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Phytophthora obscura sp. nov., a new species of the novel *Phytophthora* subclade 8d

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A new *Phytophthora* species was detected (i) in the USA, infecting foliage of *Kalmia latifolia*, (ii) in substrate underneath *Pieris*, and (iii) in Germany in soil samples underneath *Aesculus hippocastanum* showing disease symptoms. The new species *Phytophthora obscura* sp. nov. is formally named based on phylogenetic analysis, host range, Koch's postulates and morphology. *Phytophthora obscura* is homothallic with paragynous antheridia and semipapillate sporangia. It is genetically closely related to *P. syringae* and *P. austrocedrae* and together these three species define a new *Phytophthora* subclade 8d, with significant support for all genetic loci analysed including seven nuclear genes and the mitochondrial gene *coxII*. The morphological and ecological characteristics are very similar to *P. syringae*, and it is likely that *P. obscura* was not described earlier because it was identified as *P. syringae*. Artificial inoculations indicated that horse chestnut, kalmia, pieris and rhododendron might be hosts, and Koch's postulates were confirmed for kalmia from which it was isolated. This pathogen was named after its elusive nature since it has to date rarely been detected in the US and Germany.

Keywords: *Aesculus hippocastanum*, *Kalmia latifolia*, oomycete, *Pieris*, *Rhododendron*, taxonomy

Introduction

The genus *Phytophthora* is well known for repeated introductions of exotic species or strains that pose significant plant biosecurity threats. One notable introduction is that of the sudden oak death pathogen *P. ramorum* affecting oak trees and many woody ornamentals (Werres *et al.*, 2001; Grünwald *et al.*, 2008). It is now evident that this pathogen has undergone at least four global migrations and was introduced into North America at least three times (Prospero *et al.*, 2007, 2009; Mascheretti *et al.*, 2008, 2009; Goss *et al.*, 2009a,b, 2011). Recently, *P. ramorum* has been found to cause unexpected, landscape-scale dieback of Japanese larch plantations in the UK (Brasier & Webber, 2010). With continued globalization and relaxation of trade barriers, the movement of exotic pathogens to new regions and hosts is likely to accelerate the emergence of another high impact pathogen such as *P. ramorum*.

Phytophthora species continue to emerge at an accelerated rate. In the mid 1990s when *P. ramorum* was discovered, about 60 *Phytophthora* species were recognized (Erwin & Ribeiro, 1996). The devastating impact of *P. ramorum* on West coast ecosystems invigorated the search and description of *Phytophthora* species in native habitats worldwide, which has expanded the number of known species to over 100. Some notable recently described species affecting ornamentals or trees include *P. alni* (Brasier *et al.*, 2004), *P. austrocedrae* (Greslebin *et al.*, 2007), *P. bisheria* (Abad *et al.*, 2008), *P. foliorum* (Donahoo *et al.*, 2006), *P. ipomoeae* (Flier *et al.*, 2002), *P. kernoviae* (Brasier *et al.*, 2005), *P. polonica* (Belbahri *et al.*, 2006), *P. pseudosyringae* (Jung *et al.*, 2003) and *P. siskiyouensis* (Reeser *et al.*, 2007) among others.

This paper describes another newly emerging *Phytophthora* species recovered to date in Germany and the United States. This novel species was occasionally found in the Werres and Grünwald culture collections and was originally classified as *P. syringae* based on morphology (Werres *et al.*, 1995) and ITS sequencing (Grünwald *et al.*, 2011). However, preliminary amplified fragment length polymorphism (AFLP) analysis indicated that these isolates belonged to a unique cluster. In Germany the isolates originated from soil underneath a diseased horse chestnut (*Aesculus hippocastanum*) grown in an allée, displaying a bleeding canker (Werres *et al.*, 1995). Isolation of a pathogen from these cankers was attempted but unsuccessful. In Oregon, USA the new species was detected in substrate underneath diseased *Pieris japonica* and was isolated from a leaf of a diseased *Kalmia latifolia* showing typical foliar blight symptoms similar to *P. syringae*.

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The objective of this study is to formally describe the new species *Phytophthora obscura* sp. nov. based on extensive nuclear and mitochondrial DNA sequencing, AFLP, morphological characterization, and host range analysis including the closest relatives *P. syringae* and *P. austrocedrae*. Discovery of *P. obscura* has also led to the definition of a new subclade 8d in the genus *Phytophthora* with significant phylogenetic support based on DNA sequences from nuclear and mitochondrial loci.

Materials and methods

Cultures and morphological work

All isolates used in this work are listed in Table 1. Isolates of the new species from single zoospore culture were obtained from the *Phytophthora* culture collections in the Grünwald and Werres laboratories. In the Grünwald laboratory, cultures were maintained on V8 agar (100 mL V8 juice; 2 g $CaCO_3$; 30 mg L^{-1} β-sitosterol (EMD Chemicals, Inc.); 15 g agar; 900 mL deionized water). In the Werres laboratory, isolates were stored on oatmeal agar under paraffin oil at 15°C and refreshed on carrot piece agar (see below).

Morphology was determined on four media: carrot piece agar (CPA) with 50 g carrot pieces (*Daucus carota* cv. Parano, HILD) and 15 g agar per L deionized water; potato dextrose agar (PDA) with 39 g PDA (BD-Diagnostics no. 214030) per L deionized water; oat meal agar (OMA) with 40 g oat meal and 15 g agar per L deionized water; and V8 agar (V8A) prepared with V8 juice (Campbells), 1 g $CaCO_3$ and 15 g agar per L deionized water. To establish temperature response, isolates were grown in four replicates at 2, 5, 10, 15, 20, 25, 28, 30, 32, 35 and 37°C on CPA, PDA, OMA and V8A. For morphological measurements, such as spore size, isolates were grown on CPA as described in Werres *et al.* (2001). For each isolate, 100 sporangia, oogonia, oospores and antheridia were measured with the exception of *P. syringae* (only isolate BBA 12/99-5), where only 40 gametangia could be measured due to low abundance. For the calculation of the morphological data the isolate P-09-011 was excluded because it appeared damaged under the microscope, possibly due to lengthy storage in liquid nitrogen. A representative isolate, BBA 2/94-IIB, has been deposited as holotype with the Centraalbureau voor Schimmelcultures (CBS 129273) in Utrecht, The Netherlands. The new species was also submitted to Mycobank (MB 519834).

Refreshment of isolates

With the exception of *P. austrocedrae* where apples could not be infected, all isolates were refreshed for infection studies using apple cv. Boskoop. Two apples per isolate were inoculated via two holes each (diameter 0·8 cm, depth *c.* 1 cm). Each hole was filled with an agar plug from a growing colony on CPA. The incubation holes were closed with apple tissue and wrapped with Parafilm for 2 days to prevent drying. The apples were incubated in wet chambers at 15°C (12 h day) and 10°C (12 h night) until development of discoloration, but no longer than 6 weeks. For reisolation, tissue pieces from the edge of apple tissue showing symptoms were cut out and placed on CPA. If no symptoms developed, tissue pieces from the symptomless apple tissue were placed on CPA 6 weeks after inoculation.

