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Phytophthora abietivora, A New Species Isolated from Diseased Christmas Trees in Connecticut, U.S.A.

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Abstract

A number of fir species (*Abies*) are produced as Christmas trees around the world. In particular, Fraser fir (*Abies fraseri* (Pursh) Poir.) is popular as it yields high-quality Christmas trees in temperate North America and Europe. A *Phytophthora* sp. causing root rot on Fraser fir was isolated from a Christmas tree farm in Connecticut, U.S.A., and found to be new to science according to morphological and molecular phylogenetic analysis using multilocus DNA sequences from ITS, *Cox1*, β-*Tub*, *Nadh1*, and *Hsp90* loci. Thus, it was described and illustrated as *Phytophthora abietivora*. An informative Koch's postulates test revealed that *P. abietivora* was the pathogen causing root rot of Fraser fir.

Keywords: multi loci, oomycetes, pathogenicity, phylogeny, zoospore

A number of firs (*Abies* spp.) are grown as Christmas trees. Fraser fir, *Abies fraseri* (Pursh) Poir., is native to the Appalachian Mountains of the Southeastern United States (Hunt 1993) and is one of the predominant species of Christmas trees (Talgø and Chastagner 2012), producing high-quality Christmas trees in Connecticut and other states in the U.S.A., Canada, and Europe. Phytophthora root rot is a serious disease of *Abies* spp., which leads to significant losses from Christmas tree farms and conifer nurseries (Chastagner and Benson 2000; Hinesley et al. 2000; McKeever and Chastagner 2016). Fraser fir is highly susceptible to root rot disease caused primarily by *Phytophthora cinnamomi* Rands and other *Phytophthora* species (Erwin and Ribeiro 1996; Hoover and Bates 2013; Quesada-Ocampo et al. 2009) and the disease develops rapidly, with a majority of infected trees dying within 4 to 5 weeks (Chastagner and Benson 2000; Hinesley et al. 2000).

The genus *Phytophthora* has about 150 to 170 described species (Brasier 2009; Jung et al. 2016). Over 80 taxa are pathogenic causing devastating plant diseases worldwide and leading to severe economic losses each year (Judelson and Blanco 2005; Shamoun et al. 2018). Members of the genus *Phytophthora* are reported to infect more than 130 plant species and Tsao (1990) reports that *Phytophthora* spp. caused >90% of all collar rots and >66% of all fine root diseases of woody plants. Surveys by Jung et al. (2016) in Europe found 49 taxa of *Phytophthora* in nursery plants (most were healthy at the time of sampling), and 56 taxa of *Phytophthora* in forest and landscape plantings. Together, a total of 68 *Phytophthora* taxa were found. Among these 68 species, at least 47 taxa detected in nurseries and plantings were introduced species and seven were new species (Jung et al. 2016).

McKeever and Chastagner (2016) documented 13 species of *Phytophthora* causing root rot in Christmas trees of *Abies* spp. in the United States and 18 documented species on *Abies* spp. worldwide. Three additional species found to infect *A. fraseri* in North America are mentioned in the literature, for a total of 16 species: *P. cactorum* (Lebert & Cohn) J. Schröt. in Connecticut, Michigan, Pennsylvania, and Wisconsin; *P. cambivora* (Petri) Buisman in Michigan, Oregon, and Washington; *P. capsici* Leonian in Michigan; *P. cinnamomi* Rands in North Carolina and West Virginia; *P. citricola* Sawada in Michigan and North Carolina; *P. citrophthora* (R.E. Sm. & E.H. Sm.) Leonian in North Carolina; *P. cryptogea* Pethybr. & Laff. in North Carolina and Pennsylvania; *P. drechsleri* Tucker in North Carolina and Pennsylvania; *P. europaea* E.M. Hansen & T. Jung in Wisconsin; *P.* taxon '*kelmania*' in Connecticut, New York, and North Carolina (this species has not yet been formally described [Phytophthora Database 2018]); *P. megasperma* Drechsler in Michigan; *P. nicotianae* Breda de Haan in Michigan; *P. pini* (belonging to a species complex of *P. citricola*) in Michigan, North Carolina, and New York; *P. plurivora* T. Jung & T.I. Burgess (holotype MURU 433) in Connecticut, Michigan, North Carolina, and Wisconsin; *P. pseudosyringae* T. Jung & Delatour in Washington; and *P. sansomeana* E.M. Hansen & Reeser in Michigan, New York, North Carolina, and Wisconsin (Adams 1988; Grand 1985; Hoover and Bates 2013; Huang et al. 2004; Kuhlman and Hendrix 1963; Martin et al. 2014; McKeever and Chastagner 2016; Pettersson et al. 2017; Quesada-Ocampo et al. 2009; Shew and Benson 1981; Williams and Haynes 1982).

Root rot leading to death of *A. fraseri* trees was found in a Christmas tree farm in Connecticut. Samples were collected from infected trees at the farm and one pathogen isolate belonging to *Phytophthora* sp. was found to be new to science – here a novel fungal species is described and illustrated. Koch's postulates were tested to confirm the pathogenicity of the recovered *Phytophthora* spp.

