. Representative isolates from each of 21 other

TABLE 2. Isolates of *Phytophthora ramorum* used in this study; country and host plant from which isolates were recovered initially

Country	Host plant[a]							Total
	Rhododendron spp.	*Viburnum* spp.	*Quercus* spp.	*L. densiflora*	*V. ovatum*	*U. californica*	Other	
Europe								
Belgium	2	3	…	…	…	…	…	5
Germany	3	4	…	…	…	…	…	7
France	4	3	…	…	…	…	…	7
The Netherlands	30	11	…	…	…	…	…	41
Poland	3	…	…	…	…	…	…	3
Spain	3	1	…	…	…	…	…	4
Sweden	2	…	…	…	…	…	…	2
United Kingdom	6	8	…	…	…	…	…	14
Total	…	…	…	…	…	…	…	83
United States								
California	2	…	10	7	4	6	16	45
Oregon	1	…	…	1	2	1	1	6
Total	…	…	…	…	…	…	…	51

[a] Host plants: *Quercus* spp. = *Q. agrifolia*, *Q. parvula* var. *shrevei*, *Q. chrysolepis*; *Lythocarpus densiflora*; *Vaccinium ovatum*; *Umbellularia californica*; Other = *Acer macrophyllum*, *Aesculus californica*, *Arbutus menziesii*, *Corylus cornuta*, *Heteromeles arbutifolia*, *Lonicera hispidula*, *Pseudotsuga menziesii*, *Rhamnus cathartica*, *Rhamnus purshiana*, *Sequoia sempervirens*, soil, and *Trentalis latifolia.*

Phytophthora spp. also were included in the analysis (Table 1). These species were chosen based on two criteria: pathogenicity on *Rhododendron* spp. or *Quercus* spp. (hosts of *P. ramorum*) or a close clustering to *P. ramorum* in phylogenetic analyses (L. P. N. M. Kroon, *unpublished data*). The reaction mix consisted of 10 to 20 ng of template DNA, 200 µM dNTPs, 1 U of *Taq* DNA polymerase (Roche, Indianapolis, IN), 3.5 mM of $MgCl_2$, and 25 ng of each primer (CoxF4N and CoxR4N) in a reaction volume of 25 µl. Amplifications were run in a PTC200 thermocycler (MJ Research, Waltham, MA), with an initial denaturation at 94°C for 2 min, followed by 35 cycles consisting of denaturation at 94°C for 30 s, annealing at 52°C for 30 s, and extension at 72°C for 60 s. A final extension at 72°C for 10 min followed.

Based on a comparison of *Cox*1 DNA sequence information for *P. ramorum* isolates with the *Cox*1 sequences of 19 other *Phytophthora* spp., a nested PCR primer pair was developed that was specific for *P. ramorum*. Primers PrnestF 5′ TAGCTACTTTATGGGGTGGTTCA 3′ (base pairs 508 to 530 of the 972-bp fragment) and PrnestR 5′ CATTCCAACCACTCATAGCATCA 3′ (base pairs 869 to 891) were chosen to amplify a fragment of 383 bp that included the SNP site. DNA from 99 isolates of *P. ramorum* (52 EU and 47 U.S.) was used as template. Amplification conditions were identical to the procedure described for the CoxF4N and CoxR4N primer pair, with the exception of the annealing temperature, which was raised to 69°C. The detection limit for the nested PCR primer pair was determined using a dilution series (1 ng to 0.1 fg of pure *P. ramorum* DNA per reaction). DNA from other *Phytophthora*

		position 773 ▼
PD20017607	USA	GAAATTTTAGGTCAAATTCATTTTTGGTTATTTTT
PD20017608	USA	GAAATTTTAGGTCAAATTCATTTTTGGTTATTTTT
PD20019539	Germany	GAAATTTTAGGTCAAATCCATTTTTGGTTATTTTT
PD 93/56	Netherlands	GAAATTTTAGGTCAAATCCATTTTTGGTTATTTTT
PD20019535	Germany	GAAATTTTAGGTCAAATCCATTTTTGGTTATTTTT

Fig. 1. Sequence alignment of part of the 972-bp amplicon of the cytochrome C oxidase subunit 1 gene for five isolates of *Phytophthora ramorum* from the United States and Europe. The shaded area denotes the additional *Apo*I restriction site present in isolates from the United States. Isolate information is listed in Table 1.

