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The avocado subgroup of *Phytophthora citricola* constitutes a distinct species, *Phytophthora mengei* sp. nov.

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Abstract: Isolates from avocado tree cankers have been recognized as a distinct subgroup within the *P. citricola* complex since 1974, both morphologically and molecularly (isozyme and amplified fragment length polymorphism [AFLP] analyses). This subgroup is formally separated from *P. citricola* after comparative DNA fingerprinting and sequence analyses of the ITS region, as well as by morphological examinations. This new taxon is homothallic, produces plerotic oospores with paragynous antheridia and noncaducous semipapillate sporangia. Morphologically it differs from other species of Waterhouse group III by producing many large bizarre-shaped sporangia and smaller oogonia with asymmetric capitate antheridia. It belongs to clade 2 and is phylogenetically closer to *P. siskiyouensis*, *P. capsici* and *P. tropicalis* than to *P. citricola*. *P. mengei* can be easily differentiated from its relatives in the same clade and other species of this morpho-group by DNA fingerprints and sequence analysis. This new taxon is named *Phytophthora mengei* sp. nov.

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INTRODUCTION

Among six species of *Phytophthora* affecting avocado (*Persea americana* Miller) in USA (Farr et al 1989) *P. citricola* was identified to be the primary cause of trunk canker (Zentmyer 1973, Zentmyer et al 1974). Feeder roots and tree trunks are the primary infection courts (Elhamalawi et al 1995b) although the pathogen may attack main structural roots (Coffey 1987, Zentmyer et al 1974) and fruit (Koike et al 1987, Ouimette et al 1988). The resultant disease is commonly known as "citricola canker" (Elhamalawi et al 1995a). This disease first was described by Fawcett (1916) and Barrett (1917), and it has caused increasing devastation to avocado in California (Coffey et al 1988, Coffey 1987, Elhamalawi and Menge 1994).

Our knowledge about the identity of the causal agent of avocado trunk canker has evolved over time. The pathogen was identified first as a *Pythiacystis* sp. (Fawcett 1916). A year later it was redescribed as *Phytophthora cactorum* (Lebert and Cohn) J. Schrot. (Barrett 1917). *Phytophthora citricola* was separated from *P. cactorum* by Sawada (Sawada 1927, Tucker 1931). The avocado canker pathogen subsequently was identified as *Phytophthora citricola* Sawada based on its similarity in morphology and protein banding patterns with those of other isolates of *P. citricola* (Zentmyer 1973, Zentmyer et al 1974). While identifying the pathogen as *P. citricola*, Zentmyer and associates clearly acknowledged that the avocado canker pathogen produced smaller oogonia and oospores than did the type culture of this species. Also the avocado isolates more frequently produced irregular sporangia in greater variety than the type culture of *P. citricola* (Zentmyer et al 1974). This pathogen more recently was demonstrated to belong to a subgroup within *P. citricola* based on distinct patterns of isozymes (Oudemans et al 1994) and amplified fragment length polymorphisms (AFLP) (Bhat and Browne 2007).

The above morphological and molecular differences indicate that the avocado canker pathogen is a separate species. To test this hypothesis we performed a standard DNA fingerprinting technique based on single-stranded conformation polymorphism of PCR-

amplified ribosomal DNA (PCR-SSCP) (Gallegly and Hong 2008). This technique was developed by Kong and associates (2003) and since has been evaluated with the vast majority of the known species of *Phytophthora* (Hong et al unpubl data). Each different PCR-SSCP, with a few exceptions, represent a distinct species within the genus (Gallegly and Hong 2008; Kong et al 2003, 2004a). We also sequenced representative isolates and examined their morphology. This paper reports on the morphology, DNA fingerprint and sequence analysis data of this new taxon in comparison with morphologically similar species.

