

Morphological and Molecular Characterization of Two *Aphelenchoides* Endophytic in Poplar Leaves

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Abstract: During a long-term, large network study of the ecology of plant endophytes in native habitats, various nematodes have been found. Two poplar species, *Populus angustifolia* (narrowleaf cottonwood) and *Populus trichocarpa* (black cottonwood), are important ecological and genomic models now used in ongoing plant–pathogen–endophyte interaction studies. In this study, two different aphelenchid nematodes within surface-sterilized healthy leaves of these two *Populus* spp. in northwestern North America were discovered. Nematodes were identified and characterized microscopically and molecularly with 28S ribosomal RNA (rRNA) and 18S rRNA molecular markers. From *P. angustifolia*, *Aphelenchoides saprophilus* was inferred to be closest to another population of *A. saprophilus* among sequenced taxa in the 18S tree. From *P. trichocarpa*, *Laimaphelenchus heidelbergi* had a 28S sequence only 1 bp different from that of a Portuguese population, and 1 bp different from the original Australian type population. The 28S and 18S rRNA trees of *Aphelenchoides* and *Laimaphelenchus* species indicated *L. heidelbergi* failed to cluster with three other *Laimaphelenchus* species, including the type species of the genus. Therefore, we support a conservative definition of the genus *Laimaphelenchus*, and consider these populations to belong to *Aphelenchoides*, amended as *Aphelenchoides heidelbergi* n. comb. This is the first report of these nematode species from within aboveground leaves. The presence of these fungal-feeding nematodes can affect the balance of endophytic fungi, which are important determinants of plant health.

Key words: nematode ecology, phylogeny, ribosomal DNA, systematics, taxonomy.

During a long-term, large network study of the ecology of plant endophytes in native habitats, various nematodes have been found. These include fungal-feeding *Paraphelenchus acontioides* Taylor and Pillai, 1967 (Carta et al., 2011) and bacterial-feeding *Panagrolaimus artyukhovskii* Blinova and Mishina, 1975 (Baynes et al., 2012) within cheatgrass stems from Colorado. Two other species of fungal-feeding nematodes were isolated from within dicots, specifically from the leaves of two species of poplar (*P. angustifolia* and *P. trichocarpa*) originating in Utah and western Washington, respectively. These plants are important ecological and genomic models, respectively, used in ongoing plant–pathogen–endophyte interaction studies (Busby et al., 2013). Here, we characterize these two fungal-feeding nematodes microscopically and with two DNA markers for identification and phylogenetic analysis. Their potential ecological impact is also discussed.

MATERIALS AND METHODS

Microscopy: Nematodes from leaves surface-sterilized with 70% ethyl alcohol were imaged at $\times 40$ –60 on an Olympus BX51 microscope equipped with polarization optics and with a DP71 camera (Olympus America Inc., Center Valley, PA). Measurements in micrometers were taken with an ocular micrometer on a Zeiss Ultraphot II compound microscope (Carl Zeiss, Inc., Jena, Germany) with Nomarski optics before formalin fixation, and images

were also measured with the calibrated measuring tool in the imaging program CellSens version 1.6 (Olympus America Inc.). Fixed specimens were processed for permanent slides according to the formalin–glycerine method (Golden, 1990). Measurements in micrometers and morphometrics were calculated on an Excel spreadsheet.

DNA analysis: Specimens were mechanically disrupted in 20 μ l of extraction buffer (Thomas et al., 1997), then stored in polymerase chain reaction (PCR) tubes at -80°C until needed. Extracts were prepared from thawed pools by incubating the tubes at 60°C for 60 min, followed by 95°C for 15 min to deactivate proteinase K. Two microliter of the extract was used for each 25- μ l PCR reaction.

The ribosomal 28S D2–D3 expansion segment was amplified with primers D2A 5'-ACAAGTACCGTGAGGGAAAGTTG-3' and D3B 5'-TCGGAAGGAACCAGCTACTA-3' (Nunn, 1992) using a previously published amplification procedure (Ye et al., 2007). The sequence is listed in Table 1 with other 28S sequences from GenBank.