Plant inoculations

Pathogenicity studies were carried out with *A. hippocastanum*, *K. latifolia*, *Pi. japonica* and *Rhododendron catawbiense* cv. Boursault. For the pathogenicity studies with horse chestnut, 5-year -old seedlings of *A. hippocastanum* were obtained. The seedlings originated from a nursery but were cultivated for 4 years without chemical control at JKI before inoculation. In October 2009, four containerized horse chestnuts were inoculated per isolate at four different points each, two inoculation points in older stem parts and two in younger ones. In November 2009 the trees were inoculated at two further points. Inoculation was similar to grafting. Instead of the scion

Table 1 *Phytophthora* isolates used in this study

Phytophthora species	Isolate	Source/Host	Year of isolation	Origin
P. obscura sp. nov.	BBA 2/94-IIA	Soil (*Aesculus hippocastanum*)	1994	Germany
P. obscura sp. nov.	BBA 2/94-IIB (Type strain)	Soil (*Aesculus hippocastanum*)	1994	Germany
P. obscura sp. nov.	JP-09-059	Leafy debris underneath *Pieris* sp.	2009	USA
P. obscura sp. nov.	P-09-011 (469[a])	*Kalmia latifolia* leaf	–	USA
P. austrocedrae	PAU-09-001 (AG195[b])	*Austrocedrus chilensis*	2005	Argentina
P. austrocedrae	PAU-09-002 (AG203[b])	*Austrocedrus chilensis*	2005	Argentina
P. syringae	BBA 1/99-28	Soil (*Quercus* sp.)	1999	Germany
P. syringae	BBA 12/99-5	*Prunus domestica* cv. Top	1999	Germany
P. syringae	PSY-06-099 (1341231-5[c])	*Magnolia* sp.	2006	USA

[a]Original isolate name in parentheses; isolate obtained from Paul Tooley, USDA Foreign Disease-Weed Science Research Unit, USA. This isolate was originally sampled by R. G. Linderman.
[b]Original isolate name in parentheses; isolate obtained from Everett Hansen, Oregon State University, USA.
[c]Original isolate name in parentheses; isolate obtained from Lani Yakabe, University of California, USA.

wood, a plug from the edge of a growing *Phytophthora* colony was placed on the fresh wound. The inoculation points were sealed with Parafilm for 6 days. Due to safety concerns, the horse chestnuts were kept in the greenhouse. Incubation temperatures were similar to outdoor temperatures but remained above 0°C. Occurrence of disease symptoms were observed monthly during the season. In June 2010 reisolations were carried out onto CPA plates for confirmation of identity.

Whole plant, non-wounded dip inoculations were carried out on 10 plants of each of *Pi. japonica*, *K. latifolia* and *R. catawbiense* cv. Boursault. Five isolates were selected, four of *P. obscura* sp. nov. and one of *P. syringae*, for comparison with these plant species. Small plants in 4-inch containers were inoculated by dipping the above ground portion of the plant into a zoospore suspension (10 000 zoospores mL^{-1}), misted and bagged for 48 h to encourage infection. Plants were incubated in a containment chamber at 20°C with 16 h day length. Following successful lesion development, re-isolations were carried out onto V8A and PARP medium plates for confirmation of identity.

Amplified fragment length polymorphism (AFLP) genotyping

AFLP was performed on genomic DNA using the AFLP Microbial Fingerprinting protocol (Applied Biosystems (ABI)) with slight modifications as described previously (Grünwald & Hoheisel, 2006). Total genomic DNA was obtained from mycelia using the FastDNA SPIN kit (MP Biomedicals LLC). DNA (20 ng) was digested, adaptors were ligated, and preselective amplifications with *Eco*RI core primer, *Mse*I core primer and AFLP amplification core mix were performed on a GeneAmp 9700 thermal cycler (ABI) as described. Pre-amplified samples (10 μL) were diluted with 190 μL TE buffer and selective PCR was performed with *Mse*I-AC/*Eco*RI-AC and *Mse*I-CC/*Eco*RI-AA primer combinations. Products (0·5 μL) were run with 25 μL loading buffer (24 μL deionized formamide and 0·05 μL GeneScan-500 size standard, ABI) on a capillary sequencer (ABI Prism 3100). Electropherograms were analysed using GeneMapper software (v.3·7; ABI) to extract a matrix of presence and absence of alleles in a range of 50–500 bp after visual inspection. All AFLP analyses were replicated two or more times including independent DNA extractions and PCR reactions, until all polymorphic alleles could be unambiguously binned.

Genetic distance among AFLP genotypes was calculated using Nei's unbiased genetic distance (Nei, 1978) as implemented in Tools for Population Genetic Analyses (TFPGA) v.1·3 (Miller, 1997). The genetic distance matrix was imported into MEGA v.4 (Tamura *et al.*, 2007) to construct a UPGMA dendrogram. Support for isolate groupings was assessed using 1000 bootstrap samples in TFPGA.

Phylogenetic analyses

Isolates for inclusion in this study were identified to species by sequencing of the internal transcribed spacer of the rDNA (ITS) in Phytophthora-ID as described previously (Grünwald *et al.*, 2011). Eight nuclear loci and one mitochondrial DNA locus were sequenced in eight isolates for phylogenetic analysis (Table 2): the ITS, the seven nuclear genes sequenced in Blair *et al.* (2008), and *coxI*, *coxII* and the *cox* spacer region in the mitochondrial genome. Primers and PCR conditions were as previously reported for ITS (Cooke *et al.*, 2000; Grünwald *et al.*, 2011), the other seven nuclear genes (Blair *et al.*, 2008), *coxI* and *coxII* (Martin & Tooley, 2003a), and the *cox* spacer region (Martin *et al.*, 2004; Grünwald *et al.*, 2011). Samples were sequenced by the Oregon State University CGRB on an ABI Prism 3730 Genetic Analyzer (Applied Biosytems). Nucleotide sequences from other *Phytophthora* clade 8 species and outgroups *P. infestans* and *P. sojae* were obtained from GenBank. Accession numbers for sequences included are given in Table 2. All indels were removed from sequence alignments for phylogenetic analysis.

ITS and *coxII* were analysed independently using maximum likelihood, maximum parsimony and Bayesian criteria. A multilocus phylogeny of clade 8 was also conducted by concatenating the seven nuclear loci from Blair *et al.* (2008). Phylogenetic inference by maximum likelihood was conducted using PhyML 3·0 (Guindon & Gascuel, 2003). Nucleotide substitution models were selected using MODELTEST 3·7 by AIC (Posada & Crandall, 1998). Parameter values were estimated in PhyML. The starting tree for the analysis was generated using BIONJ and nearest-neighbour interchange (NNI) was used for tree searching. Branch support was assessed using 1000 bootstrap samples for ITS and *coxII*, and 500 bootstrap samples for the multilocus data set. Inference by maximum parsimony was conducted in PAUP 4·0b10 (Swofford, 2002). Heuristic search was used with 20 replicates of random stepwise addition of sequences. Bootstrap analysis was conducted using heuristic search with 1000 samples. Bayesian phylogenetic inference was conducted in MrBayes 3·1·2 (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003). Substitution models were specified based on MODELTEST 3·7 results (Posada & Crandall, 1998). For the seven concatenated loci, one substitution model was used across all seven loci. A second analysis in which sequences were partitioned by gene and evolutionary rates allowed to vary among genes, produced nearly identical results but required many more MCMC steps to obtain adequate sampling of parameters. Two simultaneous runs were conducted with four chains each, default priors, and tree sampling every 100 generations. Run lengths were 1×10^6 steps for ITS and *coxII*, and 5×10^6 steps for the unpartitioned, multilocus analysis. The first 2000 trees were discarded as burn-in for calculation of clade posterior probabilities. MCMC