MATERIALS AND METHODS

Isolation and isolate maintenance.—All cultures of the new taxon examined in this study were collected from the trunk of avocado trees in southern California. Nine of the isolates, including the type culture, were isolated by Dr John Menge of the University of California at Riverside (TABLE I). Single zoospore isolates of these cultures were obtained as described by Bhat and Browne (2007), and they were grown on V8 juice agar (Erwin and Ribeiro 1996). Blocks of fresh agar cultures were transferred into microtubes with sterile distilled water for long-term storage at 15 C. Five additional cultures representing several known species with similar morphology also were included in this study for comparison purposes.

Morphology.—Among the 11 isolates of the avocado trunk canker pathogen whose SSCP DNA fingerprints were identical, two (p340, p341) were selected for morphology studies. The detailed procedures are presented on pages 6 and 15 in Gallegly and Hong (2008). Sporangia were produced on disks of lima bean agar in 10% sterile soil extract under fluorescent light at 20–22 C. Hemp-seed agar was used for production of the sexual organs. About 20 measurements were made of each morphological stage. Microphotographs of the organs were made with Kodak Professional T-Max 100 35 mm film (Eastman Kodak Co., Rochester, New York) with 43× and 97× objectives of a Bausch & Lomb zoom microscope. Black and white negatives were converted to positive digital images with a Polaroid 35 mm scanner using Photoshop.

Physiology.—The two isolates (p340, p341) used in the morphology studies were incubated at 6, 10, 15, 20, 25, 28, 30, 33 and 35 C. Disks (4 mm diam) from 3 d old cultures were placed on lima bean agar in 60 mm Petri dishes, and diameters of colony growth were recorded after 2 d.

DNA extraction.—Isolates were grown in V8 juice broth as described in the Appendix to Chapter 3, Growth media and method (Erwin and Ribeiro 1996) at room temperature (ca. 23 C) for 10 d. DNA was extracted from each culture as instructed with the DNeasy® Plant Minikit (QIAGEN, Valencia, California).

DNA fingerprinting.—A standard fingerprinting (Gallegly and Hong 2008; Kong et al 2003, 2004a) was used. The only modification was that a smaller volume (2 μL) denatured PCR product was loaded for electrophoresis. In addition to the 11 isolates from avocado the type culture and another authentic culture (CBS 295.29) of *P. citricola*, one isolate each of *P. primulae* Tomlison, *P. pseudosyringae* T. Jung & Delatour and *P. syringae* (Klebahn) Klebahn (Gallegly and Hong 2008) were included for comparison (TABLE I).

DNA sequencing and sequence analysis.—Two isolates from avocado were sequenced in four nuclear and mitochondrial DNA regions. The ITS regions were amplified with forward primer ITS6 (Cooke et al 2000) and reverse primer ITS4 (White et al 1990). Genes encoding β-tubulin, translation elongation factor 1α and NADH dehydrogenase subunit I were amplified as described by Kroon et al (2004). Excess primer and dNTP were removed from quantified PCR products with shrimp alkaline phosphatase and exonuclease I (USB Catalog Nos. 70092Y, 70073Z). One unit of each enzyme was added to 15 μL PCR product, incubated at 37 C for 30 min, followed by heat inactivation at 65 C for 15 min. Sequencing was performed in both directions for all regions with the same primers as for PCR by the University of Kentucky Advanced Genetic Technologies Center (Lexington, Kentucky). Sequencing was repeated at least once.

Sequences from different runs were compared with the Clustal W multiple sequence alignment at http://align.genome.jp. The consensus sequence of each isolate was aligned with those from other isolates to examine inter-isolate sequence variation. Basic local alignment search tool (BLASTn, http://ncbi.nlm.nih.gov) was used to identify the closest relatives of *P. mengei* at the respective regions. The phylogenic analyses with a group of selected species was carried out in TOPALI 2.5 (Milne et al 2009) with the Felsenstein-84 nucleotide substitution plus gamma rates heterogeneity model to calculate pair-wise distances, resulting in a neighbor joining tree. A total of 21 other species representing major clades (Blair et al 2008, Cooke et al 2000, Kroon et al 2004, Martin and Tooley 2003) of genus *Phytophthora* were selected based on the availability of type culture sequence. These sequences were published by Cooke and associates (2000) and by species authorities of *P. alni* subsp. *alni* and *P. inundata* (Brasier et al 2004, Brasier et al 2003), *P. bisheria* (Abad et al 2008), *P. melonis* (Ho et al 2007), *P. quercetorum* (Balci et al 2008) and *P. siskiyouensis* (Reeser et al 2007). Sequences of other sources were double-checked by sequencing the type culture in our collection to confirm its accuracy. Several species of Cooke's clade 2 were included.

