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PCR-RFLP markers identify three lineages of the North American and European populations of *Phytophthora ramorum*

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Summary

Phytophthora ramorum, the cause of sudden oak death and ramorum blight, has three major clonal lineages and two mating types. Molecular tests currently available for detecting *P. ramorum* do not distinguish between clonal lineages and mating type is determined by cultural methods on a limited number of samples. In some molecular diagnostic tests, cross-reaction with other closely related species such as *P. hibernalis*, *P. foliorum* or *P. lateralis* can occur. Regions in the mitochondrial gene *Cox1* are different among *P. ramorum* lineages and mitochondrial genotyping of the North American and European populations seems to be sufficient to differentiate between mating types, because the EU1 lineage is mostly A1 and both NA1 and NA2 lineages are A2. In our study, we were able to identify *P. ramorum* isolates according to lineage using polymerase chain reaction-restriction fragment-length polymorphism (PCR-RFLP) of the *Cox1* gene, first by using *Apo*I to separate *P. ramorum* from other species and EU1 from North American populations, and then *Ava*I to distinguish between NA1 and NA2 genotypes. However, *P. foliorum* had the same restriction profile as *P. ramorum* NA1 isolates.

1 Introduction

Phytophthora ramorum S. Werres & A.W.A.M. de Cock (Oomycetes: Peronosporales), the cause of sudden oak death in the US and ramorum blight in Europe, causes damage on more than 70 species of trees and shrubs (USDA-APHIS 2007). Nursery stock is one of the primary modes of transmission and countries where the disease is found have quarantine measures to prevent further spread (EPPO 2006). The disease has been detected and eradicated several times from nurseries in British Columbia between the years 2003 and the present. Early detection and eradication in nurseries are critical to keeping *P. ramorum* out of the forest environment (Mascheretti et al. 2008). As the organism is microscopic and symptoms resemble those of other plant pathogens or are innocuous on some hosts, detection is problematic. Based on molecular data, there are three clonal lineages of *P. ramorum* (Ivors et al. 2006): one from Europe (EU1) and two from North America (NA1 and NA2).

Phytophthora ramorum is heterothallic, requiring two mating types (A1 and A2) for sexual reproduction. A sexually reproducing population of *P. ramorum* could give rise to new genotypes that may be more damaging than the parent strains, and have an increased host range or tolerance for more extreme environmental conditions. There is evidence for

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the occurrence of sexual reproduction in *P. ramorum* in the past, creating new clonal lineages that proliferate asexually (Mascheretti et al. 2008).

Mating type in *P. ramorum* is determined by crossing a given isolate with tester isolates of known mating type and observing the formation of sexual structures. This test is time-consuming and not always reliable because some isolates will not mate in culture. In addition, only a small number of isolates have been tested for mating type and this test is not done routinely when examining samples from nursery surveys. A molecular diagnostic test based on mating-type genes in *P. ramorum* has not yet been developed, because these genes in *Phytophthora* are poorly understood and have not yet been cloned or sequenced (Judelson 1996; Gu and Ko 2005). However, mitochondrial genotyping of the North American and European populations seems to be sufficient for differentiating between mating types, because the EU1 population is mostly A1 with a few exceptions (Werres and De Merlier 2003) and both NA1 and NA2 lineages are A2. Both mating types have the same host range, and pathogenicity to different hosts appears to be similar for a given isolate (Brasier et al. 2006).

The A1 mating type of *P. ramorum* is found in Europe and North America, but is the predominant mating type in Europe (Hansen et al. 2003; Prospero et al. 2007). All A1 isolates discovered belong to the EU1 lineage (Brasier et al. 2006). Isolates of *P. ramorum* collected from forests in California and Oregon form a separate clade based on mitochondrial DNA sequences and microsatellite data (Ivors et al. 2006; Prospero et al. 2007; Martin 2008) and all the isolates tested belong to the A2 mating type. This clonal lineage is designated 'NA1' and includes the genome-sequenced strain, Pr102 (Tyler et al. 2006). However, some isolates from North American nurseries belonging to the A2 mating type behave phenotypically like EU1 isolates, being fast growing and having similar colony morphology. These isolates have a unique mitochondrial and microsatellite genotype and belong to the NA2 clonal lineage (Ivors et al. 2006; Martin 2008). The NA2 clade is distinctly different from EU1 and NA1, and has so far only been found in North American nurseries. The differences in the A1 and A2 mating types and their the obstacles to mating in culture, as well as the lack of evidence for sexual reproduction in isolates collected to date where both mating types coexist, suggest a mating barrier in *P. ramorum* (Brasier et al. 2005). All three lineages of *P. ramorum* have been found in North American nurseries (Grunwald et al. 2008).