Table 2 *Phytophthora* species studied and the GenBank sequence accession numbers for DNA sequences characterized

Phytophthora species	Isolate	GenBank accession number								
		ITS	cox	60S	β-tub	EF1-A	Enl	HSP90	28S	TigA
This study										
P. obscura	BBA 2/94-IIA	HQ917909	HQ917877	HQ917861	HQ917869	HQ917885	HQ917893	HQ917901	HQ917853	HQ917915
	BBA 2/94-IIB	HQ917910	HQ917878	HQ917862	HQ917870	HQ917886	HQ917894	HQ917902	HQ917854	HQ917916
	JP-09-059	HQ917911	HQ917879	HQ917863	HQ917871	HQ917887	HQ917895	HQ917903	HQ917856	HQ917917
	P-09-011	HQ917912	HQ917880	HQ917864	HQ917872	HQ917888	HQ917896	HQ917904	HQ917855	HQ917918
P. austrocedrae	PAU-09-001	DQ995185	HQ917881	HQ917865	HQ917873	HQ917889	HQ917897	HQ917905	HQ917857	HQ917919
	PAU-09-002	DQ995184	HQ917882	HQ917866	HQ917874	HQ917890	HQ917898	HQ917906	HQ917858	HQ917920
P. syringae	BBA 12/99-5	HQ917914	HQ917884	HQ917867	HQ917876	HQ917892	HQ917900	HQ917907	HQ917860	HQ917922
	PSY-06-099	HQ917913	HQ917883	HQ917868	HQ917875	HQ917891	HQ917899	HQ917908	HQ917859	HQ917921
From GenBank										
P. syringae		AY787032	AY129224	EU080557	EU080558	EU080559	EU080560	EU080561	EU080562	EU080563
		AY787033	AY369366	–	–	–	–	–	–	–
		AY787034	AY369367*	–	–	–	–	–	–	–
		AF266803	–	–	–	–	–	–	–	–
P. hibernalis		AY369375	AY129199	EU079513	EU079514	EU079515	EU079516	EU079517	EU079518	EU079519
P. foliorum		EF120469	EF120470	EU079679	EU079680	EU079681	EU079682	EU079683	EU079684	EU079685
P. lateralis		AF266804	AY369360	EU079852	EU079853	EU079854	EU079855	EU079856	EU079857	EU079858
P. ramorum		AY369368	AY423303	EU080683	EU080684	EU080685	EU080686	EU080687	EU080688	EU080689
P. brassicae		AF380147	n.a.	EU080523	EU080524	EU080525	EU080526	EU080527	EU080528	EU080529
P. primulae		AF266802	n.a.	EU080823	EU080824	EU080825	EU080826	EU080827	EU080828	EU080829
P. porri		AF380150	n.a.	EU079672	EU079673	EU079674	EU079675	EU079676	EU079677	EU079678
P. sansomea		EU925375	n.a.	EU080270	EU080271	EU080272	EU080273	EU080274	EU080275	EU080276
P. drechsleri		AF266798	AY129189	EU079506	EU079507	EU079508	EU079509	EU079510	EU079511	EU079512
P. erythroseptica		AF266797	AY129191	EU079827	EU079828	EU079829	EU079830	EU079831	EU079832	EU079833
P. cryptogea		AF266796	AY129188	EU080446	EU080447	EU080448	EU080449	EU080450	EU080451	EU080452
P. medicaginis		AF266799	n.a.	EU079899	EU079900	EU079901	EU079902	EU079903	EU079904	EU079905
P. trifolii		AF266800	n.a.	EU080141	EU080142	EU080143	EU080144	EU080145	EU080146	EU080147
P. infestans		–	AY129204	EU079632	EU079633	EU079634	EU079635	EU079636	EU079637	EU079638
P. sojae		–	AY129223	EU079789	EU079790	EU079791	EU079792	EU079793	EU079794	EU079795

n.a. not available.

*Downloaded from GenBank as *P. syringae*, but shown here to be *P. obscura* sp. nov. in Figure 9.

output was visualized in TRACER 1·3 (Rambaut & Drummond, 2007) to assess convergence. Phylogenetic trees were visualized in MEGA v.4 (Tamura *et al.*, 2007), TREEVIEW 1·6·6 (Page, 1996) and GENEIOUS 4·8·2 (Biomatters Ltd).

Results

Morphology

The *P. obscura* sp. nov. isolate from *Kalmia* (P-09-011) appeared to be damaged in microscopic observations, although it was able to cause disease on *Pieris*, *Kalmia* and *Rhododendron*, but was not inoculated onto *Aesculus*, indicating that its pathogenicity was not compromised. This isolate was originally obtained by plating on selective media, and recently from liquid nitrogen storage, and either process might have damaged the isolate. It showed differing growth rates and had a high rate of distorted oospores in comparison to the other three isolates of this new species. Therefore the morphological characterization of the new species is based only on the three isolates from soil associated with *A. hippocastanum* and from substrate underneath *Pieris*.

Colonies grew smooth to slightly stellate on CPA, OMA and V8A, but cottony and slightly petaloid to stellate on PDA (Fig. 1). The vegetative growth rate varied with the medium. On CPA, *P. obscura* sp. nov. isolates grew nearly as fast as *P. syringae* but much faster than the *P. austrocedrae* isolates (Fig. 2, Table 3).

Hyphal swellings were rarely observed. Mycelium showed a relatively low rate of branching. *Phytophthora obscura* sp. nov. produced semipapillate, non-caducous sporangia on single, sympodial sporangiophores. Sporangia were generally ovoid but could also vary from globose to limoniform (Fig. 3). Spore measurements are presented in Table 3.

The species is homothallic and readily produced oogonia with paragynous antheridia in culture with one antheridium per oogonium (Fig. 3). Oogonia were usually round, and sometimes a little irregular. Antheridia were mainly globose. Oospores were mainly plerotic, but mature oospores were sometimes slightly aplerotic. Dimensions for the gametangia are presented in Table 3. Chlamydospores were not observed.