TAXONOMY

Phytophthora mengei G.T. Browne, M.E. Gallegly & C.X. Hong, sp. nov.

MycoBank MB 513047

Species nova homothallica, laevi-tunicata, faciliter in agaro "lima bean" dicto oogonia globosa numero medio 24.0 μm diam formans. Oosporae pleroticae parietibus 2.3 μm crassis, numero medio 21.7 μm diam. Antheridia claviformia ad vel prope stipitem oogonialem affixa. Antheridia admodum rotundata (9.3 × 9.9 μm). Sporangia non caduca semipapillata, saepe in formis monstruosis sed

TABLE I. Isolate origin of *Phytophthora mengei* and other species of *Phytophthora* examined in this study

Species	SSCP	Isolate code[c]				Host	Origin	Year	Alternative source
		VT	WVU	UCD	International				
P. mengei	Men	42B2	p340	M218.z1	MYA-4554	*Persea americana*	California	—	
		42B3	p341	M220.z1	MYA-4555	*P. americana*	California	—	
		46F9	—[b]	M213.z1	—	*P. americana*	California	—	
		46G1	—	M214.z1	—	*P. americana*	California	—	
		46G2	—	M215.z1	—	*P. americana*	California	—	
		46G3	—	M216.z1	—	*P. americana*	California	—	
		46G5	—	M219.z1	—	*P. americana*	California	—	
		46G7	—	M265.z1	—	*P. americana*	California	—	
		46G8	—	M266.z1	—	*P. americana*	California	—	
		46F7	—	tc-gb3588.z1	—	*P. americana*	California	—	
		46F8	—	tc-gb3590.z1	—	*P. americana*	California	—	
P. citricola	Cil	33H8[a]	p396	—	IMI 021173	*Citrus sinensis*	Japan	—	
		33J2	p375	—	CBS 295.29	*Citrus* sp.	Japan	1929	
P. primulae	Pri	29E9[a]	p286	—	CBS 620.97	*Primula acaulis*	The Netherlands	1997	WM (PD97/875)
P. pseudosyringae	Pss	30A8[a]	p284	—	CBS111772	*Quercus robur*	Germany	1997	TJ(IFB-PSEU6)
P. syringae	Syr	21H9[a]	p187	—	ATCC34002	*Citrus* sp.	California	—	

[a] Type culture or key isolates of respective species as designated by (Gallegly and Hong 2008).

[b] Data not available.

[c] VT = Virginia Tech; WVU = West Virginia University; UCD = University of California at Davis; Internationals: those beginning with ATCC and MYA are culture reference codes of the American Type Culture Collection, CBS for the Centraal Bureau voor Schimmelcultures and IMI for the International Mycological Institute.

saepissime formis ovoideis, obpyriformibus ellipsoideisque apparentia. Sympodia simplicia in agaro laxa. Magnitudo sporangialis numero medio 62.7 × 35.2 µm (in mensura tota 37.4–78.2 × 27.2–44.2 µm), ratione arithmetica longitudinis usque latitudinis 1.8. Partes inflatae hyphales atque chlamydoposporae nullae. Temperatura maxima ad incrementum coloniale 30–32 C.

Holotypus: ATCC MYA 4554.

Phytophthora mengei G.T. Browne, M.E. Gallegly & C.X. Hong, sp. nov.