Phytophthora lateralis Tucker & Milbrath is a closely related species found in the forest environment but does not cross-amplify with *P. ramorum*-specific primers in the mitochondrial *Cox1* region (Kroon et al. 2004; Martin et al. 2004). *Phytophthora foliorum* Donahoo & Lamour is a species described recently and found on azalea during nursery surveys for *P. ramorum* (Donahoo et al. 2006). *Phytophthora hibernalis* Carne, like *P. ramorum* and *P. foliorum*, is another species found in nurseries (Blomquist et al. 2005; Yakabe et al. 2007). *Phytophthora hibernalis* is a known pathogen on citrus (Goodwin et al. 1995), and was found in an Oregon nursery during surveys for *P. ramorum* on rhododendron (Osterbauer et al. 2004). Unlike *P. ramorum*, both *P. foliorum* and *P. hibernalis* are homothallic and do not require the presence of an opposite mating type to form sexual structures. Neither *P. foliorum* nor *P. hibernalis* forms chlamydospores. All three of these species belong to clade 8c (Blair et al. 2008) and produce deciduous sporangia, making airborne or water-splash dispersal possible. Long-term survival structures are asexual chlamydospores in *P. ramorum* and sexual oospores in *P. foliorum* and *P. hibernalis* (Ho and Jong 1993; Werres et al. 2001; Donahoo et al. 2006).

Many polymerase chain reaction (PCR)-based diagnostic tests for *P. ramorum* use the ribosomal ITS region to differentiate between *Phytophthora* species but are not sensitive enough to distinguish between closely related *Phytophthora* species or clonal lineages of *P. ramorum* (Kroon et al. 2004; Osterbauer et al. 2004; Blomquist et al. 2005;

Osterbauer and Trippe 2005; Donahoo et al. 2006). The mitochondrial gene *Cox1* was chosen for molecular typing in this study because of the presence of regions that are different among *P. ramorum* clonal lineages (Kroon et al. 2004; Ivors et al. 2006; Martin 2008). The *Cox1* gene is present in high copy numbers in the genome, providing a larger target concentration than single-copy genes.

The objectives of this study were to develop a polymerase chain reaction-restriction fragment-length polymorphism (PCR-RFLP) marker that would distinguish between NA1 and NA2 genotypes of *P. ramorum* and the closely related species *P. foliorum*, *P. hibernalis* and *P. lateralis*.

2 Materials and methods

2.1 Isolates

A collection of 23 isolates representing the geographic range and all three clonal lineages of *P. ramorum* were examined. These included five from Europe and 18 from North America. Other closely related *Phytophthora* species such as *P. hibernalis*, *P. lateralis* and *P. foliorum* were included for comparison (Table 1). Isolates were imported into Canada under permits P-2006-00625, P-2006-00626 and P-2006-00627 issued by the Canadian Food Inspection Agency. The collection of isolates imported from the United States included isolates from the US and Europe and was studied at the Pacific Forestry Centre, Canadian Forest Service, Victoria, BC, while isolates from Canada were studied at the Sidney Laboratory, Canadian Food Inspection Agency, Sidney, BC. All isolates were collected between 1995 and 2005, with most collected in the years 2002–2003.

2.2 Selection of a restriction enzyme for differentiation of NA1 and NA2 genotypes

Sequences of the *Cox1* gene (Ivors et al. 2006) were downloaded from GenBank and aligned using ClustalX version 1.8 (Thompson et al. 1997). Virtual restriction digests were performed using RestrictionMapper v. 3.0 (http://www.restrictionmapper.org/). Three enzymes (*Ava*I, *Sml*1 and *Xho*1) distinguished between the two North American A2 mitochondrial genotypes (NA1 and NA2) at the 373 position of the 972-bp amplicon, and of these the restriction enzyme *Ava*I was chosen (Fig. 1).

2.3 DNA extraction

Pure cultures of *P. ramorum*, *P. hibernalis*, *P. lateralis* and *P. foliorum* (Table 1) were grown in the dark for 10 to 14 days at 20°C on 20% V8 juice agar overlaid with cellophane (BioRad, Mississauga, ON, Canada). Mycelia were harvested (~100 mg fresh weight) and 50–100 mg sand (Sigma-Aldrich, Oakville, ON, Canada) was added to the mycelia in a 1.5-ml Eppendorf tube. Liquid nitrogen was added and the mycelia were ground to a powder using a micropestle. Total DNA was extracted using the Nucleospin kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions for fungi. The DNA pellet was dissolved in 100 μl of TE [10 mM Tris–HCl, pH 8.0 and 1 mM ethylenediaminetetraacetic acid (EDTA)] provided from the kit, and the solution was stored at −20°C.