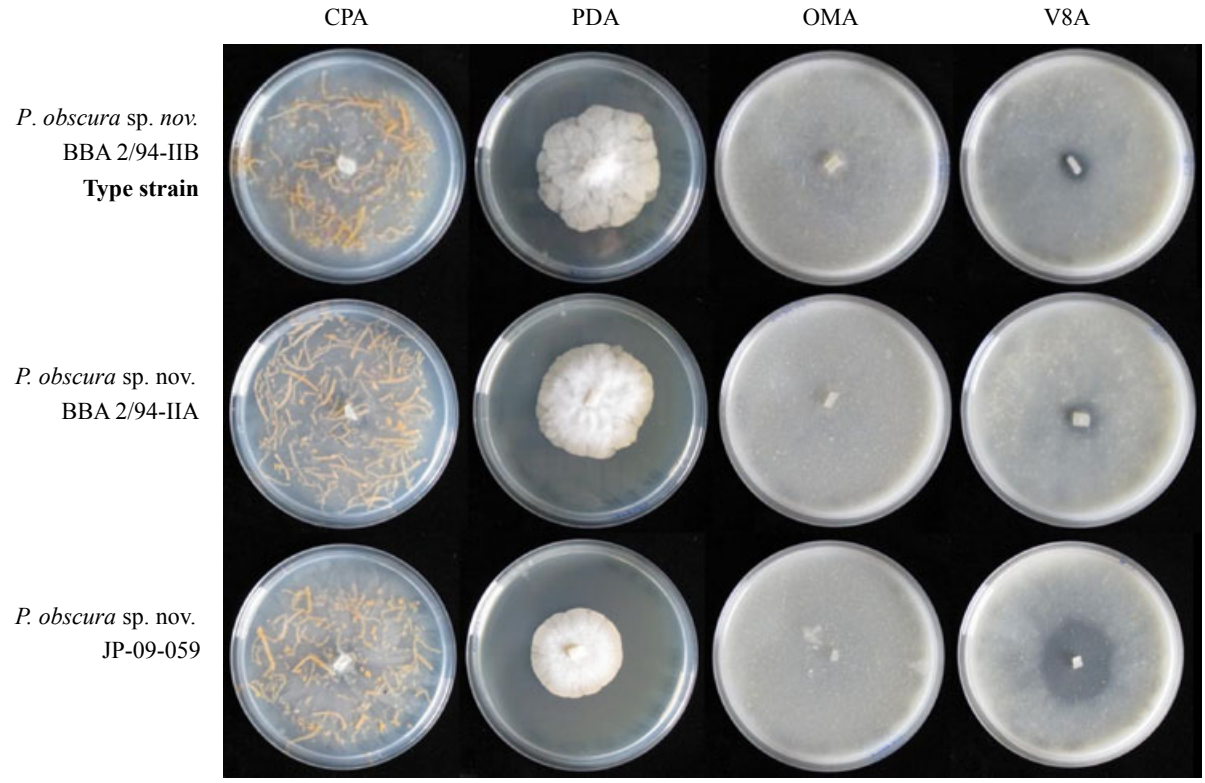

Figure 1 Characteristic colony morphology of *Phytophthora obscura* sp. nov. on various culture media. Incubation at 20°C for 18 days in the dark. Culture media shown are: CPA = carrot piece agar; PDA = potato dextrose agar, OMA = oat meal agar and V8A = vegetable juice agar.

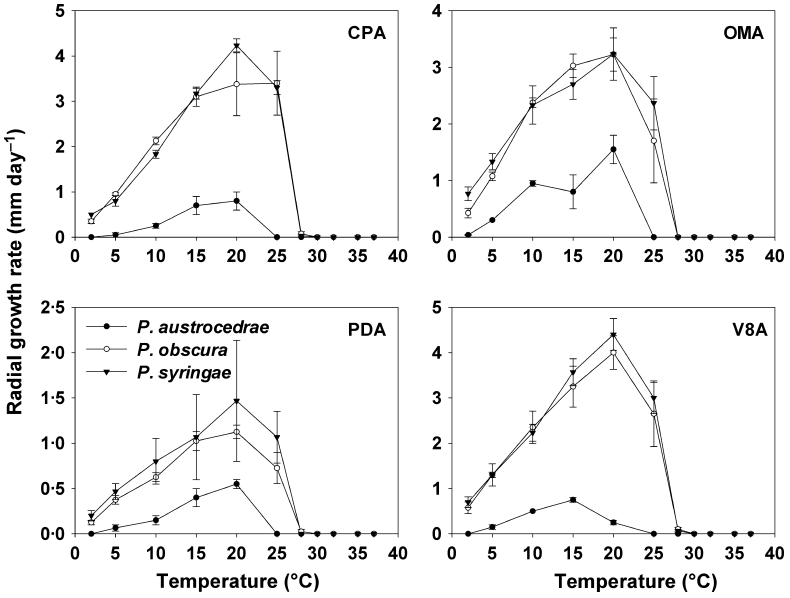

Figure 2 Vegetative growth rate of *Phytophthora obscura* sp. nov. on various culture media. Shown are means ± standard error calculated across isolates for each taxon. CPA = carrot piece agar, PDA = potato dextrose agar, OMA = oat meal agar, V8A = vegetable juice agar.

Table 3 Morphological observations for *Phytophthora obscura* sp. nov. type strain and averages observed for *P. obscura* sp. nov., *P. syringae* and *P. austrocedrae* averaged across strains included in this study

	P. obscura sp. nov. BBA 2/94-IIB (type strain)	*P. obscura* sp. nov.[a]	*P. syringae*[b]	*P. austrocedrae*[c]
Morphological group	III	III	III	IV
Vegetative growth				
Temperature (°C)				
Minimum	2	2	2	5–10
Optimum	25	20–25	20	15–20
Maximum	25	25–28	25–28	20
Growth rate at optimum temperature (mm/24 h)	4·3	4·1	4·2	0·8
Sporangia	semipapillate non-caducous globose-ovoid, dominant apical plug	semipapillate non-caducous globose-ovoid, dominant apical plug	semipapillate non-caducous ovoid-obpyriform, ellipsoid	semipapillate non-caducous ovoid, obpyriform, limoniform, ellipsoid
Length × width				
(range, μm)	30–50 × 20–32	24–50 × 14–32	26–54 × 22–48	40–82 × 28–44
(average, μm)	36·2 × 26·4	33·1 × 24·2	40·2 × 29·2	59 × 36·9
Length : width				
(range, μm)	1·1–2·1 : 1	1·1–2·3 : 1	1·0–1·9 : 1	1·1–2·2 :1
(average, μm)	1·4 : 1	1·4 : 1	1·4 : 1	1·6 : 1
Production of gametangia	homothallic	homothallic	homothallic	homothallic
Oogonia	predominantly globose, sometimes ellipsoid tapered base	predominantly globose, sometimes ellipsoid tapered base	–	globose or nearly so
Diameter (range, μm)	26–40	22–40	24–32	30–64
(average, μm)	33·4	32·6	29·4	49·6
Antheridia	paragynous	paragynous	predominantly paragynous, occasionally amphigynous	amphigynous
Length × width (range, μm)	6–16 × 6–18	6–16 × 6–18	6–16 × 4–12	10–36 × 10–20
(average, μm)	10·0 × 10·0	9·8 × 9·5	9·7 × 9·1	19·1 × 15·3
Oospores	globose, nearly plerotic	globose, nearly plerotic	globose, plerotic	globose, aplerotic
Diameter (range, m)	22–36	18–36	22–30	20–62
(average, m)	29·8	28·8	26·5	44·4
Chlamydospores	none	none	none	none

[a]On CPA, summarized data from the isolates BBA 2/94-IIA, BBA 2/94-IIB, JP-09-059 (*n* per propagule and isolate = 100); the isolate P-09-011 looked damaged, the morphological data were not used for the official description.
[b]On CPA, summarized data from the isolates BBA 1/99-28, BBA 12/99-5, PSY-06-099 (*n* per propagule and isolate = 100; *n* = 40 for gametangia of *P. syringae* isolate BBA12/99-5, the only *P. syringae* isolate that produced gametangia).
[c]On CPA, summarized data from the isolates PAU-09-001, PAU-09-002 (*n* per propagule and isolate = 100).