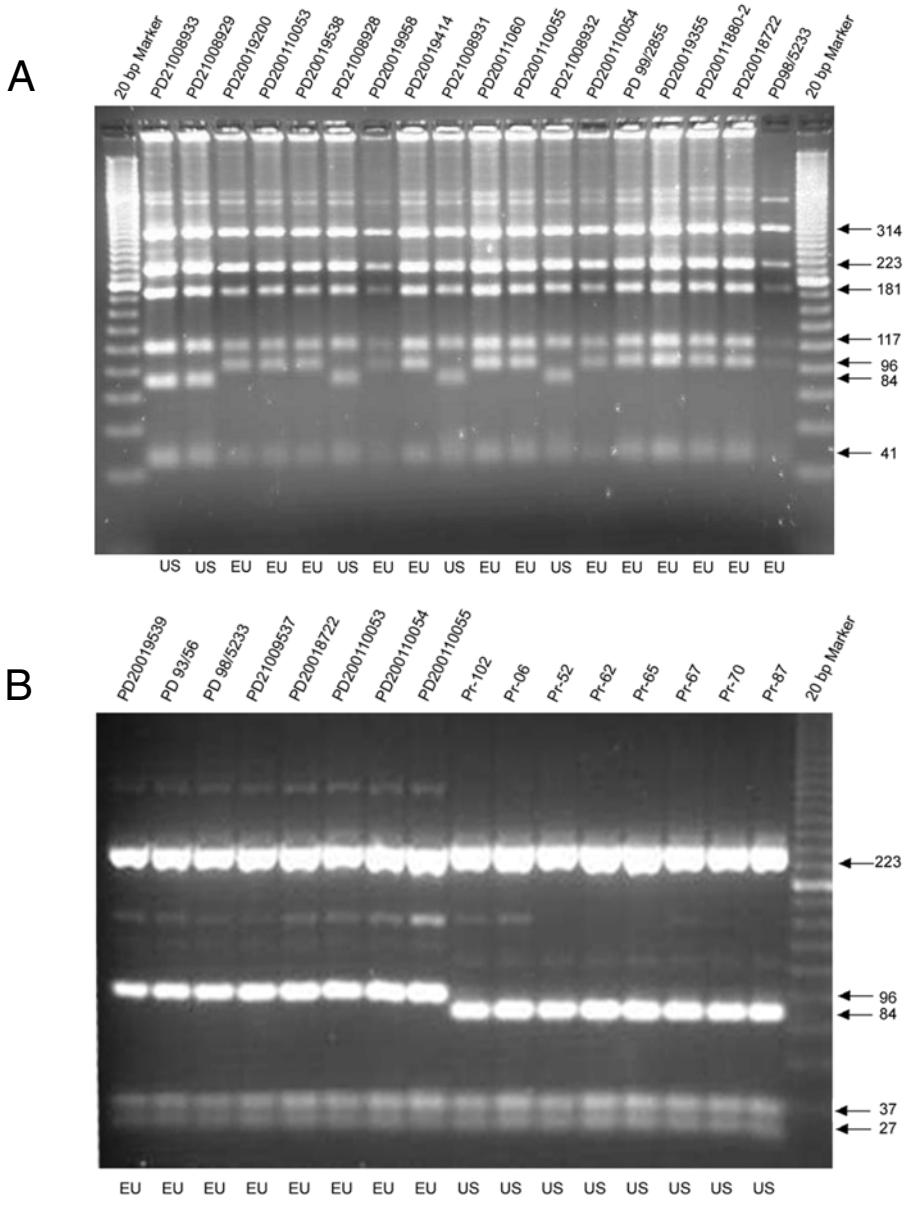

Fig. 2. Restriction fragment length polymorphism patterns for the **A,** 972-bp amplicon and **B,** 383-bp amplicon of the cytochrome C oxidase subunit 1 gene for **A,** 18 and **B,** 16 arbitrarily chosen isolates of *Phytophthora ramorum*. Isolates in **A,** lanes 1, 2, 6, 9, and 12 and **B,** lanes 9 to 16 came from the United States (US); isolates in the other lanes came from Europe (EU). Details on isolates are listed in Table 1. A 20-bp marker is used as size reference.

spp. (Table 1) was used to confirm species specificity of the nested PCR primer pair.

For amplification of the ITS region in samples from infected plants, the reaction mix consisted of 5 µl of template DNA (unknown concentration), 200 µM dNTPs, 1 U of HotStar *Taq* DNA polymerase (Qiagen, Hilden, Germany), 1.5 mM of $MgCl_2$, and 0.4 µM each primer (Phyto1 5′ CATGGCGAGCGCTTGA 3′ and Phyto4 5′ GAAGCCGCCAACACAAG 3′) (13). Amplifications were conducted in a PTC200 thermocycler, with an initial denaturation at 95°C for 15 min, followed by 35 cycles consisting of denaturation at 94°C for 15 s, annealing at 62°C for 60 s, extension at 72°C for 45 s, and a final extension at 72°C for 10 min. The presence of *P. ramorum* was confirmed by observing a diagnostic band at 687 bp using gel electrophoresis (2 h at 100 V) on 1.0% agarose gels (MP, Roche, Mannheim, Germany) in 0.5× Tris-borate EDTA (TBE) buffer. DNA fragments were visualized after staining with ethidium bromide and illuminating under UV light.