2.4 PCR amplification

For amplification of the *Cox1* gene, the CoxF4N 5′-GTATTTCTTCTTTATTAGGTGC-3′ and CoxR4N 5′-CGTGAACTAATGTTACATATAC-3′ primers were used to amplify a fragment of 972 bp (Kroon et al. 2004). The reaction mix consisted of 10 to 20 ng of template DNA, 200 μM dNTP (Amersham Biosciences, Montreal, Quebec, Canada), 1 U of Gold *Taq*

Table 1. **Isolates of *Phytophthora* species used in the study.**

Isolate number	Strain number[1]	Species	Host	Origin, year isolated[2]	Genotype[3]
CPH-0002[4]	CPH-0002[SCB]	*P. ramorum*	*Rhododendron* sp. cv. "Vulcan"	Canada 2003	EU1
CPH-16207[4]	CPH-16207[SCB]	*P. ramorum*	*Prunus lusitanica* cv. "Lolita"	Canada 2005	NA2
CPH-16391[4]	CPH-16391[SCB]	*P. ramorum*	*Rosa meidiland* cv. "Pink"	Canada 2005	NA2
CPH-17017[4]	CPH-17017[SCB]	*P. ramorum*	*Camellia sinensis* var. "Teabreeze"	Canada 2005	NA2
CPH-18753[4]	CPH-18753[SCB]	*P. ramorum*	*Rhododrendron* sp. "Edith Bosley"	Canada 2005	NA2
PFC-5038	2027[EH]	*P. ramorum*	*Lithocarpus densiflorus*	OR, USA 2002	NA1
PFC-5039[4]	03-74-D12-A, MYA-3239[NO]	*P. ramorum*	*Viburnum plicatum*	OR, USA 2003	EU1
PFC-5041	Pr 102[DR]	*P. ramorum*	*Quercus agrifolia*	CA, USA 2004	NA1
PFC-5046	2239[EH]	*P. ramorum*	*Lithocarpus densiflorus*	OR, USA 2003	NA1
PFC-5049	5449.1[EH]	*P. ramorum*	*Lithocarpus densiflorus*	OR, USA 2002	NA1
PFC-5052	03-156-6	*P. ramorum*		OR, USA 2003	NA1
PFC-5054	04-207-Q	*P. ramorum*	*Pieris japonica*	OR, USA 2004	NA1
PFC-5058[4]	wsda4175	*P. ramorum*	*Rhododendron*	WA, USA 2004	NA1
PFC-5059[4]	wsda4946	*P. ramorum*	*Pieris*	WA, USA 2004	NA1
PFC-5061	wsda1839	*P. ramorum*	*Rhododendron*	WA, USA 2003	NA1
PFC-5067[4]	PR106, CBS110956[DR]	*P. ramorum*	*Umbellularia californica*	CA, USA ?	NA1
PFC-5073[4]	RHCC-23[DR]	*P. ramorum*	*Rhododendron*	CA, USA 2005	NA2
PFC-5074[4]	RHCC-4[DR]	*P. ramorum*	*Rhododendron*	CA, USA 2005	NA2
PFC-5081	PD98/2627[HdG]	*P. ramorum*	*Rhododendron*	Netherlands 1998	EU1
PFC-5084	CSL2266, BBA9/95, CBS101553, PD20019539[HdG]	*P. ramorum*	*Rhododendron catawbiense*	Germany 1995	EU1
PFC-5086	CSL2268, P1578[CRL]	*P. ramorum*	*Rhododendron grandiflora*	UK 2002	EU1
PFC-5087	CSL2097[CRL]	*P. ramorum*	*Hamamelis*	UK 2004	EU1
PFC-5091	CSL1659, BBA12/98[CRL]	*P. ramorum*	*Rhododendron*	Germany 1998	EU1
PFC-5040[4]	LT192-010505[KHL]	*P. foliorum*	*Rhododendron* sp. (azalea) "Pink Ruffles"	TN, USA 2004	NA1
PFC-5069[4]	368[EH]	*P. lateralis*		CA, USA ?	
PFC-5071[4]	04-206C[EH]	*P. hibernalis*		OR, USA ?	

[1]Isolated by: HdG, Hans de Gruyter; EH, Everett Hansen; KHL, Kurt. H. Lamour; CRL, C. R. Lane; NO, Nancy Osterbauer; DR, Dave Rizzo; SCB, Stéphan C. Brière.
[2]Origin: location of plant host where isolate was collected.
[3]Genotype EU1, EU RFLP pattern with *Apo*I, no digestion with *Ava*I; NA1, NA RFLP pattern with *Apo*I, no digestion with *Ava*I; NA2, NA RFLP pattern with *Apo*I, digestion with *AvaI*. [4]Isolates sequenced in this study.

DQ117982.1	ACT**A**GAGCTT	*Phytophthora ramorum* isolate 509
DQ117984.1	ACT**A**GAGCTT	*Phytophthora ramorum* isolate CBS101553*
DQ117985.1	ACT**A**GAGCTT	*Phytophthora ramorum* isolate Pr102*
DQ117983.1	ACT**A**GAGCTT	*Phytophthora ramorum* isolate Pr218
DQ117986.1	ACT**C**GAGCTT	*Phytophthora ramorum* isolate PrWA0692
AY564183.1	ACT**C**GAGCTT	*Phytophthora hibernalis* isolate CBS 522.77
DQ117981.1	ACT**C**GAGCTT	*Phytophthora lateralis* isolate PL33
AY564191.1	ACT**C**GAGCTT	*Phytophthora lateralis* isolate CBS 168.42

Fig. 1. Sequence alignment of bases 370–378 of the 972 bp fragment of the cytochrome C oxidase subunit 1 gene showing the restriction site for *Ava*I at position 373 (shaded). GenBank accession numbers for each sequence are shown. *Isolates used in the present study.