Amplified fragment length polymorphism (AFLP) analysis

Two selective primer combinations were evaluated. Both yielded qualitatively similar results supporting *P. obscura* sp. nov. as a new taxonomic species. Electropherograms among *P. obscura* sp. nov., *P. austrocedrae* and *P. syringae* were distinctly different, yet close to identical within species (Fig. 4). The genetic distance between isolates from Germany and Oregon reveals polymorphism within the species and is less than that observed between the three *P. ramorum* clonal lineages (Fig. 5).

Phylogenetic analysis

All of the *P. obscura* isolates had nearly identical ITS sequences. The two Oregon isolates were heterozygous (A/G) at base 696, while the German isolates were homozygous (G). Across the seven other nuclear loci, there were six fixed nucleotide differences between the German and United States isolates. In the *cox* region, there were four single nucleotide differences between the isolates from Germany and the US: one in *coxII*, three in *coxI*, and two in the spacer (Fig. 6).

The best model for sequence evolution for ITS and the other nuclear genes was GTR+I+G. However, the estimates of the gamma shape parameter for ITS in MrBayes were large and highly variable, therefore this analysis was rerun with the GTR+I model, which gave very similar results. The GTR+I substitution model was selected for *coxII*. ITS sequences produced trees showing *P. obscura* sp. nov. isolates as a well-supported phylogenetic species (Fig. 7). Furthermore, *P. obscura* sp. nov., *P. austrocedrae* and *P. syringae* formed a well-supported clade distinct

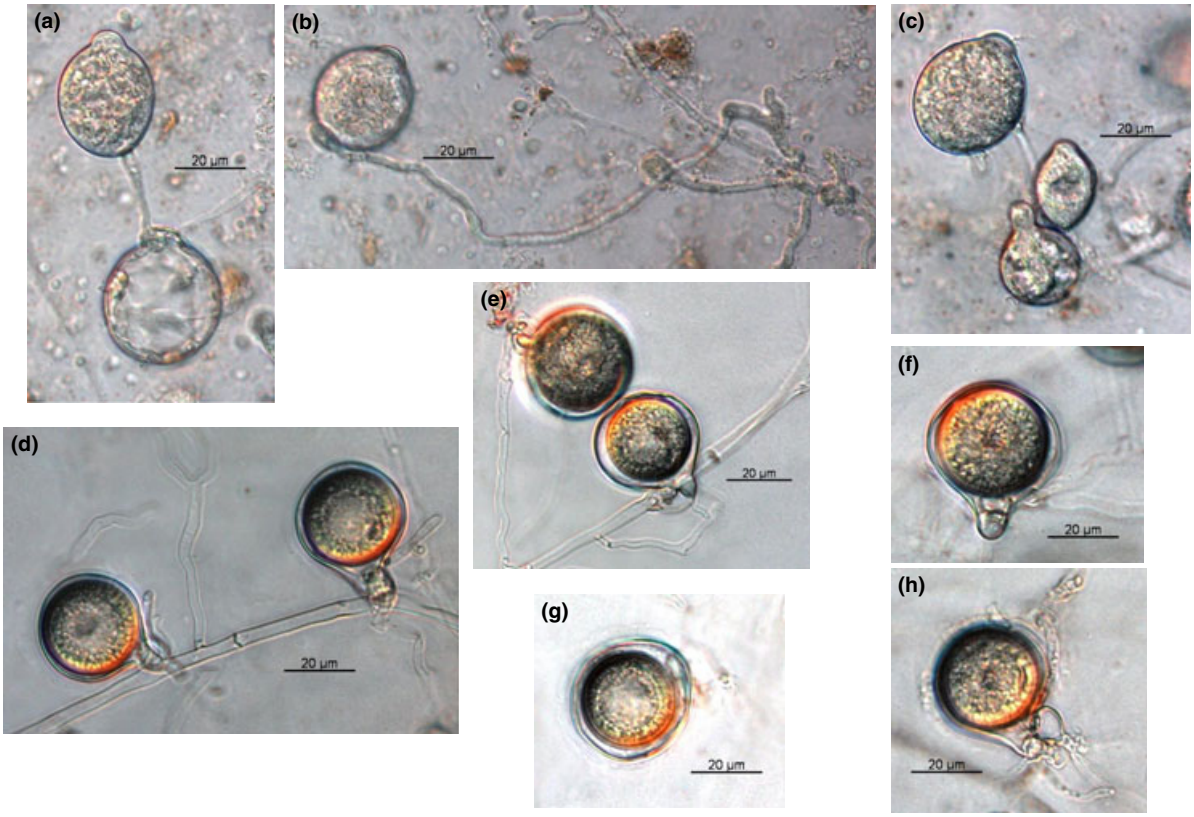

Figure 3 Typical morphological structures of *Phytophthora obscura* sp. nov. BBA 2/94-IIB. Observation on carrot piece agar under the light microscope. a–c = sporangia and empty sporangia, d-h = oogonia, antheridia and oospores.

from previously defined clades (Cooke *et al.*, 2000; Kroon *et al.*, 2004; Blair *et al.*, 2008). This clade has been labelled 8d (Figs 7–9). Maximum parsimony (MP) produced two equally parsimonious trees for ITS, which differed from each other and from the maximum likelihood tree and Bayesian consensus tree in the relative positions of species in clade 8a but were otherwise equivalent. Similar results were obtained for the seven concatenated nuclear loci (Fig. 8). *Phytophthora obscura* sp. nov., *P. austrocedrae* and *P. syringae* again formed a