For 18S sequence reaction components, per 25 μ l reaction: 17.55 μ l H₂O, 2.5 μ l 10 \times PCR buffer, 0.5 μ l dNTP mix (10 mM each dNTP), 0.75 μ l MgCl₂, 50 mM, 0.75 μ l 18S-G18S4 primer, 10 μ M, 0.75 μ l 18S-18P primer, 10 μ M, 0.2 μ l Taq (1 unit Invitrogen platinum, Carlsbad, CA), 23 μ l of the above mix + 2 μ l template DNA. Cycling conditions were 94°C for 2 min; 94°C for 30 sec, 50°C for 30 sec, 68°C for 2 min, repeating 40 times; 68°C for 10 min; hold at 4°C . Primers of Thomas et al., 1997 were used for PCR and sequencing.

28S rRNA gene PCR products were visualized with UV illumination after ethidium bromide staining. DNA was excised from the gels and purified with the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA). Clean PCR products were directly sequenced by a local vendor (Genewiz, Inc., Germantown, MD and South Plainfield, NJ). PCR products were visualized and purified within the Lonza FlashGel™ DNA system (VWR International, Radnor, PA), and sequences were generated with an ABI BigDye

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TABLE 1. Species, strains, localities, 28S ribosomal DNA sequence lengths, and GenBank accession numbers for sequences included in phylogenetic analysis.

Name	Isolate/strain	Accession no.	bp no.	Locality	Authors
<i>Laimaphelenchus heidelbergi</i>	-	EU287595	714	Australia	Gulcu, B., Hazir, S., Giblin-Davis, R. M., Ye, W., Kanzaki, N., Mergen, H., Keskin, N., and Thomas, W. K. Zhao, Z., Ye, W., Giblin-Davis, R. M., Li, D., Thomas, W. K., Davies, K. A., and Riley, I. T.
<i>Laimaphelenchus heidelbergi</i> Poplar	87F1	KT884898	723	Canada	This study
<i>Laimaphelenchus australis</i>	-	EU287600	713	Australia	Gulcu, B., Hazir, S., Giblin-Davis, R. M., Ye, W., Kanzaki, N., Mergen, H., Keskin, N., and Thomas, W. K. Zhao, Z., Ye, W., Giblin-Davis, R. M., Li, D., Thomas, W. K., Davies, K. A., and Riley, I. T.
<i>Aphelenchoides</i> sp. Japan	J8503	KF638651	783	Japan	Fang, Y., Gu, J., Wang, X., and Li, H.
<i>Aphelenchoides</i> sp. Korea	23556	KF772859	775	Korea	Gu, J., Fang, Y., Wang, X., and Li, H.
<i>Laimaphelenchus</i> sp.	RGD636L	AB368539	724		Kanzaki, N., Giblin-Davis, R. M., Cardoza, Y. J., Ye, W., Raffa, K. F., and Center, B. J.
<i>Laimaphelenchus varicaudatus</i>	-	HQ283353	761	China	Huang, R.-E., Ye, W., Liang, J., Lu, Q., and Zhao, X.-Y.
<i>Schistonchus benjamina</i>	RGD441	AB535553	725	Australia	Davies, K. A., Bartholomaeus, F., Ye, W., Kanzaki, N., and Giblin-Davis, R. M.
<i>Schistonchus altermacrophylla</i>	RGD75	AB535534	742	Australia	Davies, K. A., Bartholomaeus, F., Ye, W., Kanzaki, N., and Giblin-Davis, R. M.

Terminator v3.1 kit with sample sequence data analyzed on an ABI 3130XL Automated DNA sequencer (Applied Biosystems, Foster City, CA). The 28S sequence was determined on both strands using D2A and D3B primers.

The DNA sequences for the following genes were deposited in GenBank: *A. cf. saprophilus* 18S rDNA (KT884899), *A. heidelbergi* (n. comb.) (= original *Laimaphelenchus*) 28S rDNA (KT884898), and *Aphelenchoides cf. parietinus* 18S rDNA (KU525689).