Materials and Methods

Nine hundred 3- to 5-year-old Christmas trees of different species were planted into a field (41°45.997′N, 71°55.182′W) in Brooklyn, CT, as bare-root transplants in 2010, among which 60 trees were Fraser fir. Within three years, all Fraser firs in this field had died. In a new experiment, 5-year-old bare-root Fraser fir seedlings were replanted in 2015 into this field. Ten presumably infected *A. fraseri* Christmas trees from this field were sampled on Oct. 10, 2017. Plants chosen for sampling were identified based upon subtle signs of poor color or chlorotic foliage on the lowest whorl of branches and were dug with intact major roots from the field. Diseased trees were transported to the laboratory, the roots washed with tap water but not surface sterilized, and root crown and stem lesions identified. Slivers of diseased tissue (2 × 2 mm) from the interior of the stem were removed from the margin of lesions with a heat-sterilized scalpel and inserted

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into the flesh of apples ('Braeburn' fruits) through a slit in the skin of the fruit following the method of Tucker (1931). The apple fruits were incubated for a week at room temperature.

Lesions with brown discoloration within the apple flesh expanded outward from inoculated sites. Two isolates were obtained. The isolates were purified by cutting hyphal tips, which were cultured on PDA at 25°C for 7 days. A heat-sterilized scalpel was used to remove a 2 × 2 mm piece, less than 1 mm in thickness, which was then placed on potato dextrose agar (PDA: 39 g dried powder, 1 liter water) and subsequently grown by inoculating three equidistant points in a triangle pattern on 10% V8 agar (V8) (100 ml clarified V8 juice, 900 ml water, 1 g $CaCO_3$, 15 g agar; Ferguson and Jeffers 1999) at 15, 20, and 25°C for 7 to 30 days depending on rate of mycelial growth. To induce sporangia and zoospore formation, five V8 plates were flooded with nonsterile soil extract (Jeffers and Aldwinckle 1987) and another five were flooded and also embedded with Fraser fir root pieces 2 cm in length. A third method, flooding culture plates with soil-free root extracts, was also used to induce development of sporangia and zoospores. Live roots (<5 mm diameter) from Fraser fir and red oak (*Quercus rubra*) were washed with deionized water. Ten grams of each, chopped into 1 cm segments, were soaked in 250 ml of deionized water for 1 h, then filtered through a 0.2 μm filter and held at 4°C until used.

Pieces of colonies were mounted in 85% lactic acid on glass slides for microscopic observation. To monitor sporulation, aerial hyphae, and colony development, malt extract agar (MEA) (20 g malt, 20 g agar, and 1 liter distilled water) and V8 media were used. Colonies incubated at 25°C for a month on MEA plates were used for observing the growth, for preparing an extype, and for harvesting mycelium to conduct molecular studies. All observations and measurements of fungal structures were carried out under a compound microscope (Zeiss Imager.M2) with differential interference contrast (DIC). Photomicrographs were taken with an Axiocam 506 color camera (Carl Zeiss AG, Oberkochen, Germany). Measurements of the fungal structures were made under 40–100× objective lenses, from which means, standard deviations, and 95% confidence intervals of means were calculated using the Data Analysis Package of Excel 2016.

The holotype has been deposited in The UAMH Centre for Global Microfungal Biodiversity at University of Toronto (UAMH), Canada, and an ex-type in the NRRL Culture Collection, USDA, Peoria, IL, U.S.A.

DNA extraction, amplification, and sequencing. The isolate was grown on PDA at 25°C, and on V8 at 15, 20, 25°C for up to one month. DNA was extracted from colonies grown in Petri plates according to the procedure in ZR Fungal/Bacterial DNA MicroPrep Kit (Zymo Research, Irvine, CA, U.S.A.). Portions of the 18S ribosomal RNA including the internal transcribed spacers 1 and 2 and 5.8S ribosomal RNA gene (*ITS*), cytochrome c oxidase subunit 1 (*Cox1*), NADH dehydrogenase subunit 1 (*Nadh1*), heat shock protein 90 (*Hsp90*), and β-tubulin (*β-Tub*) loci were amplified from genomic DNA by polymerase chain reaction (PCR) using oligonucleotides *V9G* or *ITS5* with *LR1* (Van den Ende and De Hoog 1999; Vilgalys and Hester 1990; White et al. 1990), *COXF4N* and *COXR4N* (Kroon et al. 2004), *NADHF1* and *NADHR1* (Kroon et al. 2004), *HCF90_F1* and *HCF90_R1* (Blair et al. 2008), and *TUBUF2* and *TUBUR1* (Kroon et al. 2004), respectively. The parameters for the PCR amplification protocol were 94°C 3 min; 94°C 30 s; 45°C 30 s; 72°C 2 min, repeat 35×, 72°C 7 min. Resulting amplified DNA products were purified using QIA quick PCR Purification columns (Qiagen, Valencia, CA, U.S.A.) and the DNA concentrations were determined on a NanoDrop Lite Spectrophotometer (ThermoScientific, Waltham, MA, U.S.A.). DNA sequencing of the PCR amplified ITS, *Cox1*, *Nadh1*, *Hsp90*, and *β-Tub* products employed oligonucleotides (*V9G*, *ITS2*, *ITS3*, *LR1*), (*COXF4N*, *COXR4N*), (*NADHF1* and *NADHR1*), (*HCF90_F1* and *HSP90_R1*), (*TUBUF2*, *TUBUR1*, *TUB2T22* [O'Donnell and Cigelnik 1997], *TUB2REV 5′-CAGCGGGGCGAAGCCGATC-3′, TUB2FOR 5′-CCAGCTGAACT CGGACCTGCG-3′*), respectively. All DNA sequencing was performed at the W. M. Keck Biotechnology Resource Laboratory, Yale School of Medicine, New Haven, CT, U.S.A. The resulting DNA sequences were deposited into GenBank with the following accession numbers: ITS [MK163944]; *Cox1* [MK164270]; *Nadh1* [MK164269]; *Hsp90* [MK164275]; *β-Tub* [MK164274].