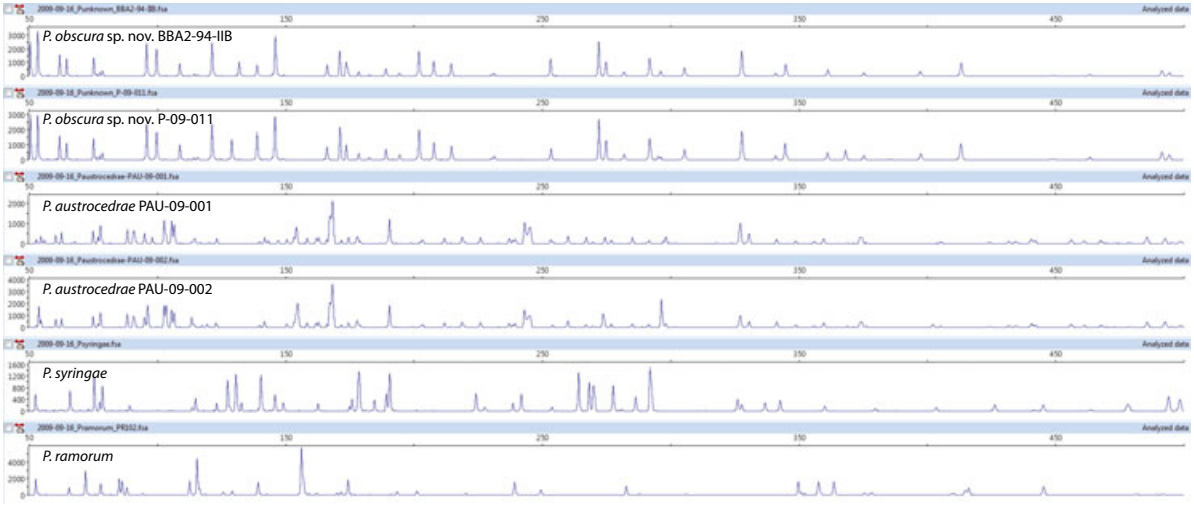

Figure 4 Electropherograms showing amplified fragment length polymorphism (AFLP) profiles for AC/AC selective primer combinations of genomic DNA for *Phytophthora obscura* sp. nov. (BBA2-94-IIB and P-09-011), *P. austrocedrae* (PAU-09-001 and PAU-09-002), *P. syringae* and *P. ramorum*.

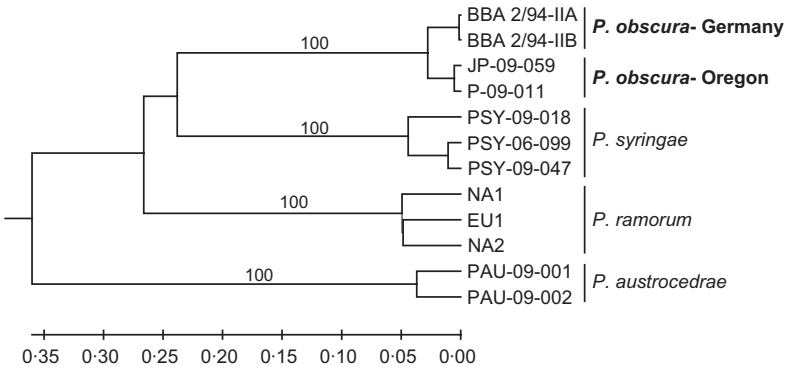

Figure 5 UPGMA dendrogram based on Nei's genetic distance (Nei, 1978) among *Phytophthora* isolates as measured by AFLP. The genetic distance between isolates from Germany and Oregon is less than that observed between the three *P. ramorum* clonal lineages. Support for isolate grouping was assessed using 1000 bootstrap samples and are shown as percentages above nodes.

well-supported clade. The new clade 8d forms a larger highly supported clade with clade 8b. *Phytophthora syringae* was previously observed to group with clade 8b in a phylogeny based on these same genes (Blair *et al.*, 2008). MP produced two tied trees, which differed in the relative positions of clades 8a and 8c. For the mitochondrial gene *coxII*, *P. obscura* isolates were also distinct from *P. austrocedrae* and *P. syringae*, and these three species clustered into a clade with 100% support across phylogenetic methods. However, clade 8b sequences were not available for this gene. One of the *coxII* sequences identified as *P. syringae* that was downloaded from GenBank (AY369367) was identical to *coxII* from the Oregon isolate from *Pieris* substrate JP-09-059. The AY369367 sequence was published by Martin & Tooley (2003b) and was obtained from an isolate collected from *K. latifolia* in a home garden in Oregon. This isolate (originally 469, here P-09-011) was kindly provided by P. Tooley and included in this study, thus the sequences labelled AY369367 and P-09-011 in Fig. 9 are from the same isolate.

Host range

Although only isolated directly from kalmia, host range was evaluated for horse chestnut, kalmia, rhododendron and pieris. On horse chestnuts, first disease symptoms developed within 4 weeks after inoculation, but only with *P. obscura* sp. nov. and not on all seedlings or

Site position	111
	478389
	984756
	554853
German isolates	CCATAG
Oregon isolates	TTTGTA

Figure 6 Nucleotide differences between *Phytophthora obscura* sp. nov. isolates from Oregon, USA and Germany across the entire *cox* region by site. There was one nucleotide difference in *coxII*, two in the *cox* spacer (underlined sites), and three differences in the *coxI* gene.

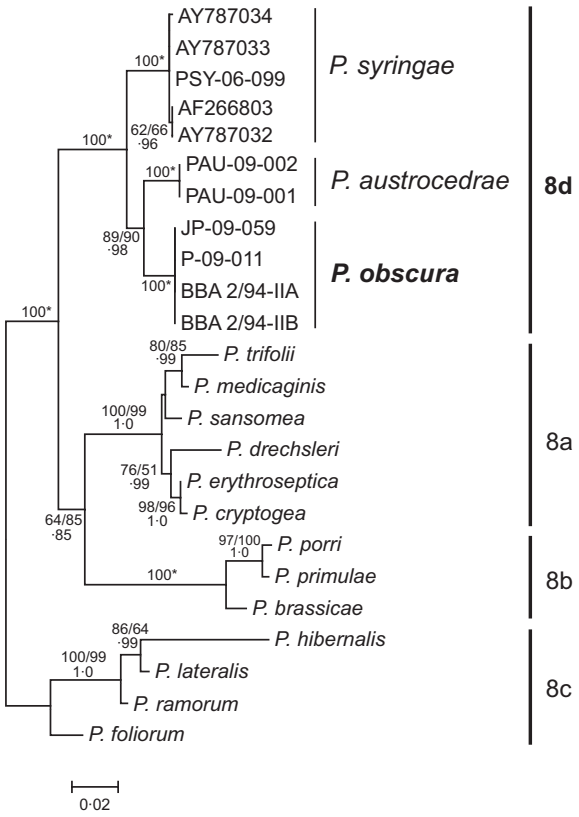

Figure 7 Maximum likelihood phylogeny for *Phytophthora* clade 8 based on ITS sequence. Bootstrap support for branches is shown for all three methods: maximum likelihood/maximum parsimony (top) and Bayesian posterior probability (below). Support values of 100/100 and 1·0 for a branch are indicated by 100*. Isolate designation is given for isolates sequenced for this study, other sequences were obtained from GenBank and accession numbers are shown for clade 8d or given in Table 2 for other species.

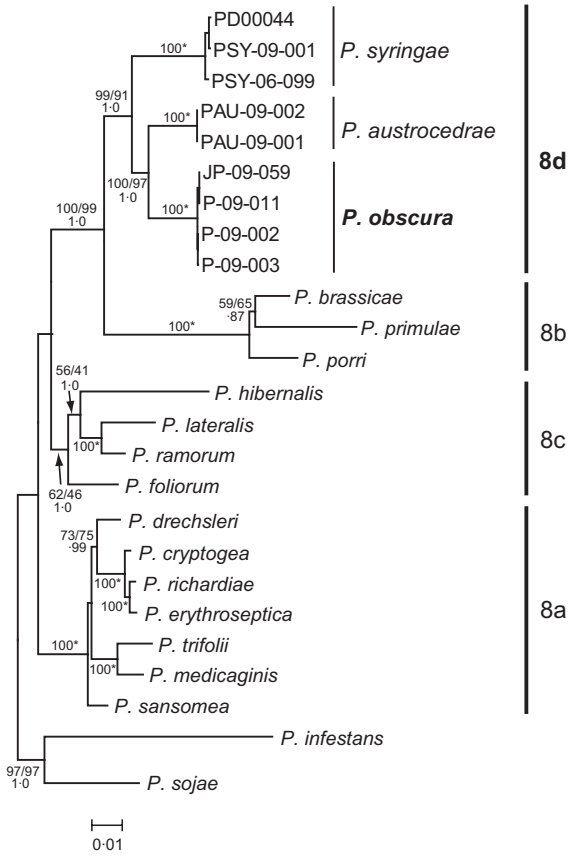

Figure 8 Maximum likelihood phylogeny for *Phytophthora* clade 8 based on the seven nuclear loci used by Blair *et al.* (2008). Branch support values are as in Figure 7. Isolate designation is given for isolates sequenced for this study plus one *P. syringae* isolate for which gene sequences were obtained from GenBank. All other sequences were obtained from GenBank and accession numbers are given in Table 2.