Phylogenetic analysis: DNA sequences were analyzed using BLASTN of nematode sequences contained in the EBI-EMBL parasite sequence database (<http://www.ebi.ac.uk/blast2/parasites.html>). Nematode sequences with highest e-values for BLAST similarity and morphologically close nematode species that had GenBank sequences were aligned in ClustalW (Thompson et al., 1994) using default parameters, as implemented in Geneious version 7.1.7 (BioMatters, Auckland, New Zealand). Markov chain was run for 1,100,000 generations, with the first 100,000 trees discarded as burn-in. The Bayesian likelihood tree was inferred using the MrBayes plugin (Huelsenbeck and Ronquist, 2001) as also implemented in Geneious. GenBank sequences (Table 1 for 18S rDNA, Table 2 for 28S rDNA) were aligned with those determined in this study.

RESULTS

SYSTEMATICS

Aphelenchoides cf. saprophilus Franklin, 1957
(Fig. 1)

Voucher description

Female ($n = 4$): L = 521 ± 34 (495–567) μm , V% = 70 ± 1 (69–72), a = 26.6 ± 3.1 (22.1–28.9), b = 10.0 ± 1.0 (8.9–10.9), c = 16.5 ± 2.7 (12.6–18.3), c' = 3.2 ± 0.2 (2.8–3.3) μm ,

stylet = 10.8 ± 1.2 (9.3–12.1) μm , body width (BW) = 20 ± 2 (18–22), tail = 32.8 ± 6.2 (28.8–42.0) μm , postvulval sac (PVS) = 57 ± 0 (56–57) μm , PVS/vulval anal distance (VAD) % = 46 ± 4 (41–51).

Male ($n = 4$): L = 534 ± 24 (509–567) μm , a = 30.0 ± 3.44 (26.1–34.3), b = 9.9 ± 0.57 (9.3–10.5), c = 15.8 ± 1.74 (14.5–18.3), c' = 3.02 ± 0.37 (2.52–3.40), stylet = $10.3 \mu\text{m}$, tail = 34.0 ± 4.0 (28.8–38.4) μm , spicule = 21.8 ± 1.6 (20.0–23.0).

Locality and host: Ogden, UT, along Weber River, *P. angustifolia* (narrowleaf cottonwood), cultured on *Trichoderma* sp. endophytic from within *P. angustifolia*.

Specimen designation and deposition: Seven slides (G-21529 to G-21535) with 10 females and 20 males were deposited in the U.S. Department of Agriculture Nematode Collection (USDANC).

Differential diagnosis: The stylet demonstrates a wider range than the type population of *A. saprophilus* (9.3–12.1 vs. 11 μm for original description where $n = 4$), and two of the four lateral incisures are very faint, but this diagnosis fits better than *Aphelenchoides pinusi* Bajaj and Walia, 2000, a species with two lateral incisures and a larger spicule size (>25 μm) than *A. saprophilus*.

Aphelenchoides heidelbergi (Zhao, Davies, Riley, and Nobbs, 2007) n. comb. (syn. *Laimaphelenchus heidelbergi* Zhao, Davies, Riley, and Nobbs, 2007)
(Fig. 2)

Voucher description

Female ($n = 3$): L = 397 ± 68 (349–445) μm , a = 38.1 ± 1.57 (37.0–39.2), b = 7.0 ± 0.08 (6.9–7.0), b' = 4.42 ± 0.29 (4.22–4.62), c = 19.0 ± 5.26 (15.3–22.7), c' = 3.55 ± 0.71 (3.04–4.05), V = 0.67 ± 0.01 (0.67–0.68), stylet = 8.7 ± 0.6 (8.2–9.1) μm , tail = 22.2 ± 9.7 (15.3–29.1) μm , PVS = 35 ± 7 (30–40) μm , PVS/BW = 3.3 ± 0.3 (3.1–3.5), PVS/VAD% = 36 ± 4 (33–42).

TABLE 2. 18S rDNA sequence numbers, lengths, and localities from GenBank for *Aphelenchoides* and *Laimaphelenchus* sequences included in phylogenetic analysis.