The aforementioned five genes/regions of DNA sequences were employed as a query for BLASTn analysis in databases at GenBank (https://blast.ncbi.nlm.nih.gov). The allied taxa and the corresponding DNA sequences of allied taxa for loci ITS, *Cox1*, *Nadh1*, *Hsp90*, *β-Tub* were obtained from GenBank (Blair et al. 2008). Sixteen additional species of *Phytophthora* from Clade 7 and *P. megasperma* from Clade 6 with 26 isolates in total were selected for phylogenetic analyses (Jung et al. 2017b). Uncultured/unidentified samples, environmental samples, and samples with questionable identifications were excluded (Wheeler et al. 2003). The sequences of the unknown pathogen generated from the present study were deposited to GenBank; the accession numbers of the sequences and accession numbers of the taxa and isolates used in our phylogenetic analysis are presented in Table 1.

Alignment and phylogenetic analysis. Sequence data from ITS, *Cox1*, *β-Tub*, *Nadh1*, and *Hsp90* were aligned independently using MUSCLE (Edgar 2004), followed by manual correction, then were trimmed and concatenated with FABOX sequence alignment joiner (http://users-birc.au.dk/palle/php/fabox/alignment_ joiner.php).

Phylogenetic analyses employed both maximum likelihood (ML) with MEGA X (Kumar et al. 2018; Tamura 1992) or Bayesian inference (BI) using MrBayes3.2.6 (Ronquist et al. 2012). The dataset had 26 concatenated nucleotide sequences of five genes/region with 4,250 positions, and all sites were treated with equal weight for the analysis. Gaps were treated as missing data. *P. megasperma* DDS3432 was designated as an outgroup. For ML analysis, a bootstrap was calculated with 1,000 replicates. For BI analysis, four Markov chains were used for one run from random starting trees for two million generations. Tree sampling frequency was 1,000 generations. The first one-tenth generations were discarded as burn-in. The majority rule consensus tree of all remaining trees was computed. Branches that received Bayesian posterior probabilities of at least 0.95 (BPP) were considered to be significantly supported. Phylogenetic trees were constructed using TreeGraph2 (Stöver and Müller 2010).

Koch's postulates test. Healthy bare-root 5-year-old *A. fraseri* transplants were obtained from a cooperating Christmas tree grower and potted into 2-gallon (7.6 liter) plastic nursery containers in composted hardwood chips in June 2018. They were maintained with twice-daily overhead irrigation of 80 ml per pot from a domestic, chlorinated water source in a nursery area with coarse crushed stone surface at the CAES Valley Laboratory. Six trees were inoculated on 6 July 2018. An additional six trees were wounded but not inoculated and held as controls. To inoculate the trees, a 5 × 5 mm flap of bark was opened with a sterile scalpel to the depth of the cambium, and *Phytophthora* mycelium and an agar piece containing oospores (2 × 2 mm, grown on 10% V8 agar for 1 month) were inserted under the bark flap. The flap was then closed and sealed with Parafilm. Controls were treated in the same manner as the inoculated trees, except that no agar or *Phytophthora* mycelium were introduced. Trees were then placed in a growth chamber with 16:8 h light/dark fluorescent lighting and temperature set to a constant 15°C. Plants were maintained without additional watering by placing the pot into a plastic bag and securing the bag around the stem to prevent evaporation from the potting medium. A binder clip held the excess plastic from the top of the bag closed but did not clamp the stem of the tree.

After 17 days (23 July 2018), the first signs of wilting were visible with one of the six inoculated trees. Two additional inoculated trees were destructively examined at this date, and all three trees had interior lesions extending upward from the point of inoculation. Three control trees were examined and had healthy tissue surrounding the wounded site. The tree experiencing wilting of foliage was used to reisolate *Phytophthora*, using apple baiting with the methods described above. At 26 days postinoculation, the three remaining inoculated trees were examined for infection and two were used to reisolate *Phytophthora* using apples. The procedure was identical to the initial isolation from the field samples, except that two tissue samples each were taken from above and below the inoculation point along the

margin of the lesion, with four samples (in total) from each tree being used to inoculate each apple.

Apples were incubated in the growth chamber for 2 weeks, whereupon the brown lesions had developed sufficiently for pieces to be removed with a sterile scalpel and cultured on 10% V8 agar.

Cultures of *Phytophthora* were allowed to grow on the 10% V8 for 2 weeks to allow production of oospores for measurement and confirmation of identity.

Results

The results of phylogenetic analyses employing maximum likelihood or Bayesian inference using ITS, *Cox1*, *β-Tub*, *Nadh1*, and *Hsp90* sequences were highly similar and led to the same conclusion. *P. abietivora* is a sister species to *P. flexuosa*, and is in the same subclade as *P. europaea*, while other Clade 7 *Phytophthora* species reside in different subclades (Fig. 1).

Taxonomy. *Phytophthora abietivora* D.W. Li, N.P. Schultes, J. A. LaMondia, R. S. Cowles sp. nov. MycoBank # MB 830083. Oogonia, oospores and antheridia: Oogonia developed on V8. Oogonia were borne terminally or laterally and globose to subglobose, colorless to golden-yellow (Fig. 2a, b), infrequently with a tapering base, (32.1) 41.5–51.9 (55.5) × (30.6) 38.1–47.5 (50.5) μm (mean ± SD: 46.7 ± 5.2 × 42.8 ± 4.7 μm, n = 35). Oospores were plerotic to slightly aplerotic, globose and often contained 1–3 ooplasts, colorless to brownish yellow (Fig. 2a, b), (26.5) 33.7–41.3 (42.5) μm (mean ± SD: 37.5 ± 3.8 μm, n = 35), thick walled (3.5) 4.4–6.4 (7.1) μm (5.4 ± 1 μm, n = 35), colorless to pale brownish yellow, smooth to verruculose. Antheridia were formed terminally or laterally, were subglobose, ovoid to irregular shaped and mostly paragynous (Fig. 2b) or occasionally amphigynous unicellular (Fig. 2b), (8.6) 10.5–15.5 (18.2) × (7.6) 8.2–13.2 (17.4) μm (mean ± SD: 13 ± 2.5 × 10.7 ± 2.5 μm, n = 23).