Phytophthora mengei is homothallic and forms sex bodies readily in lima bean and hemp-seed agars (FIG. 1). The oogonia average 24.0 µm diam (range 20.4–27.2 µm) and some have tapered stalks, sometimes with a slight hook in the taper. Oospores are mostly plerotic with 2.3 µm thick walls (range 1.5–3.0 µm) and average 21.7 µm diam (range 18.7–24.5 µm). Oospores in tapered oogonia appear aplerotic as the taper draws away from the round oospores. The diclinous antheridia are paragynous and commonly asymmetrically capitate (FIG 1B). The end of the club is approximately 10 µm diam. On tapered oogonia the antheridia are located on the taper, commonly at the bottom. When placed in water or soil extract, mycelia on lima bean agar disks from 3 d old cultures form abundant semipapillate sporangia in fewer than 8 h under fluorescent light at room temperature. The apical thickened area is 2.4–3.4 µm deep. Bizarre shapes of sporangia occur (sickle, boomerang, sombrero and bluntly ellipsoid). Bluntly ellipsoid sporangia, sometimes with a constriction in the middle, average about 95 × 35 µm. Smaller ovoid sporangia are about 49 × 34 µm. Overall sporangia average 62.7 × 35.2 µm (range 37.4–95.0 × 27.2–44.2 µm). The length to width ratio is 1.8. Width of the papillae pores vary, 6.6–13.5 µm. Sporangia are noncaducous. Also a few sporangia are formed on lima bean agar where they are mostly ovoid and of about the same size as the ovoid ones formed in water, but those formed in agar sometimes appear to be papillate. Empty sporangia have a small plug at the point of pedicel attachment. Simple sympodia occur but sometimes a single sporangium on a long pedicel is seen. Hyphal swellings and chlamydospores have not been seen, but knobby hyphae are common.

HOLOTYPE: ATCC MYA-4554 here designated; a cryopreserved specimen of M218.z1 originally isolated from *Persea americana* by J. Menge (USA, California). GenBank EU748545.

Additional strains examined.—TABLE II.

Etymology.—"*mengei*" refers to the originator (Dr John Menge) of the type culture and eight additional avocado isolates used in this study.

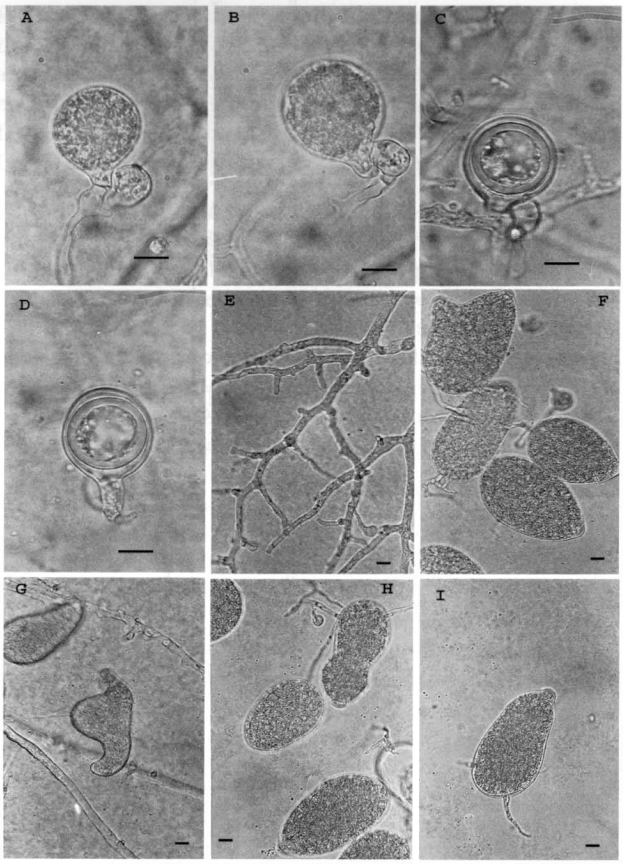

FIG. 1. Diagnostic morphological characters of *Phytophthora mengei*. A, B. Young oogonia and antheridia showing asymmetric capitate antheridia. C, D. Mature sexual bodies with plerotic oospores. E. Hyphae with knobs and short projections. F. Ovoid and bluntly ellipsoid semipapillate sporangia, one with two papillae. G. A bizarre sombrero-shaped sporangium. H. Ellipsoid sporangia, one with a constriction. I. A single bluntly ellipsoid sporangium. Bars = 10 µm.