Sequence analysis. PCR products were purified on Sephadex plates (Multiscreen HV, Millipore, Bedford, MA) to remove excess primers and nucleotides. Products were sequenced with the primers CoxF4N or CoxR4N using the BigDye sequencing kit (Applied Biosystems, Foster City, CA) and an ABI3700 DNA Analyzer (Applied Biosystems). The trace files originating from the ABI-sequencer were transferred to the SeqMan 5.0 module of DNASTAR (DNASTAR Inc., Madison, WI). Forward and reverse sequences were coupled in Editseq 5.0 (DNASTAR). The sequences were aligned using the ClustalW algorithm in the program MegAlign 5.0 (DNASTAR). Restriction patterns of isolates from the United States and Europe were compared in MapDraw 5.00 (DNASTAR) and differential restriction sites were identified. This program also was used to compare restriction profiles of isolates of *P. ramorum* with those of isolates of 21 other species of *Phytophthora* included in this study (Table 1).

SNP analysis. The 972-bp PCR fragment of the *Cox*1 gene, or the 383-bp nested fragment, was digested with the restriction enzyme *Apo*I ([A/G]AATT[C/T]) (New England Biolabs, Beverly, MA) for 1 h at 50°C according to the manufacturer's instructions. The resulting fragments were separated by gel electrophoresis on 3% REsult LE agarose gels (Biozym, Hess. Oldendorf, Germany) in 0.5× TBE buffer run at 100 V for 3 h. The DNA fragments were visualized after staining with ethidium bromide and illuminating under UV light.