Sporangia, hyphal swellings, and chlamydospores. Sporangia of *P. abietivora* were not observed on solid PDA agar and V8 flooded with soil extract and/or imbedded with root pieces on 20°C and room temperature but developed on 10% V8 plates flooded with root extract at 15°C and held under fluorescent lighting for 1 week. Sporangia were developed terminally on unbranched sporangiophores solitarily or in a cluster with two to three sporangia, ovoid or ellipsoid, hyaline or pale yellow, noncaducous and nonpapillate, flat or rounded at the apex with a lenticular plug (Fig. 2c, d). Sporangia were (27.1) 29.8 ± 37.2 (41.8) × (17.7) 22.2 ± 28.0 (30.8) μm (mean ± SD: 33.5 ± 3.7 × 25.1 ± 2.9 μm, n = 30). The length/breadth ratio of the sporangia averaged 1.34 ± 0.13. Occasionally a nesting sporangium developed through the empty preceding one (Fig. 2e). Zoospores were (10.6) 12.5–15.1 (16.7) × (9.8) 10.2–12.2 (13) μm (mean ± SD: 13.8 ± 1.3 × 11.2 ± 1 μm, n = 30), discharged through a wide exit pore (6–7.5 μm wide). They were primarily pyriform, also oval, globose to reniform, motile, becoming globose on encystment. (Fig. 2f, g). Chlamydospores were present, globose or subglobose, colorless to pale yellow, smooth, 7–11.5 μm (Fig. 2h). In solid agar flooded with soil extract, irregular coralloid hyphal swellings were formed (Fig. 2i).

Holotype. U.S.A., Connecticut, Brooklyn, Allen Hill Farm (41°45.997′N 71°55.182′W), from infected root of a Christmas tree of *Abies fraseri* (Pursh) Poir., 10 October 2017, R.S. Cowles, UAMH 12075 (RC2017-1). Holotype specimen is a living specimen being maintained via lyophilization at UAMH Centre for Global Microfungal Biodiversity, The Gage Research Institute, Toronto, Canada. Extype is NRRL66892.

Etymology. Latin, abies, referring to conifer genus *Abies* and -vora, ones that eat.

Comments. P. abietivora develops rather large oospores with very thick walls (37.5 ± 3.8 μm in size, 5.4 ± 1 μm in thickness), which differentiate it from morphologically and phylogenetically related species, *P. flexuosa* (32.2 ± 2.7 μm in size, 3.1 ± 0.5 μm in thickness), and *P. europaea* (33.2 ± 5.3 μm in size, 2.5 ± 0.7 μm in

Table 1. Taxa, isolates, their sequences, and GenBank accession numbers used in the phylogenetic analysis[a]