Habitat.—This pathogen is found in the trunks, main structural roots and fruit of infected avocado trees in southern California, USA.

RESULTS

Temperature-growth relation.—Daily growth rates on lima bean agar for p340 and p341 were respectively 0 mm at 6, 33 and 35 C, 5 mm at 10 C, 5 and 8 mm at 15 C, 13 and 11 mm at 20 C, 15 and 13 mm at 25 C, 6 mm at 28 C and 5 mm at 30 C. Thus the minimum temperature for colony growth was 6–10 C, the optimum about 25 C, and the maximum 30–33 C.

SSCP analysis.—The 11 isolates of *P. mengei* produced an identical PCR-SSCP pattern that was distinct from four reference species (FIG. 2). Comparatively both top and bottom bands of *P. mengei* were faster

TABLE II. Morphological characters of *Phytophthora mengei* and other homothallic-paragynous-semipapillate species of *Phytophthora*

	P. mengei	*P. citricola*	*P. primulae*	*P. pseudosyringae*	*P. siskiyouensis*	*P. syringae*
Number of isolates or reference	11	2	(Gallegly and Hong 2008)	(Gallegly and Hong 2008)	(Reeser et al 2007)	(Gallegly and Hong 2008)
Oogonium (μm)	26	31	38	30	27.8	28
Antheridia	10	10.5	14	10	8.6–11.6 × 9.5–13.3	10
Oospore						
Fitness	Plerotic	Plerotic	Aplerotic	Plerotic	Aplerotic	Plerotic
Mean diameter (μm)	22	28	30	27	24.6	26
Diameter range (μm)	—	—	25.5–34.0	—	22.5–25.8	—
Wall thickness (μm)	2.3	1.5	2.5	1.0	—	4.0
Sporangia						
l × w mean (μm)	62.7 × 35.2	56.3 × 37.8	57.1 × 44.8	40.8 × 30.6	55 × 36	42.0 × 31.0
l × w range	37–95 × 27–44	35–78 × 27–49	53–65 × 39–48	37–42 × 26–33	46–70 × 30–51	30–56 × 22–37
l/w ratio	1.78	1.48	1.26a	1.44	1.5	1.35
Caducity[b]	–	–	–	–	+	–
Hyphal swelling[c]	–	+	++	+	–	+
Temperature-growth relation						
Maximum (C)	30–33	31	< 27	< 27	30	23
Optimum (C)	25	25–28	15–20	20	25	15–20
Growth rate at optimum (mm d^{-1})	14.5	—	—	4.6	6.2–8.5	—
Phylogenetic clade[d]	2	2	8	3	2	8

[a] Only ovoid sporangia measured, bizarre sporangia up to 300 μm occasionally occur.
[b] – = non-caducous, + = caducous.
[c] – = not present, + = not common, ++ = common.
[d] According to Cooke et al (2000).

moving than bands of the other *Phytophthora* spp. used as references.

Sequence analysis and phylogenetic position of P. mengei.—Both isolates of *P. mengei* had identical complete sequences in ITS1-5.8S-ITS2 region of rDNA (EU748545, EU748546, 753 bp), and partial sequences of β-tubulin gene (899 bp), translation elongation factor 1α (869 bp) and NAHD dehydrogenase subunit I (792 bp). Sequence alignments of the ITS regions indicated that this new species is clustered with *P. botryosa* Chee, *P. colocasiae* Racib. and *P. meadii.* McRae (FIG. 3). Phylogenetically this species is closer to *P. siskiyouensis* Reeser and E.M. Hansen, *P. capsici* Leonian, *P. tropicalis* Aragaki and J.Y. Uchida than *P. citricola.* Compared to *P. siskiyouensis P. mengei* has two insertions at sites 108 and 406, one deletion at 15 and five substitutions at 136, 143, 401, 531 and 689 respectively. Similarly this new species has one insertion at site 400 and seven substitutions at 94, 96, 124, 142, 419, 725 and 737 when compared to *P. tropicalis.* In contrast *P. mengei* differs from *P. citricola* by having a long gap at sites 59–69, three insertions at 414, 415 and 730 and nine substitutions at 58, 70, 133, 134, 153, 411, 429, 443 and 748 respectively. Sequence alignments of the other three regions (data not shown) support the phylogenetic analysis of the ITS region.