RESULTS

PCR amplification. Using primers CoxF4N and CoxR4N, a 972-bp fragment from the *Cox*1 gene was amplified from a total of 33 isolates of *P. ramorum*, including 27 isolates from Europe and 6 isolates from the United States. With inner primers PrnestF and PrnestR, a 383-bp fragment was amplified in 99 samples of DNA from isolates of *P. ramorum*, including 52 isolates from Europe and 47 isolates from the United States. Of these 99 samples, 11 samples from Europe and 3 samples from the United States were tested in both the 972-bp and 383-bp protocol. Specificity of the primers PrnestF and PrnestR was tested for 22 isolates of 17 different *Phytophthora* spp. (Table 1). By increasing the annealing temperature to 69°C, amplification of the 383-bp product was restricted to isolates of *P. ramorum*. Only *P. hibernalis* showed limited cross-amplification under these stringent conditions (data not shown). By using the inner primers in a second PCR amplification (nested PCR), the sensitivity of detection was increased considerably. A dilution series of DNA from *P. ramorum* amplified with single or nested PCR detected 100 pg and 10 fg of DNA, respectively.

SNP analysis. The *Cox*1 fragment that was amplified in DNA of isolates of *P. ramorum* contained an SNP at position 773 of the 972-bp amplicon (position 266 of the 383-bp amplicon) that distinguished between isolates from Europe and the United States (Fig. 1). Isolates from Europe have a cytosine residue at position 773, whereas U.S.-type isolates show a thymine residue at this site. The restriction enzyme *Apo*I was used to develop an SNP assay that can discriminate between isolates of *P. ramorum* from Europe and those from the United States. In a blind test of 18 arbitrarily selected isolates of *P. ramorum* from Europe and the Unites States, five isolates were found to share the additional *Apo*I restriction site characteristic of the U.S. population (Fig. 2A), whereas the other 13 isolates had the restriction profile characteristic of the European population. All isolates of *P. ramorum* were correctly and consistently assigned to their geographic region of recovery based on the SNP assay. For the smaller 383-bp amplicon, a less complex RFLP pattern was obtained, clearly distinguishing isolates from Europe and the United States (Fig. 2B). In all, 33 isolates were tested using the 972-bp *Cox*1 amplicon for the *Apo*I digest (27 from Europe and 6 from the United States) while 99 isolates were analyzed using the 383-bp amplicon (52 from Europe and 47 from the United States).

Infected material. DNA extracted from inoculated or naturally infected plants also was analyzed with this SNP assay. SNP restriction patterns from eight inoculated plant samples are shown in Figure 3. DNA from plants artificially infected with isolates of *P. ramorum* from Europe produced an RFLP pattern similar to the one produced by European isolates of *P. ramorum* and DNA samples from plants artificially infected with isolates from the United States gave the U.S. RFLP pattern. DNA samples from naturally infected plants from Europe first were screened for the presence of *P. ramorum* using the ITS species-specific primers and 36 samples were identified as positive (data not shown). In 21 of these 36 samples, the presence of EU-type isolates was determined using the SNP assay on the 972-bp fragment. No cross-amplification of *Q. rubra*, *Rhododendron* spp., or *Viburnum* spp. DNA was observed in experiments on infected plant material.

Comparison of *P. ramorum Apo*I restriction profiles with other *Phytophthora* spp. Using primers CoxF4N and CoxR4N,