Clade	Taxa	Type	Culture no.	Host	Location	GenBank accession numbers					Reference
						ITS	Cox1	β-Tub	NADH1	HSP90	
Clade 7a	*Phytophthora abietivora*	holo-type	UAMH 12075	Christmas tree of *Abies fraseri*	U.S.A.	MK163944	MK164270	MK164274	MK164269	MK164275	This study
Clade 7a	*P. attenuata*	ex-type	CBS 141199	*Castanopsis carlesii*	Taiwan	KU517154	KU517148	KU899277	KU899519	KU899434	Jung et al. 2017a
Clade 7b	*P. cinnamomi*	ex-type	CBS 144.22	*Cinnamomum burmannii*	Indonesia	KU899160	KU899315	KU899233	KU899475	KU899390	Scanu et al. 2014
Clade 7a	*P. europaea*	ex-type	CBS 109049	*Quercus robur*	France	HQ261556	KU681022	EU079482	KU899469	EU079485	Jung et al. 2002
Clade 7a	*P. europaea*		CBS 109051	*Quercus sp.*	France	KU899157	KU899312	KU899229	KU899470	KU899384	Jung et al. 2002
Clade 7a	*P. flexuosa*		CBS 141202	*Fagus hayatae*	Taiwan	KU899193	KU899348	KU899271	KU899513	KU899428	Jung et al. 2017b
Clade 7a	*P. flexuosa*		TW79	*Fagus hayatae*	Taiwan	KU899220	KU899375	KU899303	KU899545	KU899460	Jung et al. 2017b
Clade 7a	*P. flexuosa*	ex-type	CBS 141201	*Fagus hayatae*	Taiwan	KU517152	KU517146	KU899302	KU899544	KU899459	Jung et al. 2017a
Clade 7a	*P. formosa*		CBS 141204 TW14	*Q. glandulifera*	Taiwan	KU899201	KU899356	KU899280	KU899522	KU899437	Jung et al. 2017a
Clade 7a	*P. formosa*	ex-type	CBS 141203	*Araucaria cunninghamii*	Taiwan	KU517153	KU517147	KU899270	KU899512	KU899427	Jung et al. 2017a
Clade 7a	*P. formosa*		TW13	*Quercus glandulifera*	Taiwan	KU899199	KU899354	KU899278	KU899520	KU899435	Jung et al. 2017b
Clade 7a	*P. fragariae*		ATCC 36057	*Fragaria xananassa*	U.K.	HQ261564	KU681021	EU079744	KU899548	EU079747	
Clade 7a	*P. fragariae*	ex-type	CBS 209.46	*Fragaria*	U.K.	HQ643230	–	–	–	–	C. J. Hickman, *unpublished*
Clade 7a	*P. intricata*	ex-type	CBS 141211	*Quercus tarokoensis*	Taiwan	KU517155	KU517149	KU899284	KU899526	KU899441	Jung et al. 2017a
Clade 6	*P. megasperma*		DDS3432	Soil *Banksia sp.*	Australia	HQ012949	HQ012867	JN547608	KM883175	HQ012906	Jung et al. 2011
Clade 7b	*P. niederhauserii*		CBS 124086	*Chamaecyparis lawsoniana*	Hungary	GU230789	GU477617	GU477613	GU477619	KU899389	Józsa et al. 2010
Clade 7a	*P. rubi*	ex-type	CBS 967.95	*Rubus idaeus*	U.K.	AF139370	DQ674736	KU899234	KU899476	KU899391	Man In't Veld 2007
Clade 7a	*P. uliginosa*	ex-type	CBS 109054	*Quercu robur*	Poland	AF449495	KU681023	EU080012	KU899471	EU080014	Jung et al. 2002
Clade 7a	*P. uliginosa*		CBS 109055	*Quercus petraea*	Germany	HQ261722	–	EU079693	–	EU079696	Jung et al. 2002
Clade 7a	*P. uniformis*	ex-type	IMI 392315	*Alnus glutinosa*	Sweden	GU259293	–	–	–	–	Brasier et al. 2004
Clade 7a	*P. uniformis*		WPC P10565	*Alnus glutinosa*	Hungary	KU899221	KU899376	KU899304	KU899546	KU899461	Ioos et al. 2006
Clade 7a	*P.* x *alni*	ex-type	IMI 392314	*Alnus glutinosa*	U.K.	KU681013	KU681017	KU899238	KU899480	KU899395	Brasier et al. 2004
Clade 7a	*P.* x *cambivora* (A2)	neo-type	CBS 141218	*Quercus pubescens*	Italy	KU899179	KU899334	KU899255	KU899497	KU899412	Jung et al. 2017b
Clade 7a	*P.* x *heterohybrida* (A2)	ex-type	CBS 141207	Baiting; tributary of Ha-pen River	Taiwan	KU517151	KU517145	KU899290	KU899532	KU899447	Jung et al. 2017a
Clade 7a	*P.* x *incrassata* (A2)	ex-type	CBS 141209	Baiting; tributary of Ha-pen River	Taiwan	KU517156	KU517150	KU899286	KU899528	KU899443	Jung et al. 2017a
Clade 7a	*P.* x *multiformis*	ex-type	IMI 392316, WPC P16202, PD_01913	*Alnus glutinosa*	Netherlands	AF139368	KU681018	KU899239	KU899481	KU899396	Brasier et al. 2004

[a] Abbreviations of isolates and culture collections: ATCC = American Type Culture Collection, Manassas, U.S.A.; CBS = The Westerdijk Fungal Biodiversity Institute (formerly Centraalbureau voor Schimmel cultures), Utrecht, the Netherlands; IMI = CABI Bioscience, U.K.; PD = Phytophthora Database (http://www.phytophthoradb.org); WPC = World Phytophthora Collection, University of California Riverside, U.S.A.; other isolate names and numbers are as given by the collectors and on GenBank, respectively.

thickness) (Jung et al. 2002, 2017b). *P. uliginosa* is another species sharing some similarities in morphology, but its oospores are bigger with thinner walls (41.3 ± 6.1 μm in size, 4.2 ± 0.7 μm in thickness) (Jung et al. 2017b). The sporangia of *P. uliginosa* (67.0 ± 8.5 × 42.4 ± 6.4 μm) are much bigger than those of *P. abietivora* (33.5 ± 3.7 × 25.1 ± 2.9 μm) (Jung et al. 2017b). The size of zoospores of *P. abietivora* (13.8 ± 1.3 × 11.2 ± 1 μm) are similar to those of *P. flexuosa* (13.3 ± 1.3 μm), smaller than those of *P. europaea* (15.3 ± 2.0 μm), but larger than those of *P. uliginosa* (11.9 ± 1 μm) (Jung et al. 2017b). All four of these species develop hyphal swellings.

P. abietivora and *P. uliginosa* share high similarities in ITS sequences. However, *P. abietivora* and *P. uliginosa* are in different phylogenetic clades in the phylogenetic analysis using five loci (Fig. 1).