Name	Isolate/strain	Accession no.	bp no.	Locality	Authors
<i>Bursaphelenchus xylophilus</i>	186	AY508034	1,706	Japan	Ye, W., Giblin-Davis, R. M., Braasch, H., Morris, K., and Thomas, W. K.
<i>Aphelenchoides stammeri</i>	NK218	AB368535	1,699		Kanzaki, N., Giblin-Davis, R. M., Cardoza, Y. J., and Ye, W.
<i>Laimaphelenchus belgradiensis</i>	VO-2014	KF881745	1,142	Serbia	Oro, V.
<i>Laimaphelenchus penardi</i>	LaimPen1	AY593918	1,697		Helder, J., Van den Elsen, S. J. J., Bongers, A. M. T., Wurff, A. W. G., and Bakker, J.
<i>Laimaphelenchus preissi</i>	467a/467	EU287590	1,706	Australia	Gulcu, B., Hazir, S., Giblin-Davis, R. M., Ye, W., Kanzaki, N., Mergen, H., Keskin, N., and Thomas, W. K.
<i>Laimaphelenchus heidelbergi</i> = <i>Aphelenchoides heidelbergi</i> n.comb.		EU287587	1,673	Australia	Gulcu, B., Hazir, S., Giblin-Davis, R. M., Ye, W., Kanzaki, N., Mergen, H., Keskin, N., and Thomas, W. K.
<i>Aphelenchoides macronucleatus</i>		FJ235883	884		Huang, L.
<i>Aphelenchoides paradalianensis</i>	HR3	GU337993	1,707		Cui, R., Zhuo, K., Wang, H., and Liao, J.
<i>Aphelenchoides fujianensis</i>	XP1	FJ520227	1,552		Zhuo, K., Cui, R., Ye, W., Luo, M., Wang, H., Hu, X., and Liao, J.
<i>Aphelenchoides</i> sp.	US02	GU337997	1,707		Cui, R., Zhuo, K., and Liao, J.
<i>Aphelenchoides</i> sp.	US01	GU337998	1,683		Cui, R., Zhuo, K., and Liao, J.
<i>Aphelenchoides</i> sp.	HB	GU337999	1,680		Cui, R., Zhuo, K., and Liao, J.
<i>Aphelenchoides</i> sp.	YN	GU337996	1,680		Cui, R., Zhuo, K., and Liao, J.
<i>Aphelenchoides</i> sp.	Be	GU337995	1,696		Cui, R., Zhuo, K., and Liao, J.
<i>Aphelenchoides</i> sp.	KP	GU337994	1,711		Cui, R., Zhuo, K., and Liao, J.
<i>Aphelenchoides</i> sp.	23556	KF772858	888	Korea	Gu, J., Fang, Y., Wang, X., and Li, H.
<i>Aphelenchoides</i> sp.	JB012	DQ901553	1,439		Chizhov, V. N., Chumakova, O. A., Subbotin, S. A., and Baldwin, J. G.
<i>Aphelenchoides</i> sp.	H1 WY-2008	EU287591	1,708	Australia	Gulcu, B., Hazir, S., Giblin-Davis, R. M., Ye, W., Kanzaki, N., Mergen, H., Keskin, N., and Thomas, W. K.
<i>Aphelenchoides</i> sp.	K1 WY-2008	EU287589	1,674	Australia	Gulcu, B., Hazir, S., Giblin-Davis, R. M., Ye, W., Kanzaki, N., Mergen, H., Keskin, N., and Thomas, W. K.
<i>Aphelenchoides</i> sp.	2137	FJ040412	1,552		Holterman, M. H. M., van den Elsen, S. J. J., Mooijman, P. J. W., Pomp, R., van Megen, H. H. B., and Helder, H.
<i>Aphelenchoides</i> sp.	2134	FJ040411	1,660		Holterman, M. H. M., van den Elsen, S. J. J., Mooijman, P. J. W., Pomp, R., van Megen, H. H. B., Helder, H.
<i>Aphelenchoides</i> sp.	2130	FJ040410	1,659		Holterman, M. H. M., van den Elsen, S. J. J., Mooijman, P. J. W., Pomp, R., van Megen, H. H. B., and Helder, H.
<i>Aphelenchoides</i> sp.	SC	DQ901552	1,479	United States	Chizhov, V. N., Chumakova, O. A., Subbotin, S. A., and Baldwin, J. G.
<i>Aphelenchoides parvius</i>	57F12	KU525689	1,578		This study
<i>Aphelenchoides saprophilus</i>	E2_500F1108	KT884899	565	Ogden, UT.	This study
<i>Aphelenchoides clarus</i>	Konza ICC13	AY911887	632		Powers, T. O., Mullin, P. G., Harris, T. S., and Sutton, L.
<i>Aphelenchoides</i> cf <i>bicaudatus</i>	2145	FJ040407	1,546		Holterman, M. H. M., van den Elsen, S. J. J., Mooijman, P. J. W., Pomp, R., van Megen, H. H. B., and Helder, H.
<i>Aphelenchoides bicaudatus</i>	AChoBic	AY284643	1,657		Helder, J., Van den Elsen, S. J. J., Bongers, A. M. T., Wurff, A. W. G., and Bakker, J.
<i>Aphelenchoides ritzemabosi</i>		DQ901554	1,559	Russia	Chizhov, V. N., Chumakova, O. A., Subbotin, S. A., and Baldwin, J. G.
<i>Aphelenchoides besseyi</i>	98	AY508035	1,714		Ye, W., Giblin-Davis, R. M., Braasch, H., Morris, K., and Thomas, W. K.
<i>Aphelenchoides saprophilus</i>	2140	FJ040408	1,660		Holterman, M. H. M., van den Elsen, S. J. J., Mooijman, P. J. W., Pomp, R., van Megen, H. H. B., and Helder, H.
<i>Aphelenchoides blastophthorus</i>		AY284644	1,679		Helder, J., Van den Elsen, S. J. J., Bongers, A. M. T., Wurff, A. W. G., and Bakker, J.
<i>Aphelenchoides fragariae</i>	AChoFra1	AY284645	1,676		Helder, J., Van den Elsen, S. J. J., Bongers, A. M. T., Wurff, A. W. G., and Bakker, J.