DISCUSSION

Comparative DNA fingerprinting and sequence analyses, as well as morphological examinations, indicated that the avocado subgroup of *P. citricola* constitutes a new, separate species, and we named it *Phytophthora mengei* sp. nov. This new species is phylogenetically closer to *P. siskiyouensis, P. capsici* and *P. tropicalis* than *P. citricola.* Separation of *P. mengei* from *P. citricola* is supported by studies of morphology (Zentmyer et al 1974), isozymes (Oudemans et al 1994) and AFLP (Bhat and Browne 2007). *Phytophthora mengei* can be easily differentiated from its close relatives by sequence analysis. Also it can be easily distinguished from these relatives and other

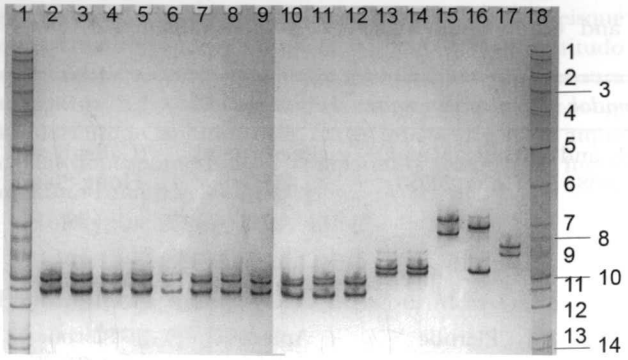

FIG. 2. Polyacrylamide gel electrophoresis of amplified ribosomal DNA internal transcribed spacer 1 region with primers ITS6 and ITS7 of 11 isolates of *Phytophthora mengei* (Lanes 2–12) and two authentic isolates of *Phytophthora citricola* (Lanes 13 and 14) and one isolate of *P. syringae, P. primulae* and *P. pseudosyringae* (Lanes 15–17 respectively). Lanes 1 and 18 are single-stranded DNA (ssDNA) ladders.

morphologically similar species of Waterhouse group III by DNA fingerprints (Gallegly and Hong 2008; Kong et al 2003, 2004a, b).

Phytophthora mengei will be placed in the homothallic-paragynous-semipapillate group of the morphological key by Gallegly and Hong (2008). The other species in this group are *P. citricola, P. syringae, P. pseudosyringae, P. primulae* and *P. porri.* Based on its original description *P. siskiyouensis* will be placed in this group. The differences of these species are that *P. mengei* has smaller oogonia, no hyphal swellings, and has asymmetric capitate antheridia on many of the oogonia. The sporangia of *P. mengei* are similar to those of *P. primulae* and differ from those of the other species in the group by forming many large, bizarre shapes. However the oogonia of *P. mengei* are much smaller than those of *P. primulae* (24 vs. 38 μm) and the maximum temperature for colony growth is higher (31 vs. < 27 C). This new species also can be easily separated from *P. capsici* and *P. tropicalis. Phytophthora mengei* is homothallic and produces noncaducous semipapillate sporangia, whereas *P. capsici* and *P. tropicalis* are heterothallic and produce conspicuously papillate sporangia. In addition sporangia of *P. tropicalis* are caducous with long pedicels.

P. mengei and *P. siskiyouensis* are mostly similar morphologically. The oospores of *P. mengei* are plerotic, whereas those of *P. siskiyouensis* are aplerotic. Also the oogonial sizes of these two species differ