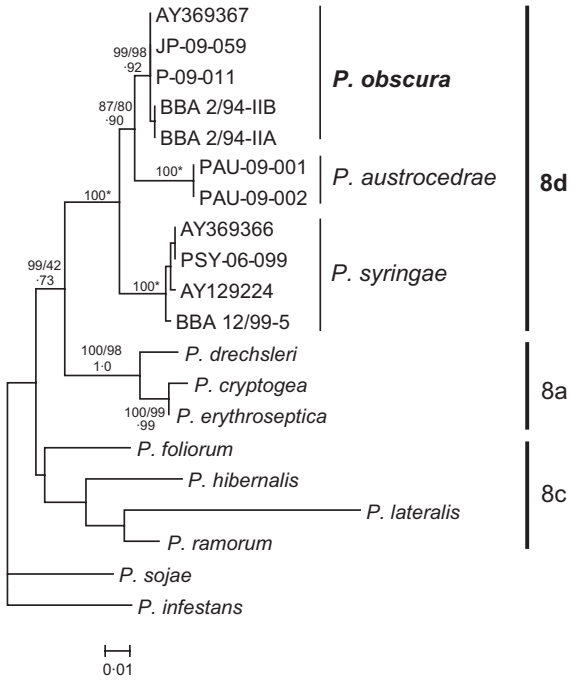

Figure 9 Maximum likelihood phylogeny for *Phytophthora* clade 8 based on the mitochondrial *coxII* gene. Branch support values are as in Figure 7. Isolate designation is given for isolates sequenced for this study, other sequences were obtained from GenBank and accession numbers are shown for clade 8d or given in Table 2 for other species. *CoxII* sequences for clade 8b were not available.

inoculation points. New symptoms at other inoculation points and further development of existing symptoms appeared mostly after the winter. Ten months after inoculation, in June 2010, the horse chestnuts inoculated with *P. obscura* sp. nov. showed severe lesions including some dead branches and bleeding stem cankers (Fig. 10). One seedling was completely killed. *Phytophthora obscura* caused foliar blight on *K. latifolia*, *Pi. japonica* and *R. catawbiense* cv. Boursault (Fig. 11). Koch's postulates could be confirmed for *K. latifolia* isolate P-09-011 obtained from plant tissue. Reisolations and successful identification using ITS sequencing confirmed pathogenicity for four isolates of *P. obscura* sp. nov. on *K. latifolia*, *A. hippocastanum*, *Rhododendron* and *Pieris*. The damage with the *P. syringae* isolates was moderate to minor on all hosts and mainly restricted to the surrounding tissue of the infection points (Fig. 11). The two *P. austrocedrae* isolates did not cause any disease symptoms on any of the hosts (pictures not shown).

Taxonomy

Phytophthora obscura Grünwald & Werres, sp. nov.

Etym.: *obscura*, obscure: referring to the cryptic nature and the fact that it has (to date) not often been found in nature.

Coloniae in agaris fragmentorum dauci (CPA), farinae avenae (OMA), et "V8" (V8A) laeves vel parum stellatae, in agaro dextrosi Solani tuberosi (PDA) gossypinae et petaloideae vel stellatae visae. Optima temperatura in CPA 25°C, medio incremento diurno radiali 4·3 mm. Tumores hypharum nulli vel rari. Mycelium in CPA tarde ramificans. Sporangia semipapillata, non caduca, terminalia in sporangiophoro, media longitudine latitudineque 36·2 × 26·4 μm, media proportione longitudinis:latitudinis 1·4:1, copiose facta in CPA post inundationem extracto humi sphagnosi. Species homothallica. Antheridia paragyna, fere globosa, media longitudine latitudineque 10·0 × 10·0 μm. Oogonia sphaerica diametro medio 33·4 μm, non ornata. Oosporae in cultura copiose prodientes, plerumque sphaericae et pleroticae vel fere apleroticae, diametro medio 29·8 μm. Nullae chlamydosporae facta.

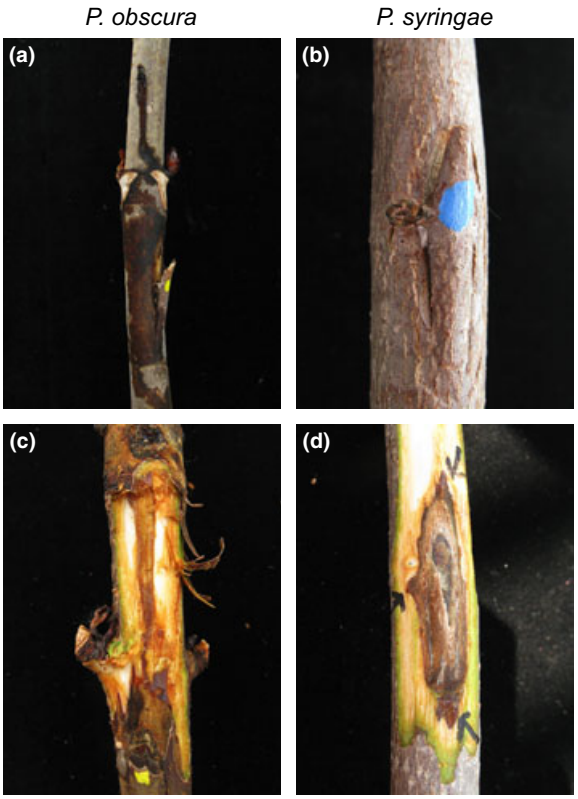

Figure 10 Lesion-inoculated stems showing distinct lesions caused by (a,c) *Phytophthora obscura* sp. nov. and (b,d) *P. syringae* on *Aesculus hippocastanum*. Symptoms (a,b) before and (c,d) after peeling away the bark. Successful inoculation was confirmed using reisolation and DNA sequencing.

Typus: **Germany:** isol. ex soil from *Aesculus hippocastanum* in an allée, S. Werres (holotypus: BBA 2/94-IIB; cultura ex tip CBS 129273; MB 519834).