DNA polymerase (Biocan Scientific Inc., Montreal, Quebec), 3.5 mM $MgCl_2$, 1X PCR buffer and 25 ng of each primer (CoxF4N and CoxR4N) in a reaction volume of 25 μl. Amplifications were run in an (Applied Biosystems, Foster City, CA, USA) GeneAmp PCR System 9700 thermocycler using conditions described in Kroon et al. (2004): 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.

A nested PCR primer pair specific for *P. ramorum*, which amplifies a segment located within the 972-bp fragment amplified by the CoxF4N and CoxR4N primers, was also used (Kroon et al. 2004). Primers Prnest F 5′-TAGCTACTTTATGGGGTGGTTCA-3′ (base pairs 508 to 530 of the 972-bp fragment) and PrnestR 5′-CATTCCAACCACTC-ATAGCATCA-3′ (base pairs 869 to 891) amplify a fragment of 383 bp within the *Cox1* gene including a single nucleic polymorphism site that distinguishes between EU and NA populations of *P. ramorum* (Kroon et al. 2004). The PCR reaction mix and amplification conditions were identical to those for CoxF4N and CoxR4N, except that the annealing temperature was increased to 69°C.

2.5 RFLP analysis

The 972-bp PCR fragment of the *Cox1* gene, amplified using the CoxF4N and CoxR4N primer pair, was digested with the restriction enzyme *Ava*I (C[C/T]CG[A/G]G) (New England Biolabs, Beverley, MA, USA) for 2 h at 37°C according to manufacturer's instructions. The 383-bp PCR fragment of the *Cox1* gene, amplified using the Prnest F and Prnest R primer pair, was digested with the restriction enzyme *Apo*I ([A/G]AATT[C/T]) (New England Biolabs) for 2 h at 50°C according to the manufacturer's instructions. The resulting fragments were separated by gel electrophoresis on 2% and 3% agarose (BioRad Certified Molecular Biology Agarose, Cat. no. 161-3102) in 1X TBE buffer (Sigma, Cat no. T4415-4L) run at 130 V for 2 and 2.25 h, respectively. The DNA fragments were visualized after staining with ethidium bromide and illumination under UV light. The image was produced using Syngene's GeneSnap System (Version 6.05.01; Synoptics Ltd, Frederick, MD, USA). Exposure time was 0.44 s. The sharpen tool was used once, which increases fine details (the high spatial frequencies) by adding the difference between the original image and a locally averaged version (Synoptics Ltd).

2.6 Sequence analysis

Polymerase chain reaction fragments of representative EU1, NA1 and NA2 (Table 1) were gel-purified and extracted using Qiagen's MiniElute Gel Extraction kit (Qiagen, Mississauga, ON, Canada). Once purified, the addition of 3′-A-overhangs were performed using the TOPO TA cloning kit (Invitrogen, Burlington, ON, Canada) and these PCR

amplicons were then ligated into the pCR 2.1-TOPO vector using the TOPO TA cloning kit as per the manufacturer's instructions (Invitrogen). Products were sequenced by automated fluorescent DNA cycle sequencing on a LI-COR 4200 DNA Sequencer (LI-COR Biotechnology, Lincoln, NB, USA) at The Centre for Biomedical Research DNA Sequencing Facility at the University of Victoria. The DNA-sequencing kit used was the EPICENTRE (EPICENTRE Biotechnologies, Madison, WI, USA) SequiTherm EXCEL II DNA sequencing kit and the primers (M13F and M13R) were fluorescently labelled.

2.7 Phylogenetic analyses of partial *Cox1* sequence

Partial *Cox1* nucleotide sequences from this study and other accessions of *Phytophthora* species were aligned using ClustalX, Version 1.83 (Thompson et al. 1997). Default multiple parameters were used to generate the alignment. A phylogenetic tree was generated within ClustalX using the Neighbour Joining method with 1000 bootstrap replicates and visualized using NJPlot (Perrière and Gouy 1996).

3 Results

3.1 PCR amplification

Using primers CoxF4N and CoxR4N, a 972-bp fragment from the *Cox1* gene was amplified from all 23 *P. ramorum* isolates and from the *P. foliorum*, *P. lateralis* and *P. hibernalis* isolates. The inner primers PrnestF and PrnestR amplified a 383-bp fragment from all 23 *P. ramorum* isolates mentioned above. The isolate of *P. hibernalis* showed cross-amplification using inner primers PrnestF and PrnestR, as did the isolate of *P. foliorum*. The *P. lateralis* isolate did not amplify using inner primers PrnestF and PrnestR.