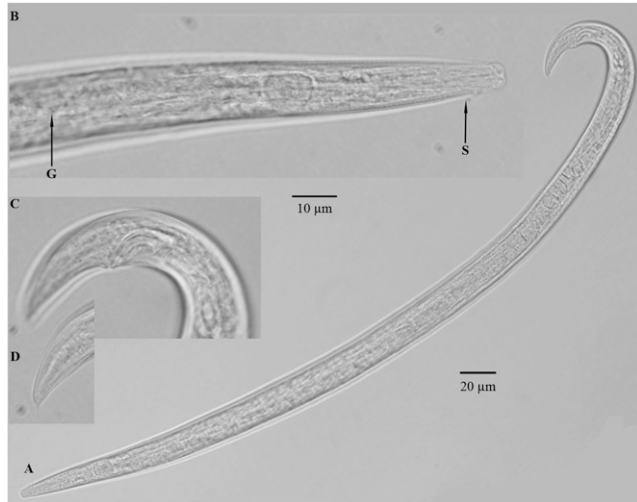


FIG. 1. *Aphelenchoides* cf. *saprophilus* male. A. Body, lateral view. B. Pharynx with stylet (S), median bulb, and pharyngeal gland (G). C. Tail with spicule. D. Tail tip with mucro, endophytic from within healthy, surface-sterilized narrow leaf cottonwood leaves, *Populus angustifolia*.