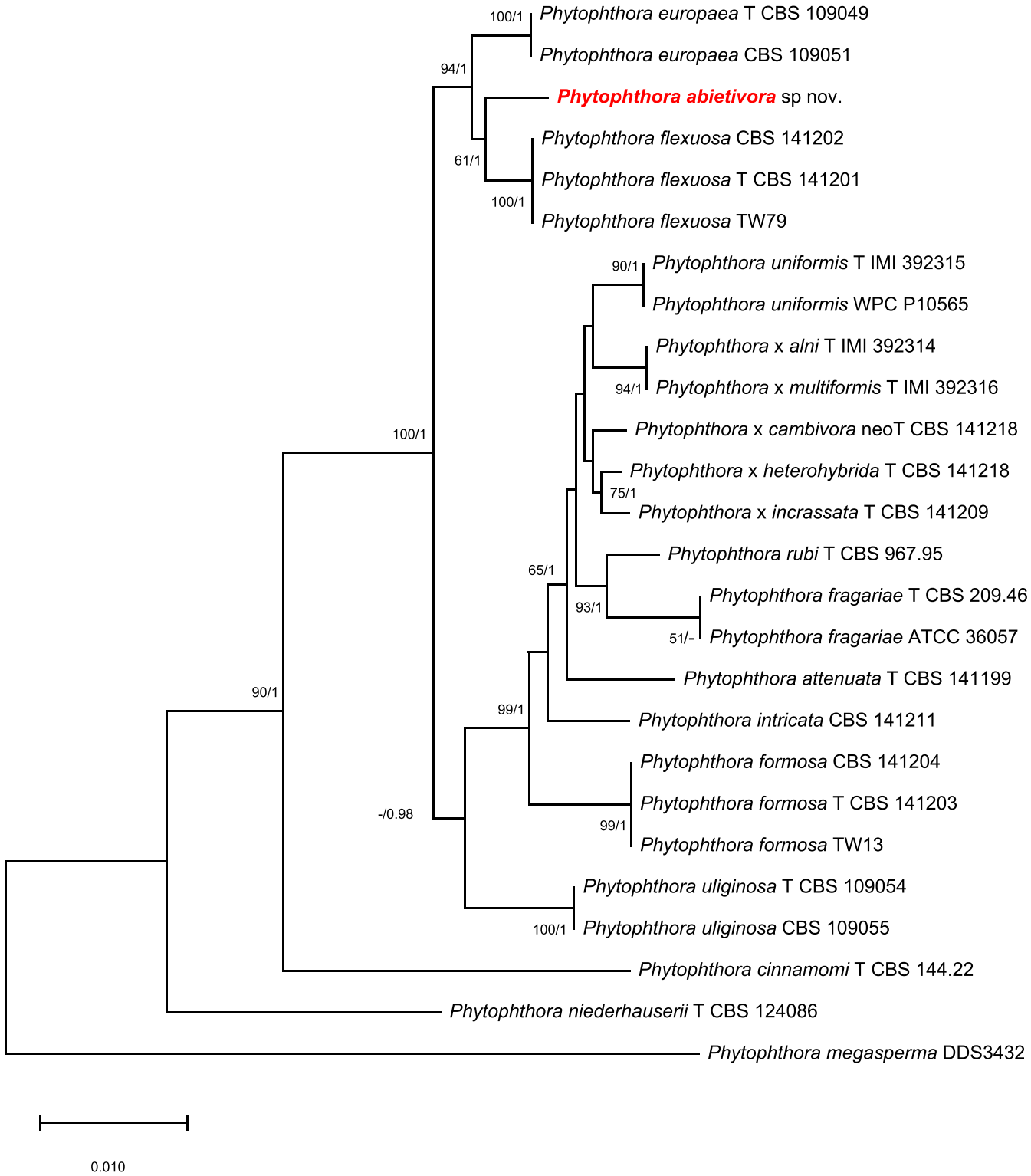

Fig. 1. Maximum likelihood analysis of *Phytophthora abietivora* (ex-type) and allied taxa based on concatenated ITS, *Cox1*, β-*Tub*, *Nadh1*, and *Hsp90* sequence data. *Phytophthora megasperma* DDS3432 is included as outgroup. The bootstrap test was conducted with 1,000 replicates. Bootstrap values >50% (before the slash) and Bayesian posterior probabilities (>0.90) (after the slash) were indicated at the nodes. The scale bar indicates the number of expected changes per site. T indicates the ex-types used in the analysis.

Symptoms in the field. All 60 Fraser firs planted in 2010 were dead by 2013 due to root rot infection, while very few mortalities occurred among the remaining 840 Christmas trees of other species planted in the same year. Following replanting in 2015, each succeeding year had trees with symptoms of the foliage turning reddish brown and current-year shoots bending downward. When the root crowns were cut open, the cambium and cortical layer had turned red (Fig. 3A, B).

Koch's postulates test. All inoculated trees became diseased. Symptoms included wilting of terminals (two of six inoculated trees) and necrotic tissue in the root crown and stem portions of the plants (all six inoculated trees) (Fig. 3). Chlorosis of the current season's needles was too subtle to quantify. *Phytophthora* was recovered from all three trees from which reisolation was attempted. None of the reisolation attempts from samples taken from the upper margin of the lesion, on the stem of the tree, were successful. However, reisolations were successful for five of six samples taken from the lower margin of the lesions. There was no disease in any of the control trees.