3.2 RFLP analysis

An SNP at position 706 of the 972-bp amplicon (position 199 of the 383-bp amplicon) was found after sequence analysis of the *Cox1* fragment that was amplified with DNA from representative EU1, NA1 and NA2 isolates of *P. ramorum* (Fig. 2). This contributed to a change in the *Apo*I RFLP profile of this fragment allowing distinction of EU1 from North American (NA1 and NA2) isolates (Fig. 3a,b). One isolate (CPH-0002) from Canada and

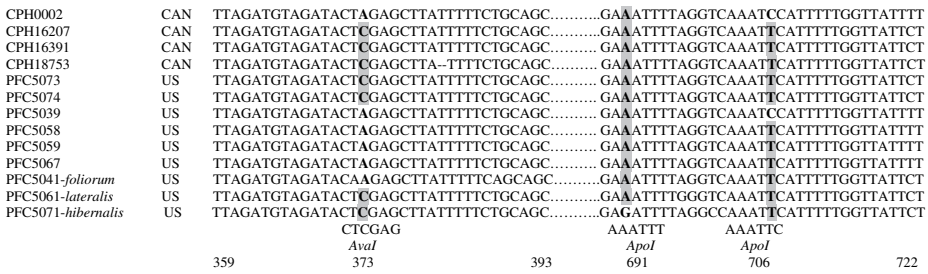

Fig. 2. Partial sequence alignment of the 972-bp amplicon (bases 359–722) of the cytochrome C oxidase subunit 1 gene for 10 isolates of *Phytophthora ramorum* from the United States, Europe and Canada, and one isolate each of *P. foliorum*, *P. hibernalis* and *P. lateralis*. Shaded areas include restriction sites for *Ava*I at position 373, which distinguishes between NA1 and NA2 genotypes of *P. ramorum* and the *Apo*I restriction site at position 706 present in NA *P. ramorum* isolates, as well as *P. foliorum*, *P. hibernalis* and *P. lateralis*. Isolate information is listed in Table 1.

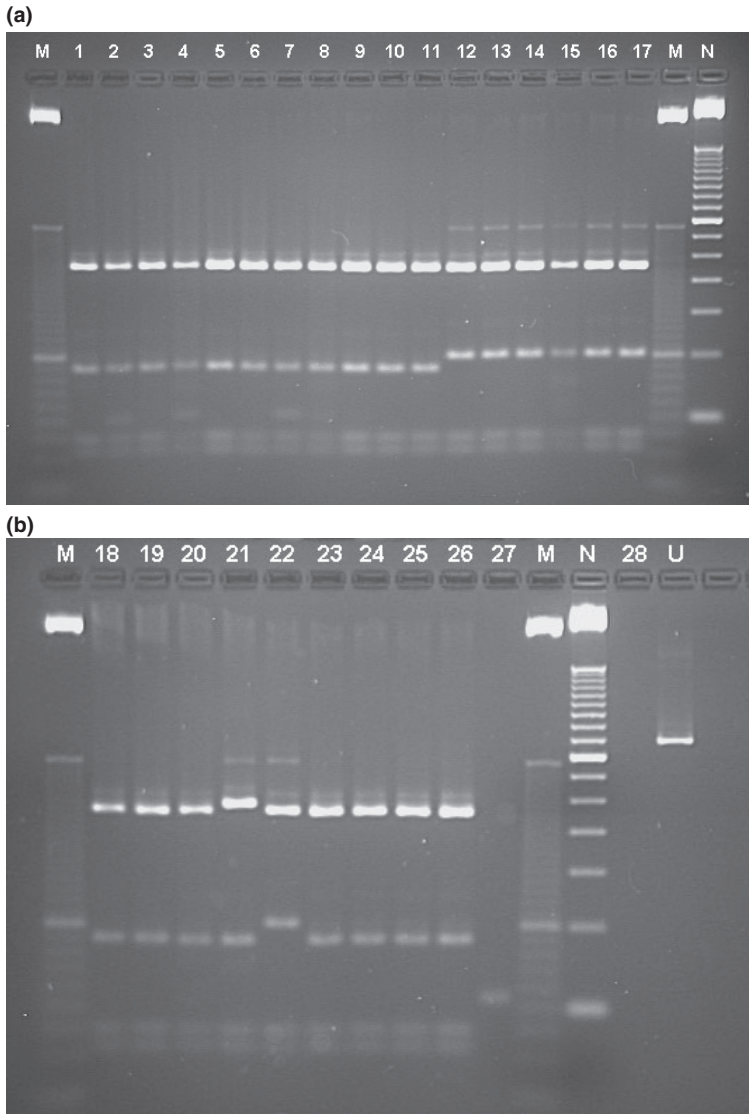

Fig. 3. (a,b) Restriction fragment-length polymorphism patterns for the inner 383-bp amplicon of the cytochrome C oxidase subunit 1 gene for 23 samples of DNA from isolates of *Phytophthora ramorum* from Europe (lanes 12, 13, 14, 15, 16, 17), the US (lanes 1–11, 18, 19) and Canada (lanes 22, 23, 24, 25, 26), *Apo*I digests from an isolate of *P. foliorum* (lane 20) and *P. hibernalis* (lane 21). Lane U = undigested PCR product. Details on isolates are listed in Table 1. A 100-bp (lane M) and 50-bp marker (lane N) is used as size reference. Lane 27 is a no template PCR control. Lane 28 is empty.