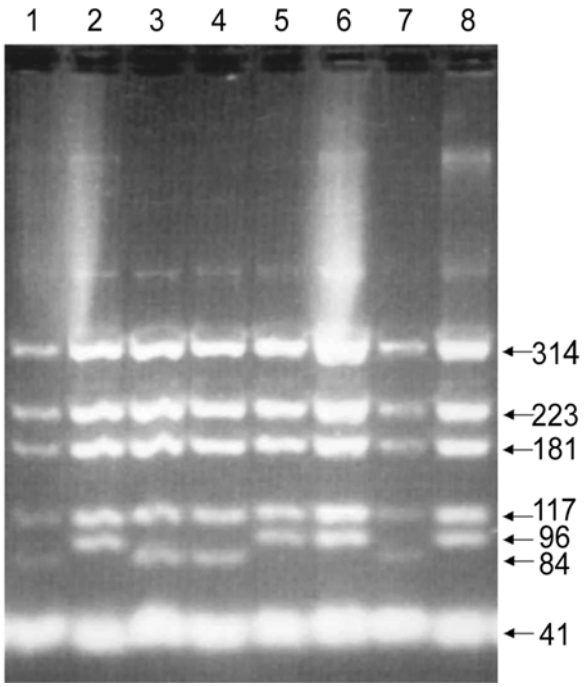

Fig. 3. Restriction fragment length polymorphism patterns for the 972-bp amplicon of the cytochrome C oxidase subunit 1 gene for eight samples of DNA from infected plants inoculated with *Phytophthora ramorum* isolate PD20017608 (US-type, lanes 1, 3, 4, and 7) or PD20019539 (EU-type, lanes 2, 5, 6, and 8). Lanes 1 and 2 = *Quercus rubra*; lane 3 to 6 = *Rhododendron* sp.; lanes 7 and 8 = *Vaccinium* sp.

the 972-bp fragment was amplified in isolates of 21 other species of *Phytophthora* (Table 1) that are closely related to *P. ramorum* or are pathogenic on hosts common with *P. ramorum* (data not shown). Although amplification efficiency varied considerably among species, *Apo*I restriction profiles could be determined for most species and these profiles corresponded with predicted fragment size from sequence analysis for all species analyzed (data not shown). For five species for which no clear RFLP profile could be obtained (Table 1), restriction profiles were based on sequence information only. In total, 9 *Apo*I sites were found throughout the sequences and 16 distinct RFLP profiles were observed (Table 3). European isolates of *P. ramorum* possessed a unique profile, but isolates from the United States had a profile similar to that of *P. brassicae*. The latter species, however, consistently showed additional fragments resulting from partial digestion.

Using the nested primers PrnestF and PrnestR, the 383-bp *Cox*1 fragment was amplified only from *P. ramorum*, with some cross-amplification for *P. hibernalis*. However, digestion of the 383-bp amplicons with *Apo*I generated restriction profiles that could differentiate these species. Fragment sizes for *P. hibernalis* were 235, 84, 37, and 27 bp, compared with 223, 84, 37, 27, and 12 bp for European isolates and 223, 96, 37, and 27 bp for U.S. isolates of *P. ramorum*.

DISCUSSION

Historically, identification of species of *Phytophthora* has been based on morphological characteristics and growth on selective media, which can be time consuming and laborious. In addition, considerable expertise is needed to clearly differentiate among closely related species. When dealing with quarantine pathogens, suspicious material should be evaluated quickly and reliably for the presence of the quarantined organism. Quick evaluation methods will avoid deterioration of product quality as well as expensive storage of arboriculture trade materials. Reliable evaluation methods are essential to take adequate phytosanitary measures in trade and forestry, and may prevent the spread of quarantine organisms to new areas. More and more molecular techniques are being deployed for rapid detection and identification of plant pathogens (8,23). For *Phytophthora* spp., ITS sequences of ribosomal DNA have been shown to be useful for species identification, although some related species share identical ITS sequences (12). For *P. ramorum*, an ITS-based PCR detection method is available (13), but it cannot discriminate between isolates from Europe and isolates from the United States.

The present test, based on the mitochondrial *Cox*1 gene, is the first to differentiate between European and U.S. isolates of the pathogen. A single point mutation in this gene distinguished isolates of *P. ramorum* from Europe from those from the United States. Based on this point mutation, we developed a single nucleotide polymorphism-based method, in which a multicopy marker is amplified in *P. ramorum*, followed by a digestion with restriction enzyme *Apo*I. Although the SNP assay initially was designed to be used on in vitro cultures of *P. ramorum*, additional tests demonstrated the potential for using this assay to detect *P. ramorum* in planta.