Male ($n = 3$): $L = 445 \pm 213$ (264–679) μm , $w = 11 \pm 3$ (8–14) μm , $e = 55 \pm 12$ (41–61) μm , $\text{testis} = 266 \pm 173$ (133–462) μm , $t = 30.8 \pm 4.6$ (25.6–34.4) μm , $a = 40.8 \pm 14.33$ (24.4–50.9), $b = 8.3 \pm 3.55$ (4.3–11.1), $b' = 4.4 \pm 1.7$ (2.4–5.7), $c = 14.4 \pm 5.9$ (8.1–9.7), $c' = 3.5 \pm 1.5$ (2.5–4.5),

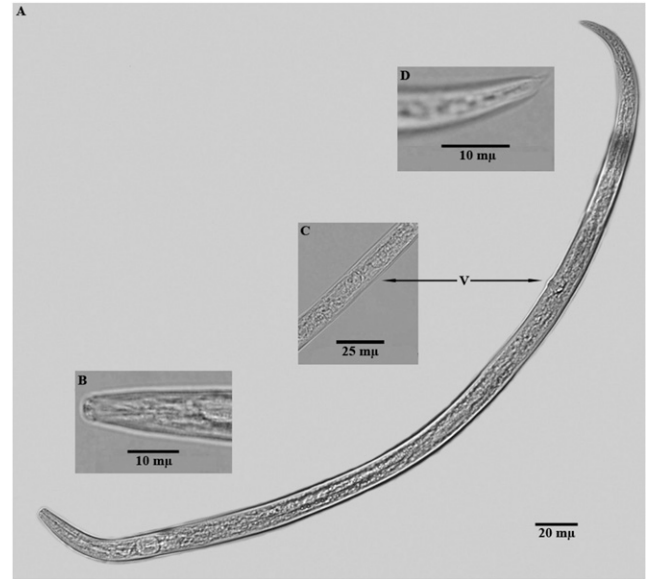


FIG. 2. *Laimaphelenchus heidelbergi*/*Aphelenchoides heidelbergi* n. comb. female from within healthy, surface-sterilized black cottonwood leaves, *Populus trichocarpa*. A. Body, lateral view with vulva (arrow). B. Vulva, ventral view (arrow). C. Offset tail tip with single tubercle.

stylet = 10.9 ± 0.6 (10.2–11.2) μm , spicule = 14.9 ± 1.3 (14.0–15.8) μm .

Locality and host: An alluvial floodplain of the Nisqually River west of the crest of the Cascade Mountains in