one from the US (PFC-5039) have a cytosine residue at position 706, similar to the European isolates reported by Kroon et al. 2004. These isolates are designated 'EU1'. All NA1 and NA2 isolates, as well as *P. foliorum*, *P. hibernalis* and *P. lateralis* have a thymine residue at position 706.

Using the internal primers PrnestF and PrnestR, the 383-bp *Cox1* fragment was amplified only from *P. ramorum*, *P. foliorum* and *P. hibernalis*, but not from *P. lateralis.* In total, four *Apo*I sites were found throughout the sequences and four distinct RFLP profiles were observed (Fig. 3a,b). EU1 isolates of *P. ramorum* possessed a unique profile, but NA1 and NA2 isolates shared a similar profile with *P. foliorum.* Although cross-amplification for *P. hibernalis* occurred, digestion of the 383-bp amplicons with *Apo*I generated restriction profiles that could differentiate *P. hibernalis* from *P. ramorum*. Fragment sizes for *P. hibernalis* were 235, 84, 37 and 27 bp, compared with 223, 97, 37 and 27 bp for EU1 isolates and 223, 84, 37, 27 and 12 bp for NA1 and NA2 isolates and *P. foliorum.*

A second SNP was found at position 373 of the 972-bp amplicon that contributed to a change in the *Ava*I RFLP profile, allowing distinction of NA1 isolates (PFC-5058, PFC-5059, PFC-5067) from NA2 isolates (PFC-5073, PFC-5074, CPH-18753, CPH-16207, CPH-16391) (Fig. 4a,b). Isolates belonging to the NA1 lineage (PFC-5058, PFC-5059, PFC-5067) have an adenine residue at position 373, which is shared by two EU1 isolates: CPH-0002 from Canada, PFC-5039 from the US, and *P. foliorum* (PFC-5040). NA2 isolates have a cytosine residue at position 373 (Fig. 2).

The 972-bp *Cox1* fragment was amplified from *P. ramorum*, *P. foliorum*, *P. hibernalis* and *P. lateralis* using primers CoxF4N and CoxR4N. Only one *Ava*I site was found among the sequences and two distinct RFLP profiles were observed (Fig. 4a,b). NA2 isolates of *P. ramorum*, *P. hibernalis* and *P. lateralis* shared the same profile (fragment sizes of 534 and 438 bp). The 972-bp *Cox1* fragment of the NA1 and EU1 isolates of *P. ramorum* and *P. foliorum* was not digested by *Ava*I.

3.3 Phylogenetic analyses of partial *Cox1* sequence

GenBank accession numbers for isolates sequenced in this study and for sequences obtained from GenBank are shown in Fig. 5. Based on Neighbour Joining analyses of *Cox1* sequences from our *P. ramorum* isolates and other accessions, three distinct clades were observed (Fig. 5). Within the NA1 clade, isolates PFC-5058 and PFC-5059 grouped with other similar genotypic representative members. Isolates CPH-16207, CPH-16391 (two sequences 16391-a and 16391-b), CPH-18753, and PFC-5074 grouped together with PrWA0692 (accession DQ117986.1) together forming the NA2 clade. From this study, members of the EU1 clade included PFC-5039 and CPH-0002 and were grouped together with other EU1 *Cox1* genotypes. Based solely on the partial *Cox1* sequence, PFC-5067 and PFC-5073 did not show a distinct grouping within any clade.

Sequences generated from isolates of *P. hibernalis* and *P. lateralis* from this study grouped with conspecific accessions. *P. foliorum* formed a distinct branch. Other species (*P. heveae*, *P. cryptogea*, *P. citricola*, *P. megasperma*, *P. katsurae* and *P. megakary*) also formed distinct species groupings.