In order to be reliably used in a regulatory setting, the potential for false positives, caused by the presence of other *Phytophthora* spp., must be ruled out. Therefore, *Phytophthora* spp. with a host range similar to that of *P. ramorum*, and closely related species (based on a phylogeny of the genus inferred from parsimony analysis of mitochondrial and nuclear DNA; L. P. N. M. Kroon, *unpublished data*) were screened for RFLP patterns of the *Cox*1 gene product with the *Apo*I restriction enzyme. Most species possessed unique RFLP profiles that could differentiate them from *P. ramorum*. Although European isolates of *P. ramorum* shared a unique and distinct profile, isolates from the United States had a different profile that was the same as the predicted profile for *P. brassicae*. However, it is unlikely that *P. brassicae* and *P. ramorum* would be found in the same ecological niche; *P. brassicae* typically is found only on nonwoody hosts that mainly are in the family *Amaryllidaceae* (25). Moreover, in RFLP analysis, the *P. brassicae* isolate showed additional, uncut

TABLE 3. *Apo*I restriction fragment lengths estimated from sequence analysis for the 972-bp amplicon of the Cytochrome c oxidase subunit 1 gene for isolates of *Phytophthora ramorum* from Europe (EU) and the United States (US) and additional species of *Phytophthora*

	Restriction fragment size (bp)[a]																	
Phytophthora spp.	436	355	314	235 237	223	199 201	181	153	143	117 119 120	96	84	80	41[b]	36[b]	28[b]	24[b]	12[b]
P. ramorum EU	…	…	+	…	+	…	+	…	…	+	+	…	…	+	…	…	…	…
P. ramorum US	…	…	+	…	+	…	+	…	…	+	…	+	…	+	…	…	…	+
P. brassicae	…	…	+	…	+	…	+	…	…	+	…	+	…	+	…	…	…	+
P. cactorum	…	+	…	…	…	…	+	…	…	+++	…	…	+	…	…	…	…	…
P. cinnamomi	…	…	+	+	…	+	+	…	…	…	…	…	…	+	…	…	…	…
P. citricola	…	…	+	…	+	+	+	…	…	…	…	…	…	+	…	…	…	+
P. citrophthora	…	…	+	…	…	…	+	…	…	++	…	+	+	+	…	…	+	+
P. cryptogea	…	…	+	…	+	+	+	…	…	…	…	…	…	+	…	…	…	+
P. drechsleri	…	…	+	+	…	+	+	…	…	…	…	…	…	+	…	…	…	…
P. gonapodyides	…	+	…	…	…	+	+	…	…	+	…	…	+	…	…	…	+	+
P. heveae	…	…	+	…	+	++[c]	+	…	…	…	…	…	…	+	…	…	+[c]	+
P. hibernalis	…	…	+	+	…	…	+	…	…	+	…	+	…	+	…	…	…	…
P. hybrid alder	…	…	+	…	…	+	…	+	…	+	…	…	+	+	+	+	…	…
P. ilicis	…	…	+	…	…	…	+	…	+	+	+	…	+	+	…	…	…	…
P. infestans	…	+	…	…	…	+	+	…	…	+	…	…	+	…	…	…	+	+
P. lateralis	…	+	…	…	+	…	+	…	…	+	…	+	…	…	…	…	…	+
P. megasperma	…	…	+	…	…	+	+	…	…	+	+	…	…	+	…	…	+	…
P. nemorosa	…	…	+	…	…	…	+	…	+	+	…	+	+	+	…	…	…	+
P. nicotianae	…	+	…	…	…	+	+	…	…	+	…	+	…	…	+	…	…	…
P. palmivora	…	+	…	…	…	+	+	…	…	+	…	+	…	…	+	…	…	…
P. pseudosyringae	…	…	+	...	…	…	+	...	+	+	+	...	+	+	...	…	…	…
P. pseudotsugae	…	+	…	…	…	…	+	…	…	+++	…	…	+	…	…	…	…	…
P. syringae	+	…	+	…	…	…	+	…	…	…	…	…	…	+	…	…	…	…

[a] Restriction fragments are pooled in one column if the size difference between the fragments is not distinguishable on gel.
[b] These fragments are difficult to visualize due to low intensity or co-migration of primers in the gel.
[c] Alternate restriction profile because of a variable *Apo*I restriction site in the 223-bp fragment.