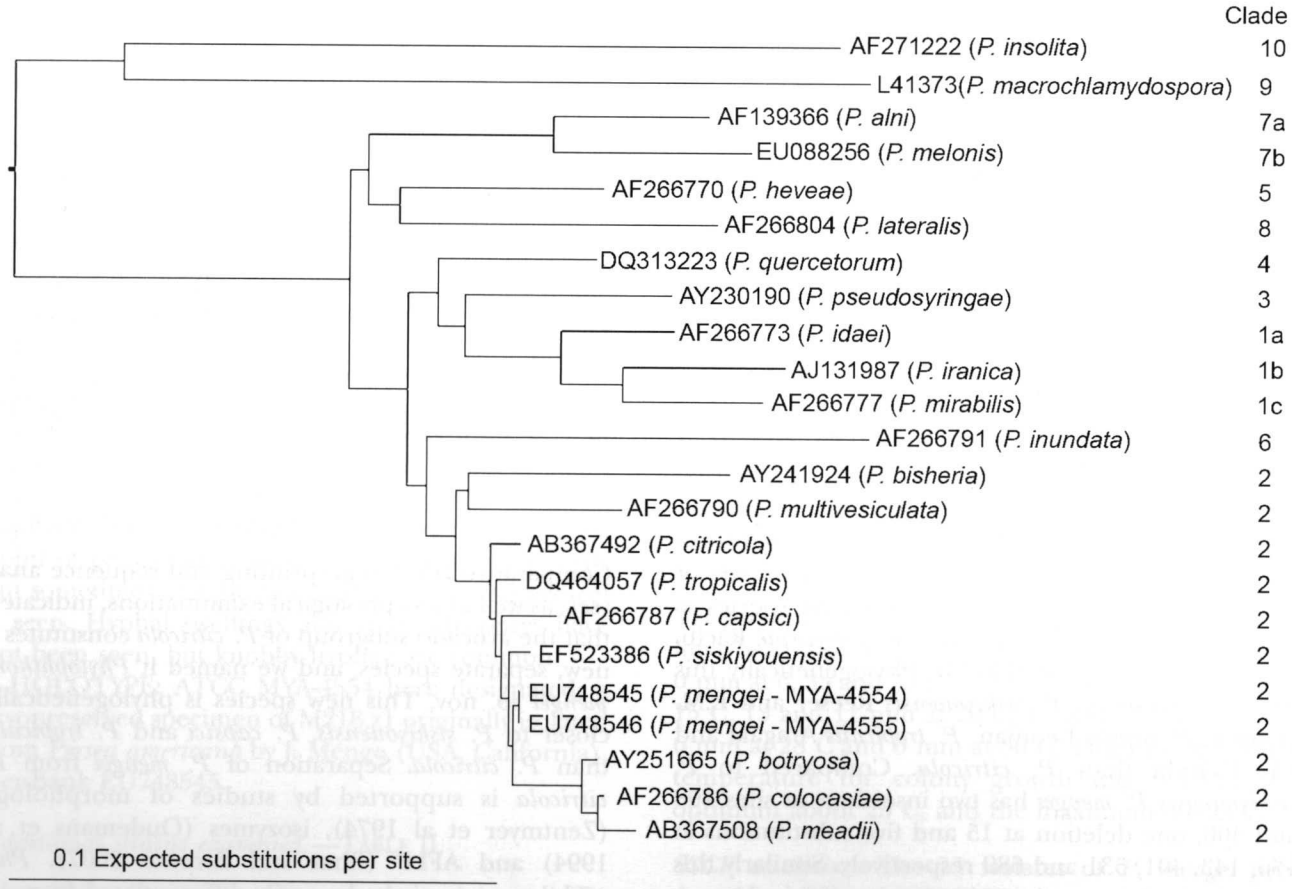

FIG. 3. Neighbor joining phylogenetic tree based on the sequences of the ITS regions of *Phytophthora mengei* with its close relatives and other species representing different clades of genus *Phytophthora.*

slightly (26.0 vs. 27.8 μm). The antheridia of *P. mengei* and those of *P. siskiyouensis* are described respectively as being asymmetrically capitate and capitate. Also sporangia of these two species are similar but those of *P. mengei* appear to be more irregularly shaped. The maximum growth temperature for *P. mengei* is slightly higher than that for *P. siskiyouensis*, whereas the minimum temperature for growth is lower for *P. siskiyouensis*. Variability among isolates of these two species could render the above discussion moot.

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