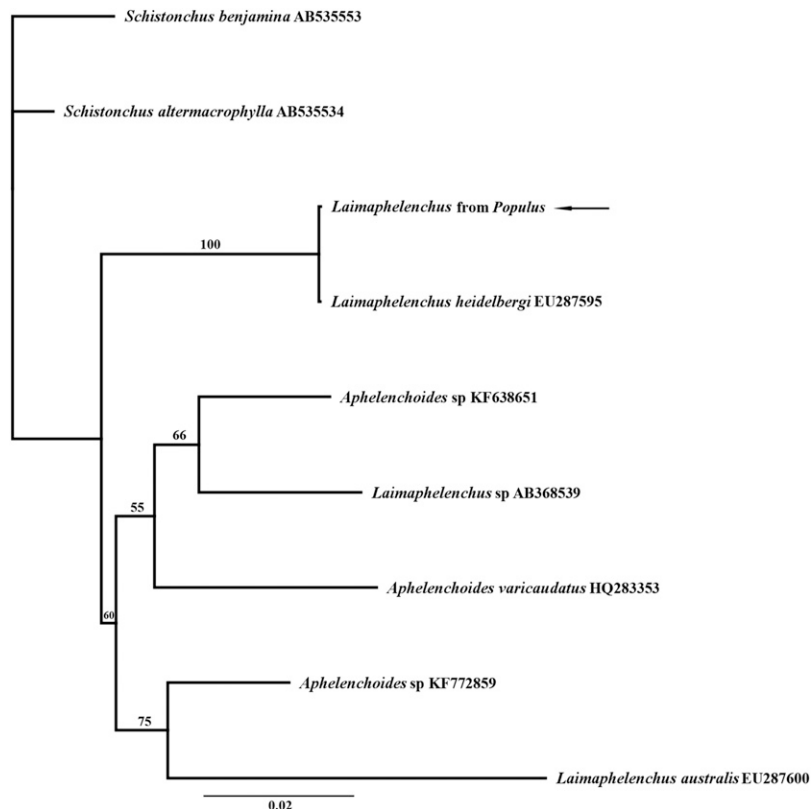


FIG. 3. Bayesian phylogenetic tree of *Aphelenchoides* and *Laimaphelenchus* species inferred from an 806-bp fragment of 28S ribosomal DNA (rDNA). Sequences were aligned in ClustalW. In analysis, Markov chain was run for 1,100,000 generations, with first 100,000 trees discarded as burn-in. LnL mean/run = -4184.6. Node support is given above branches to left of nodes. Sequence generated in this work is indicated by an arrow.

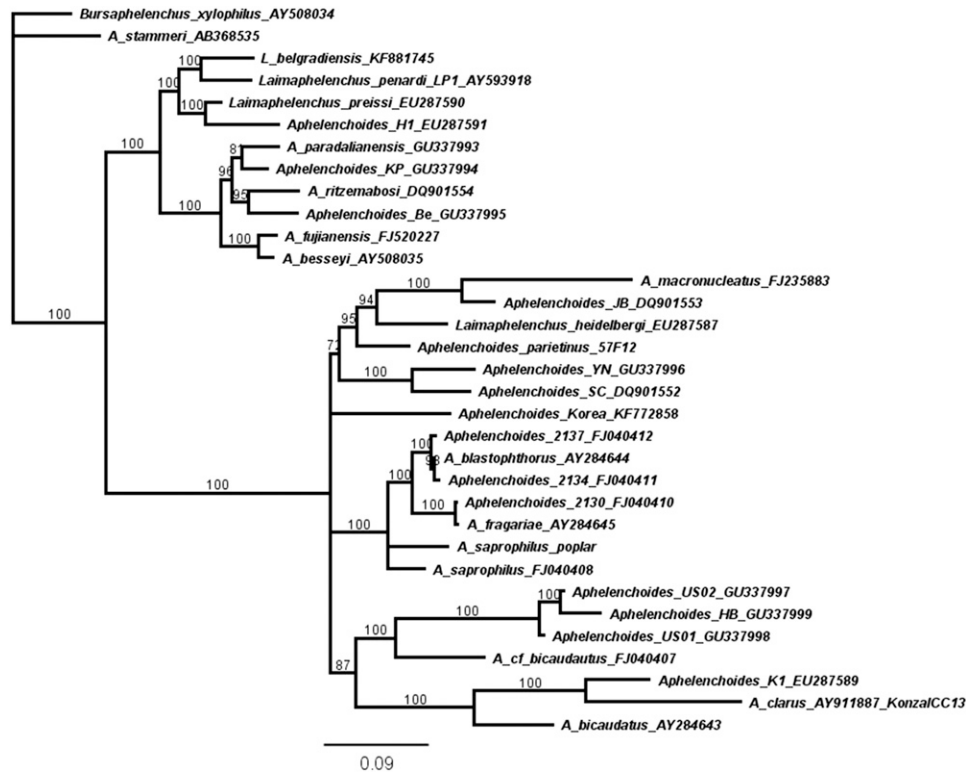


FIG. 4. Bayesian phylogenetic tree of *Aphelenchoides* and *Laimaphelenchus* species inferred from an 1846-bp fragment of 18S rDNA. Sequences were aligned in ClustalW. Sequences were analyzed under a Tamura–Nei model. Markov chain was run for 1,100,000 generations, with first 100,000 trees discarded as burn-in. LnL mean/run = -13413.144 , -13413.594 . Node support is given above branches to left of nodes. Sequences generated in this work are indicated by arrows.

western Washington (latitude and longitude: 46.84808 N, -122.31585 W), *P. trichocarpa*.

Specimen designation and deposition: Four slides (G-21546 to G-21549) with five females and four males (one juvenile) were deposited in the USDANC.

Differential diagnosis: Both the stylet and “b” ratio are slightly smaller in this population than in Australian (Zhao et al., 2007) or Portuguese (Maleita et al., 2014) populations of *L. heidelbergi*.