*Apo*I fragments and, thus, was distinguishable from *P. ramorum* isolates. The recently described species *P. nemorosa* and *P. pseudosyringae*, which share an overlapping geographic distribution with *P. ramorum* in California (16,27), also were included in this analysis. These species clearly were distinguishable from *P. ramorum* in RFLP analysis; thus, the presence of these species in California will not compromise the accuracy of the assay.

Intraspecific sequence variation may result in altered *Apo*I restriction profiles. For some species showing a profile similar to that of *P. ramorum*, this potentially could lead to mis-identification as *P. ramorum* isolates. However, a combination of the SNP assay with the species-specific ITS assay (13) or use of the nested *Cox*1-PCR will overcome this lack of specificity. The nested *Cox*1-PCR combines an increased detection limit, species specificity for *P. ramorum*, and a clear distinction between U.S. and European populations of *P. ramorum* after *Apo*I digestion. There is some cross-amplification for the closely related species *P. hibernalis*; however, in side-by-side comparisons, the *Apo*I restriction profile should differentiate these species.

Preventing the introduction of U.S.-type isolates of *P. ramorum* into Europe, or European-type isolates into the United States, is of key importance. Although progeny between the two populations is hard to obtain, it recently has been demonstrated that there is a chance of the establishment of a sexual reproducing population (35). However, the vitality and genetic content of the oospores produced in these experiments has not been tested. Studies with other species of *Phytophthora* (e.g., *P. infestans*) (10,32) indicate that oospores can survive for several years, even under unfavorable conditions. The occurrence of oospores could lead to additional problems, by providing the pathogen with new opportunities for survival and dispersal, thus thwarting eradication and control strategies for *P. ramorum*. If the two populations differ greatly in genetic content and virulence factors, giving them the opportunity for meiotic recombination would open the way for the generation of new genotypes of the pathogen.

In Europe, one isolate has been recovered that had the A2 mating type and the European SNP profile for *Cox*1 (33). This isolate clustered with European isolates in AFLP analysis (L. P. N. M. Kroon and P. J. M. Bonants, *unpublished data*). This observation may be explained by the process of selfing in A1-type isolates under influence of other species of *Phytophthora* (22) or chemicals (14). In this way, A2-type isolates may be formed with a genetic content and SNP profile similar to that of the European A1-type isolates. The reported presence of A1-type isolates in the Unites States (15) with the European type SNP profile may be explained by a secondary introduction of *P. ramorum* isolates, which show similarity with the European population based on AFLP analysis.

In the event that the populations of *P. ramorum* from the United States and Europe occur in the same region and form sexual progeny, the SNP assay described in this article no longer can be applied to distinguish these populations. The mitochondrial DNA, on which the *Cox*1 gene is situated, will be maternally inherited in the progeny and the association between mating type and mitochondrial type will be lost. Findings of both populations in Oregon nurseries has resulted in extensive eradication measures and monitoring at these sites (15).

In Europe, *P. ramorum* recently has been isolated from oak (*Q. rubra*) and this isolate was characterized as EU-type with the A1 mating type. This finding may indicate that the host range of the European population is not limited to *Rhododendron* and *Viburnum* spp. Inoculation experiments using isolates from European and U.S. populations on various host plants are in progress (9). Further studies will be performed to investigate the populations on both continents in more detail. The present *Cox*1-based SNP assay offers an attractive test for differentiating isolates at the population level, both for import quarantine purposes and for national surveys for *P. ramorum* biotypes.

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