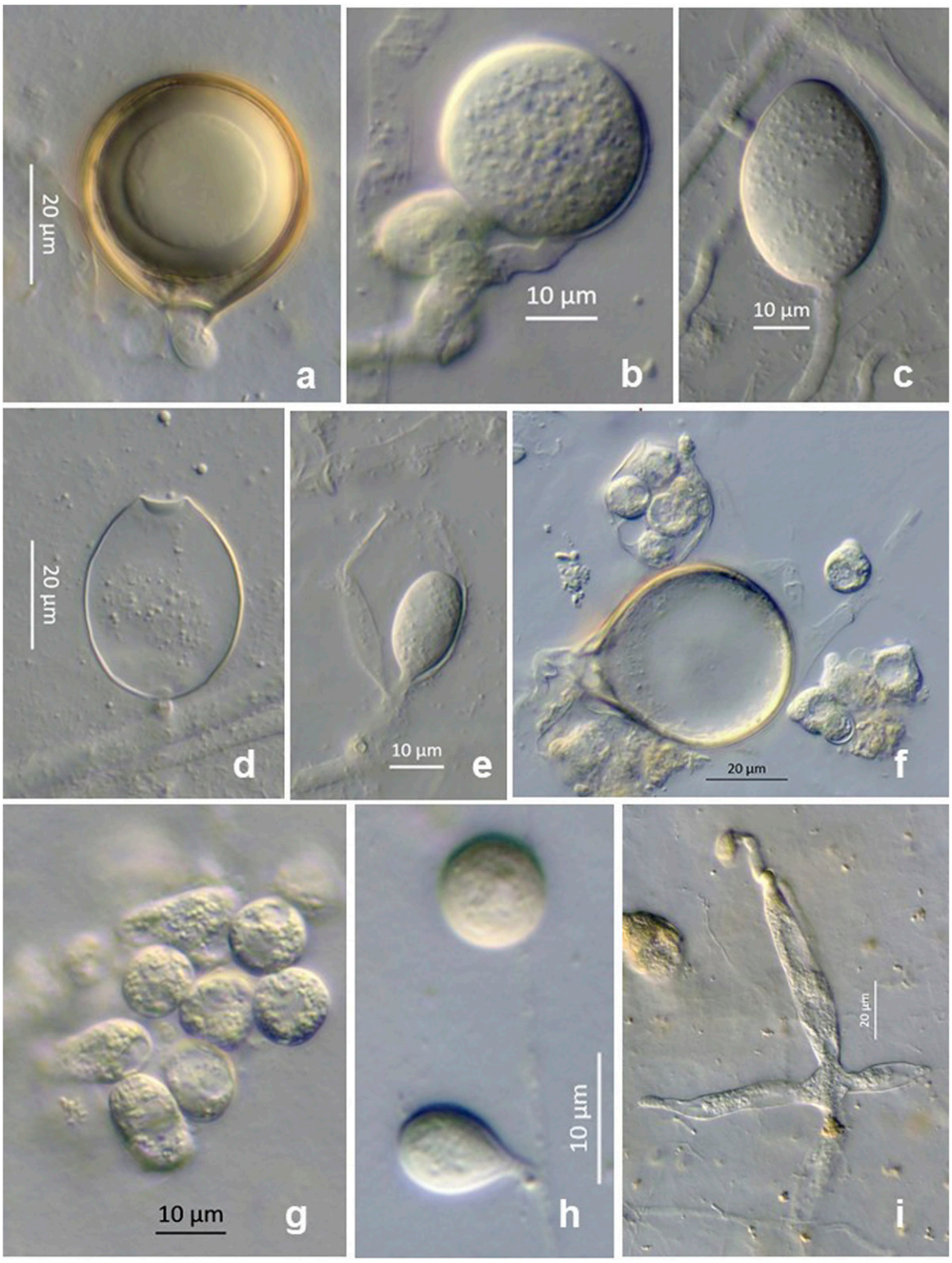

Fig. 2. *Phytophthora abietivora* (Holotype UAMH 12075). **a,** Oogonium and oospore. **b,** Immature oogonium and antherium. **c,** Sporangium. **d,** Empty sporangium. **e,** Nesting sporangium. **f,** Zoospores, sporangium, and oogonium. **g,** Zoospores. **h,** Chlamydospores. **i,** Irregular coralloid hyphal swellings.

Microscopic examination of the culture determined that the oospores had the same dimensions and morphological characters as the original isolate.

Discussion

It has been estimated that there are 100 to 500 species of *Phytophthora* unknown to science (Brasier 2009). Jung et al. (2018) indicated that a potentially high number of undescribed *Phytophthora* species may exist and the unknown origin of many known aggressive *Phytophthora* species are a huge threat to the health and sustainability of forests, ecosystems, managed forests, and crop production systems worldwide. The discovery of a new species, *P. abietivora*, in Connecticut suggests that it might be the same scenario in the U.S.A. Nine species of *Phytophthora* have been reported from Connecticut