4 Discussion

As more information about *P. ramorum* has accumulated, it has become clear that it is important to identify *P. ramorum* not only to the species level, but also to the clonal lineage and mating type level. In addition to sexual reproduction, new genotypes can arise by horizontal transmission of genes between different asexually reproducing populations of *Phytophthora*. The mechanisms by which this occurs may include heterokaryosis (Judelson and Yang 1998), viruses (Tooley et al. 1989) or transposable elements (Goodwin et al. 1995; Pritham et al. 2007). Because *P. ramorum* has two mating types, sexual reproduction can occur only when both mating types are present in the same location. In most cases, these mating types are restricted to different continents. The European lineage (EU1, mostly A1 mating type) has been found consistently in Europe,

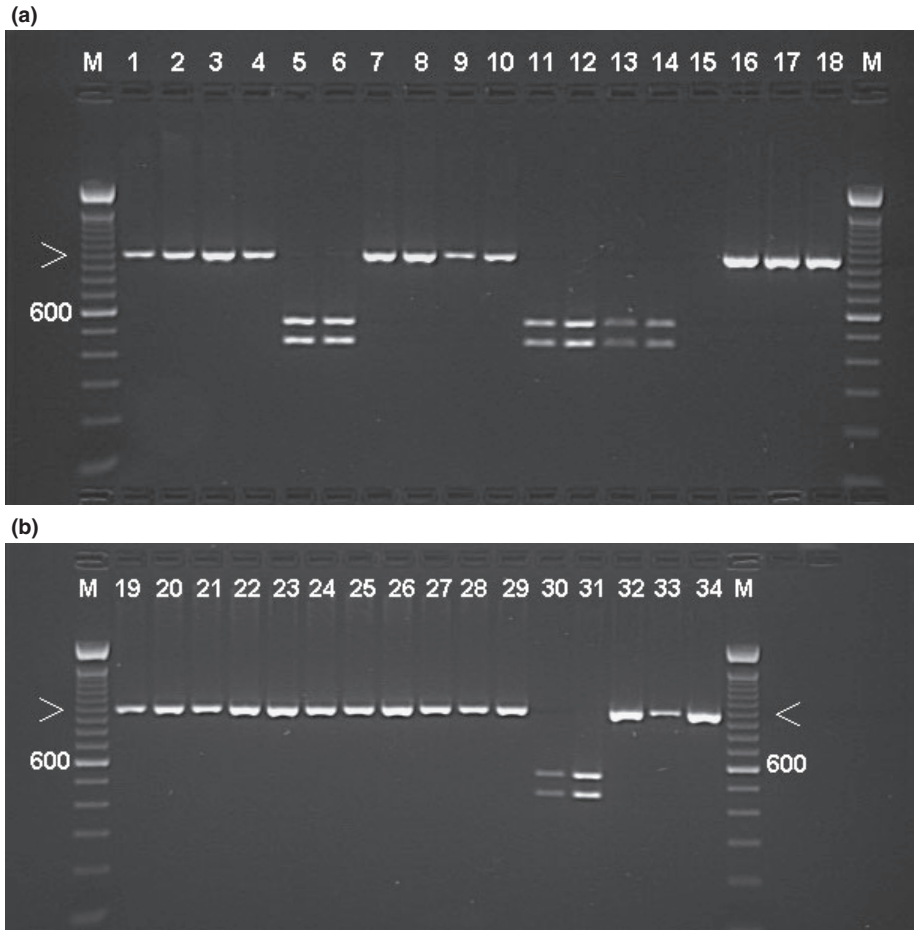

Fig. 4. (a,b) Restriction fragment-length polymorphism patterns for the 972-bp amplicon of the cytochrome C oxidase subunit 1 gene for 24 samples of DNA from isolates of *Phytophthora ramorum* from Europe (lanes 19, 20, 21, 22, and 23), the United States (lanes 1–9, 24–28) and Canada (lanes 10, 11, 12, 13, 14), *Ava*I digests from one isolate each of *P. foliorum* (lane 29), *P. lateralis* (lane 30) and *P. hibernalis* (lane 31). Lanes 16, 17 and 18 represent undigested products from PFC0-5041, PFC-5091 and CPH-16391, respectively. Lanes 32, 33 and 34 represent undigested products from PFC-5040, PFC-5069 and PFC-5071, respectively. Lane 15 is empty. A 100-bp marker is used as size reference. The arrow denotes the size of undigested product, 972 bp; '600' denotes the size of the 600-bp size marker. Details on isolates are listed in Table 1.

and occasionally in North American nurseries (Garbelotto et al. 2005; Bilodeau et al. 2007; Grunwald et al. 2008). The North American lineages (NA1 and NA2, all A2 mating type) have not been found in Europe at present.

Molecular detection methods allow for the processing of larger numbers of samples from nursery and field surveys and are less time-consuming than cultural methods. The enzyme-linked immunosorbent assay (ELISA) is commonly used to screen for *Phytophthora* species, and then a PCR test for *P. ramorum* is performed. Specific primers have been