Colonies grew smooth to slightly stellate on CPA, OMA and V8A and appeared cottony and petaloid to stellate on PDA. Temperature optimum on CPA at 25°C. Daily incremental radial growth average of 4·3 mm on CPA at 25°C. Hyphal swellings absent to rare. Low branching rate of mycelium on CPA. Sporangia semipapillate, non-caducous, born terminally on the sporangiophore. Sporangia average 36·2 × 26·4 μm with an average L:B ratio 1·4:1. Sporangia mainly ovoid. Sporangia abundantly formed on CPA after flooding with peat extract. Species homothallic. Abundant oospore production in culture. Oogonia not ornamented. Antheridia paragynous, nearly globose 10·0 × 10·0 μm in average. Oogonia spherical with 33·4 μm average diameter. Oospores mainly spherical and plerotic to nearly aplerotic, with 29·8 μm average diameter. No chlamydospores produced.

Discussion

Phytophthora obscura sp. nov. causes foliar blight on *K. latifolia*, *Rhododendron* and *Pieris*, and bleeding cankers on *A. hippocastanum*. To date, attempts to isolate *P. obscura* from bleeding cankers of the naturally infected *A. hippocastanum* have failed and inoculation studies conducted in the 1990s also failed (Werres *et al.*, 1995). The latter may be due to the very short incubation period after inoculation and the high incubation temperatures used at the time (Werres *et al.*, 1995). Recent experience indicates that the new *Phytophthora* species might require a long incubation period and low temperatures to infect trees and to establish in the tissue. Thus, it is not yet clear whether *P. obscura* was responsible for the bleeding cankers observed on horse chestnuts in Germany in the 1990s. One isolate was originally obtained from *Kalmia* and Koch's postulates were confirmed for this species. *Phytophthora obscura* causes foliar blight reminiscent of *P. syringae* on *Kalmia*, *Rhododendron* and *Pieris* (Fig. 11). *Aesculus*, *Kalmia*, *Rhododendron* and *Pieris* are highly susceptible to two other clade 8 species including the sudden oak death pathogen *P. ramorum* and *P. syringae* (Werres *et al.*, 2001; Parke *et al.*, 2004; Grünwald *et al.*, 2008; Grünwald & Parke, unpublished). All three pathogens are foliar pathogens on *Kalmia*. In a recent intensive sampling effort in Oregon, *P. obscura* was only sampled once in Oregon nurseries among *c.* 700 isolates sampled from *Rhododendron*, *Kalmia*, *Pieris*, substrate and water (Grünwald *et al.*, 2011). To the best of the authors' knowledge, this pathogen has not been observed anywhere else and is not found in GenBank or any other database. Thus, although found in Oregon once on *Kalmia* and in substrate in the US or soil in Germany, this species at this point appears to be rare and/or difficult to isolate. Given the similarity to *P. syringae*, some *P. obscura* isolates in culture might also be misidentified as being *P. syringae*.

Morphologically, *P. obscura* is more similar to *P. syringae* than to *P. austrocedrae*. *Phytophthora obscura* can easily be distinguished from *P. austrocedrae* mainly because of the slower growth, the bigger sporangia, and the different size and shape of the gametangia of the latter. *Phytophthora obscura* and *P. syringae* cannot be distinguished based on morphology alone. Ecologically, *P. obscura* might be comparable to *P. syringae* and considered to cause mostly foliar blight, twig blight and stem cankers on ornamentals or cankers on trees. All clade 8d species known to date are characterized as having semipapillate, non-caducous sporangia and being homothallic. Homothallism indicates that these taxa might be inbreeding species.

This work also provides support for a novel clade 8d in the genus *Phytophthora*. Detection of novel taxa, and hence deeper taxon sampling, allowed definition of this new subclade 8d based upon the clade structure established by Blair *et al.* (2008). Blair *et al.* grouped *P. syringae* (and *P. austrocedrae* by association) with clade 8b. The analysis here provides phylogenetic support for *P. syringae*, *P. austrocedrae* and *P. obscura* as being distinct phylogenetic species. Support was also observed for a larger clade encompassing 8b and 8d. However, 8d itself also exhibited strong bootstrap support and the genetic

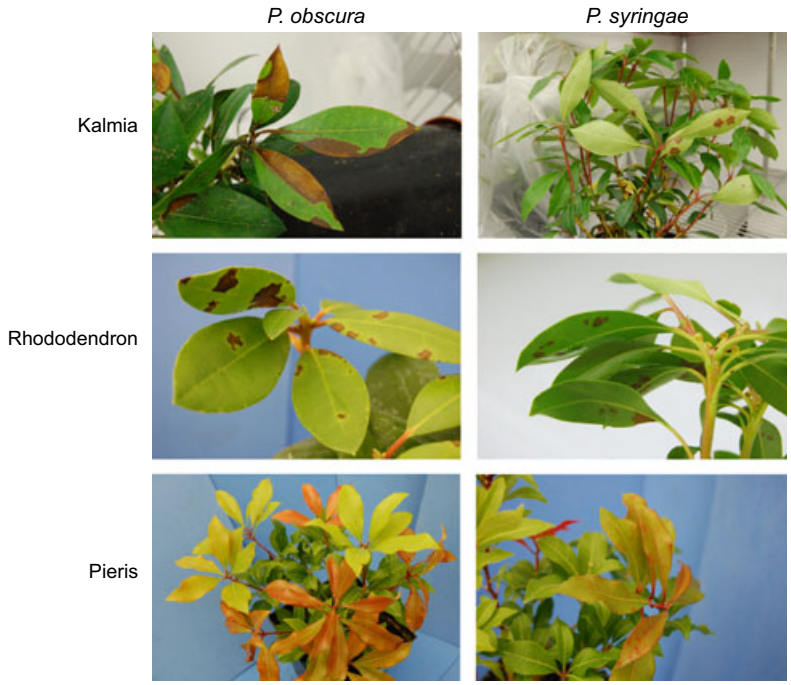

Figure 11 Whole plant, dip-inoculated plants showing symptoms caused by *Phytophthora obscura* sp. nov. and *P. syringae* on *Kalmia latifolia*, *Rhododendron catawbiense* cv. Boursault, and *Pieris japonica*. Successful inoculation was confirmed using reisolation and DNA sequencing.

distance between the 8d and 8b clades clearly indicates two separate clades. AFLP analysis, eight nuclear loci and the *coxI-cox* spacer-*coxII* mitochondrial locus support this revised structure in clade 8.

The origin of *P. obscura* is not known. While *P. ramorum* is now clearly recognized as an exotic pathogen (Mascheretti *et al.*, 2008, 2009; Grünwald & Goss, 2009; Grünwald *et al.*, 2009; Goss *et al.*, 2011), it is not clear at this point whether *P. syringae* and/or *P. obscura* are introduced or exotic pathogens. Based on the AFLP data shown here, *P. obscura* shows some genetic diversity and two apparent clades for the US and German populations. To date, *P. obscura* has only been found rarely in nurseries (USA) or in association with horse chestnuts in urban plantations (Germany) most likely because it was mistaken for *P. syringae*, well known as having the same four hosts studied here. At this point the threat posed by *P. obscura* to woody plants and tree species is unclear and further research is necessary to assess if this pathogen is introduced and what the demographic history of it is (Grünwald & Goss, 2011). The full host range of this taxon also remains to be established. *Phytophthora obscura* might be involved in causing some of the horse chestnut cankers in Europe, but further work is needed to establish support for this fact.

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