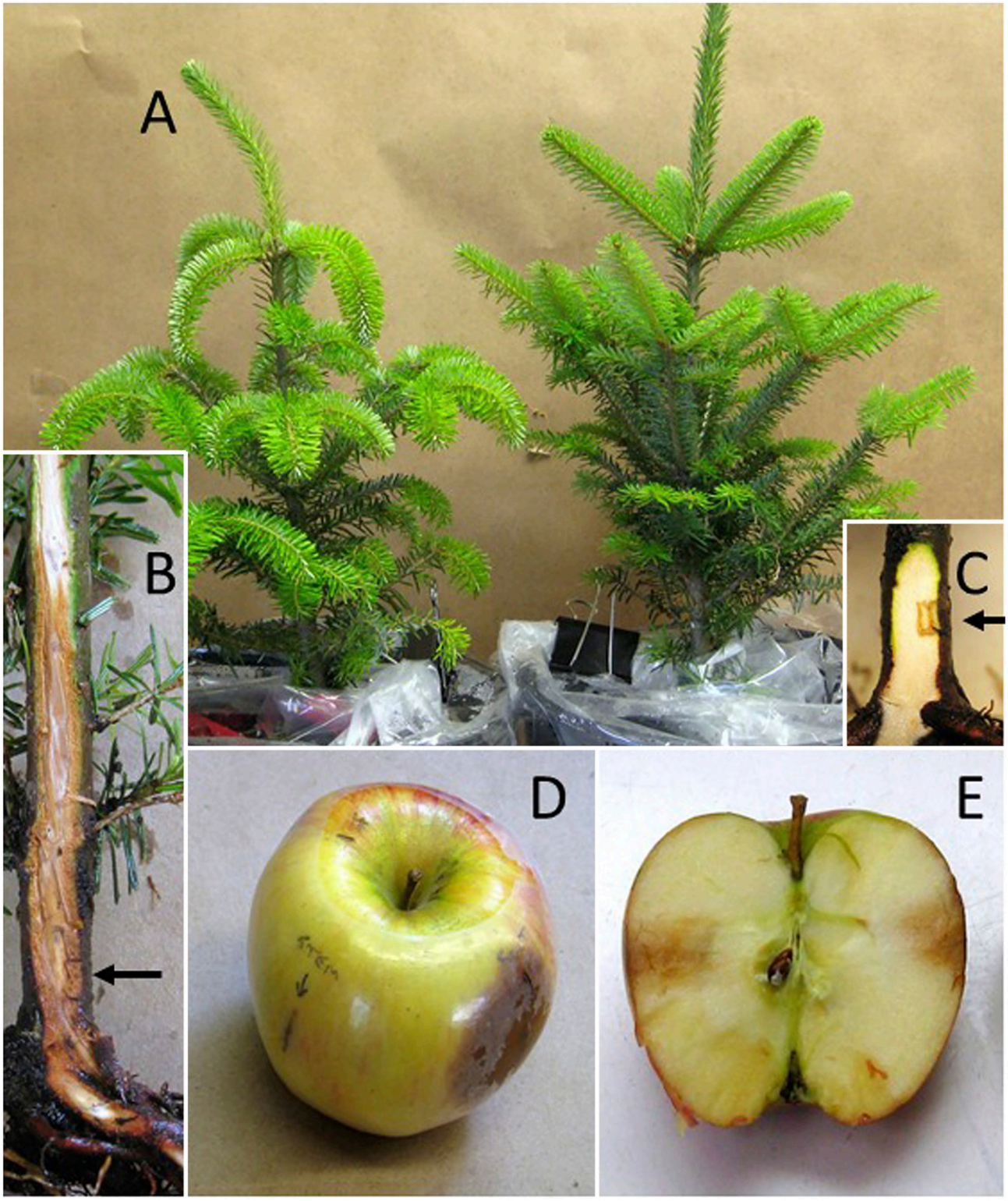

Fig. 3. Representative potted *Abies fraseri* and inoculated apples used to test Koch's Postulates. **A,** Inoculated (left) and uninoculated control (right) fir trees at 17 days post inoculation. The location of the bark flaps, marked with arrows, shows interior discoloration in lesions extending from the inoculation site in (**B**), but not in the stem of the uninoculated control tree (**C**). Brown discoloration of the flesh of an apple (**D**) developed about 2 weeks after inoculation with infected tissue taken from the roots (right side) but not when taken from the upper margin of the lesion on the stem (left side). The bisected apple (**E**) shows brown discoloration developing from inoculation slits made on opposite sides of the same apple, both originating from infected root tissue.

to date (Farr et al. 2019). It is likely that the number of *Phytophthora* spp. in Connecticut is significantly underestimated.

The results of our phylogenetic analysis were in agreement with an analogous study by Jung et al. (2017b), which showed *P. europaea* and *P. uliginosa* to be affiliated with the same subclade in Clade 7a, yet distinct and separate from other subclades. The new species, *P. abietivora*, shared the same subclade with *P. europaea* and *P. uliginosa*, but segregated distinctly from these two species (Fig. 1). Both morphological studies and phylogenetic analyses using the sequences of five loci support the same conclusion that *P. abietivora* constitutes a distinct species.

Phylogenetic analyses using each individual locus were conducted prior to multilocus analysis. ITS alone cannot differentiate *P. abietivora* from *P. flexuosa* and two other closely related species. *Cox1* and *Nadh1* cannot either, due to low significance levels. *β-Tub* and *Hsp90* seem to be able to differentiate them, but they are less reliable than multiple loci for identification purposes. Overall, our analyses of *P. abietivora* sequences confirms findings by Blair et al. (2008) that multilocus analysis is crucial to achieve adequate, species-level phylogenetic resolution within *Phytophthora*.

Our results also showed that *P. abietivora* is a pathogen causing root rot disease of Fraser fir Christmas trees. Observation in the field indicated that *P. abietivora* caused a significant loss in the Fraser fir Christmas tree farm where the pathogen was discovered. However, its host range remains unknown. Zoospores and sporangia of *P. abietivora* were not readily observed and multiple attempts described herein were required. Not all species or isolates of the same species develop sporangia on sterile media under laboratory conditions (Werres et al. 2001) and therefore, zoospores are not always observed. Limited production of zoospores may make species similar to *P. abietivora* difficult to detect through standard environmental surveys for *Phytophthora* spp. by using baits in streams (e.g., Klotz et al. 1959), and as such, they may be underrepresented. Sporangia may germinate directly to develop secondary sporangia (Domsch et al. 1980) or hyphae to infect hosts. Formation of sporangia of some species need to be induced with nonsterile soil extract, specific bacteria, or flooding with water (Domsch et al. 1980; Werres et al. 2001; Zentmyer and Erwin 1970). *P. cinnamomi* failed to develop sporangia in soil extract treated at 40–50°C for 10 min or in soils steamed at 60°C for 30 min (Broadbent and Baker 1974). Under sterile conditions, sporangia of *P. cinnamomi* only developed by a thorough washing with a salt solution (Zentmyer and Erwin 1970). Sporangia production of *P. fragariae* was induced by nonsterile pond water (Hickman and Goode 1953). In our study, colonies flooded with nonsterile soil extract with or without root pieces or flooded with distilled water developed hyphal swellings but no zoospores; *P. abietivora* only developed sporangia and zoospores on agar plates containing 10% V8 that were flooded with root extract solution at 15°C. A number of species of *Phytophthora* develop hyphal swellings, such as *P. cinnamomi* and *P. plurivora* (Domsch et al. 1980; Jung and Burgess 2009). The function of hyphal swellings remains to be studied.

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