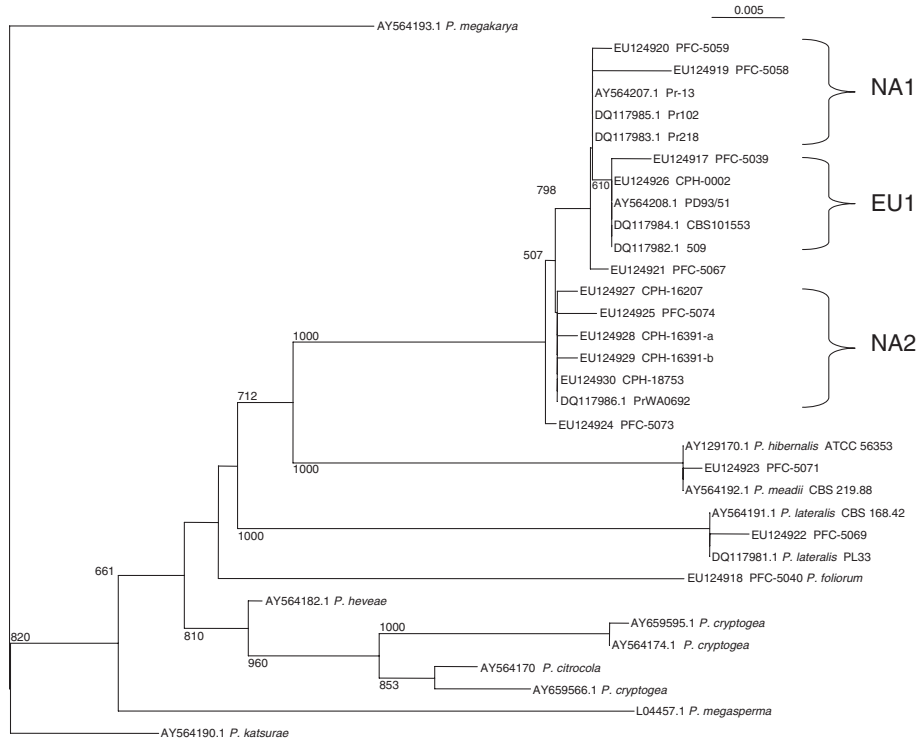

Fig. 5. Neighbour Joining phylogenetic tree based on partial cytochrome C oxidase subunit 1 gene sequences. *Phytophthora ramorum* genotypes are grouped into three distinct clades. From this study, representatives of the NA1 clade include PFC-5058 and PFC-5059. Representatives of the EU1 clade include PFC-5039, CPH-0002, and PFC-5067. Within the NA2, isolates included CPH-16207, PFC-5074, CPH-16391 (two sequences), CPH-18753, and PFC-5073. Various *Phytophthora ramorum* accessions (isolate notation in parenthesis) as well as other *Phytophthora* species are included. PFC-5071 grouped with *P. hibernalis* (accession AY129170.1) and *Phytophthora meadii* (accession AY564192.1). PFC-5069 grouped with two accessions of *Phytophthora lateralis*, AY564191.1 and DQ117981.1. Isolate PFC-5040, *Phytophthora foliorum*, grouped on its own. Bootstrap values are shown at tree branches (of 1000).

developed that will detect *P. ramorum* in plant material collected from the field, as well as other species that occur in the same ecological niche (Martin et al. 2004). Real-time PCR methods detect very small amounts of DNA from field samples, are rapid and can differentiate between isolates based on sequence because melting temperatures will differ for similarly sized products that have slight differences in sequence (Belbahri et al. 2007; Bilodeau et al. 2007). Simpler methods such as PCR-RFLP and nested PCR can be performed in most laboratories and do not require specialized equipment.

Most molecular tests currently available for detecting *P. ramorum* distinguish between *Phytophthora* species but not clonal lineages (Martin et al. 2004; Osterbauer et al. 2004; Blomquist et al. 2005; Bilodeau et al. 2007). In tests that use the ITS region, cross-reaction with other closely related species such as *P. hibernalis*, *P. foliorum* or *P. lateralis* can occur. We had access only to one isolate each of *P. hibernalis* and *P. foliorum*, but sequence data are provided for additional isolates of *P. lateralis* and *P. hibernalis* that agrees with our results. The only isolate of *P. foliorum* that has been sequenced in the *Cox1* region was done in this study, and additional data about this species would be informative.

Bilodeau et al. (2008) found SNPs in β-tubulin and CBEL genes that distinguish between EU1 and NA populations of *P. ramorum*, but do not differentiate between NA1 and NA2, similar to the results obtained by Kroon et al. (2004) using RFLP of the *Cox1* gene. Microsatellite markers can provide a more precise identification of *P. ramorum* populations (Ivors et al. 2006; Prospero et al. 2007).

The molecular tests presented here involving the *Cox1* region are suitable for differentiating between the three lineages of *P. ramorum*; however, *P. foliorum* cannot be separated from the NA1 group using this method. Visual examination of a culture will distinguish between the two species, because *P. ramorum* forms chlamydospores and *P. foliorum* forms oospores. It is possible to identify which lineage an isolate of *P. ramorum* belongs to using PCR-RFLP of the *Cox1* gene, first using *Apo*I to separate *P. ramorum* from other species and EU1 from North American populations, and *Ava*I to distinguish between NA1 and NA2 genotypes. These markers could be adapted for use in other assays, such as real-time PCR.

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