Molecular alignment and phylogeny: 28S rDNA: The sequence from this population was 99.7% identical to that of Australian *L. heidelbergi* Zhao, Davies, Riley, and Nobbs, 2007 from pine (EU287585; Zhao et al., 2008), with G-A transitions at 106 and 305/688 alignment pair positions. The sequence differed from that of a Portuguese cork bark population (KJ564293) only at the first position (99.8% similar). In the tree (Fig. 3), *L. heidelbergi* did not share a clade with *Laimaphelenchus* sp. and *L. australis* Zhao, Davies, Riley and Nobbs, 2006, a species having the tail and vulval features of the *Laimaphelenchus* type species *Laimaphelenchus penardi* Steiner, 1914.

18S rDNA: In the tree (Fig. 4), *L. heidelbergi* did not share a clade with three other *Laimaphelenchus* species, including the type species of the genus. Instead, it was inferred to be much closer to *A. cf. parietinus* Franklin, 1957 (slightly lower a ratio and higher c' ratio than the type population of *A. parietinus*) from Idaho *Pinus strobus* var. *monticola* taproots with galls of undetermined origin (USDANC G-21546 to

G-21549). The relatively short sequence for *A. cf. saprophilus* had 94.1% similarity (536/570 bp similarity of aligned nucleotides) compared to the 1,660-bp sequence for *A. saprophilus* isolate 2140 (FJ040408) from the laboratory at Wageningen, The Netherlands (Holterman et al., 2006).

DISCUSSION

Laimaphelenchus spp. are polyphyletic among *Aphelenchoides* and *Schistonchus* in molecular phylogenetic trees of ribosomal and *cytochrome oxidase I* genes (Kanzaki et al., 2009; Zeng et al., 2010; Wang et al., 2013), consistent with our findings (Figs. 3,4). This isolate of putative *L. heidelbergi* does not have the four-pronged tail, the sclerotized vagina, or the vulval flap present in *L. penardi*, the type species of the genus. Therefore, we support the conservative definition of Hirling (1982, 1986) for *Laimaphelenchus*, and we amend species *L. heidelbergi* to *A. heidelbergi* n. comb. *sensu* Hirling, 1986. The divergent position of *L. heidelbergi* from other *Laimaphelenchus* species that are more congruent with the original genus description was also noted in other phylogenetic analyses of Aphelenchida (Oro, 2015). *Laimaphelenchus heidelbergi* is not closely related to poplar leaf endophyte *A. saprophilus* (i.e., a nonpathogenic nematode that lives inside surface-sterilized plant leaves), but the latter species is closely related to another potential root endophyte, *A. cf. parietinus* (Fig. 4). Therefore, the association with leaves does not seem to be limited

to close phylogenetic relatives. Possibly all *Aphelenchoides* are capable of living endophytically, but further sampling and experimentation are necessary to support this hypothesis.

Laimaphelenchus and *Aphelenchoides* have been found in lichens (Hirling, 1982), moss, and algae from coniferous trees (Hirling, 1986), from twigs and bark (on and under) of trees (Negi and Ye, 2011), and insect frass from trees (Bajaj and Walia, 2000). However, to our knowledge, there have been no reports of *L. heidelbergi* or *A. saprophilus* from within leaves independent of soil. Likewise, neither nematode species has been reported to cause damage to plants. The presence of fungal-feeding nematodes undoubtedly affects the balance of endophytic fungi within a leaf community, and incidence of disease can depend as much or more on composition of endophyte species than on plant genotype (Raghavendra and Newcombe, 2013). Furthermore, the ability of an *Aphelenchoides* species to cause plant disease may depend on the composition of different endophytic fungi within a given host. Although epiphytic organisms were not the focus of this paper, and thus were not sampled, based on our finding here it would not be surprising to find *Aphelenchoides* living epiphytically as well, especially in humid habitats. Further characterization of nematode abundance, geographical distribution, variability, life history, and culturability of these nematodes is worth pursuing to better understand plant–microbe relationships within the poplar microbiome (Hacquard and Schadt, 2015).

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