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First record of *Bursaphelenchus hildegardae* Braasch *et al.*, 2006 (Nematoda) in New Zealand with updated information on morphology, sequencing and a key to species of the *eggersi*-group

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Abstract

Bursaphelenchus hildegardae Braasch *et al.*, 2006 was collected from pine wood (*Pinus radiata*) growing in Kaingaroa Timberlands, and a bark beetle, *Hylastes ater* Paykull, 1800 in New Zealand. This is a new record for *B. hildegardae,* occuring in New Zealand, and the second report from the southern hemisphere in addition to Australia. In general, the New Zealand isolate of *B. hildegardae* corresponds well with the description of *B. hildegardae* given by Braasch *et al.* (2006) from Germany. The New Zealand isolate is characterized by having an adult body length of 807–1190 µm, medium a ratios (47.5–58.5 for female and 44.6–60.1 for male), b ratios of 9.8–14.5 (female) and 10.2–12.7 (male), c ratios of 18.8–25.2 (female) and 21.6–32.4 (male), c' ratios of 4.0–4.4 (female) and 2.1–2.7 (male), and is characterised by having three incisures in the lateral fields, thorn-shaped spicules with a distinctly dorsally-bent thin hook-like condylus, and a dorso-ventally visible terminal bursa. In addition, molecular phylogeny using near full length small subunit (SSU), D2/D3 expansion segments of the large subunit (LSU) and the internal transcribed spacer region (ITS1 and 2) of the ribosomal rDNA supports the identification. A key to *Bursaphelenchus* species in the *eggersi*-group is given.

Key words: distribution, molecular, morphology, morphometrics, new record, phylogeny, taxonomy

Introduction

The genus *Bursaphelenchus* Fuchs, 1937 is considered an important group for quarantine status globally because of two devastating plant parasitic nematodes, *B. xylophilus* (Steiner & Buhrer, 1934) Nickle, 1970 and *B. cocophilus* (Cobb, 1919) Baujard, 1989. To date, more than 130 species of *Bursaphelenchus* have been described to science (Kanzaki *et al*., 2021; Kanzaki & Giblin Davis, 2018; Ryss *et al.*, 2005; Ryss & Subbotin, 2017). Two of them, *B. eggersi* Rühm, 1956 and *B. fungivorus* Franklin & Hooper, 1962 have been recorded from New Zealand (Dale 1967, 1971; Knight *et al*. 1997; Yeates, 2010).

Dale (1967) investigated nematodes associated with the pine-bark beetle, *Hylastes ater* (Paykull, 1800) in New Zealand and found five species: *Plectonchus molgos* Massey, 1974 (=*Anguilluloides zondagi* Dale, 1967 synomised by Abolafia *et al.* (2006)), *B. eggersi* (Rühm, 1956) Goodey, 1960, *Micoletzkya thalenhorsti* (Rühm, 1956) Goodey, 1963, *Parasitorhabditis ateri* (Fuchs, 1937) Rühm, 1956 and *Parasitylenchus hylastis* (Wfilker, 1923) Filipjev, 1934. Among these species, *B. eggersi* was isolated from its beetle vector in eight of 14 sites across the North and South Islands of New Zealand, suggesting that it has been established in the country.

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In March 2019, an unexplained mortality of *Pinus radiata* trees was observed in Kaingaroa Forest in the central North Island of New Zealand during a Forest Health Assessment survey. Close examination in the field of a dead tree failed to determine the cause: the tree trunk was found to be excessively wet, however no obvious fungal disease symptoms or insect damage were found. Subsequently, a group of three trees and a single dead tree were felled and wood disks showing sapstain and heavy waterlogged sections, along with beetles, were sampled for nematode diagnostics. Nematodes were isolated from the wood disks and beetle vector, *H. ater*.

The New Zealand Ministry for Primary Industries (MPI) initiated a biosecurity investigation and a delimiting survey within Kaingaroa Forest to establish the area of spread and host range of the nematodes. At the early phase of investigation, Kaingaroa Timberlands Ltd. ceased all operational activities and closed off a significant area of forest around the detection site to ensure that any potential biosecurity risk was contained. This action also supported the field survey operations.. The present study provides results, adds to the morphological and molecular diagnostics of the nematode species established and comments on the status in the country.

Material and Methods

The initial sample was collected during a Forest Heath Assessment Survey conducted by SPS Biota Ltd. The sampling was organised in order to study the possible reasons for the observed tree mortality of *Pinus radiata* in Kaingaroa Forest in March 2019. Subsequently, material for the present study was collected mainly in April 2019. In total, more than 158 wood discs from 158 trees and 50 beetles were collected within a 3.8 km radius from the initial detection site. In addition to fresh material, 20 dried specimen of *H. ater* collected prior 2002 were obtained from SCION New Zealand collections and tested for the presence of nematodes.

NEMATODE COLLECTION

Nematode extraction and specimen processing. Nematodes were extracted from pine wood samples using a variant of the Whitehead & Hemming (1965) method. Dried specimens of *H. ater* were dissected for extraction of nematodes. For morphological study, nematodes were heat killed and mounted on slides (Davies & Giblin-Davis, 2004). Some were mounted in water as temporary specimens and others were mounted in glycerol as permanent specimens. Morphological characteristics of these nematodes were studied using interference contrast microscopy (Nikon, Eclipse 90*i*). Measurements of material mounted in glycerol were made using a NIS-Elements Basic Research microscope (Nikon, Version 2.32). Maximum body diameter was measured at mid-length for males and female, respectively. Body length was measured along the mid-line. De Man's ratios were determined, and a camera attached to the microscope (Nikon Camera Head DS-Fi1) was used to take a series of digital images of the specimens (Zhao *et al*., 2015).

MOLECULAR METHODS

DNA extraction. Nematodes isolated from Kaingaroa Forest were studied for molecular. A single juvenile, dauer larvae, a male, and a female were each used separately for the extraction of DNA. The method of Zheng *et al.* (2002) was followed for DNA extraction. Total genomic DNA from each nematode was extracted using worm lysis buffer containing proteinase K (Williams *et al.,* 1992).

Polymerase chain reaction (PCR), PCR product purification and DNA sequencing. The internal transcribed spacer (ITS), rDNA D2/D3 expansion segments of the large subunit (LSU) and two fragments of rDNA small subunit (SSU) were amplified and sequenced using the primer pairs ITS5 + ITS26 (White *et al*., 1990), D2A + D3B (Nunn, 1992), 1096F + 1912R (Holterman *et al*. 2006), and 1813F + 2646R (Holterman *et al*. 2006), respectively. The 20 µl PCR reactions contained 10 µl 2x Go Tag® Green Master Mix (Promega Corporation, Madison, WI, USA), 1 µl (5 µM) each forward and reverse primer and 2 µl of DNA template. The thermal cycling programme was: denaturation at 95ºC for 3 min, followed by 40 cycles of denaturation at 95ºC for 15 sec, annealing at 55ºC for 15 sec and extension at 72ºC for 30 sec. A final extension was performed at 72ºC for 7 min. The amplicons

were electrophoresed on 1.2% agarose gel (in 1x TAE) stained with SYBR® safe, and visualised using a Gel Doc Software system (BioRad, Hercules, CA, USA). Successfully amplified products were sequenced bi-directionally using the amplification primers by EcoGene® (Auckland, New Zealand). The obtained DNA sequences were edited and aligned using Geneious Pro 7.1.5 (Biomatters, Auckland, New Zealand), and a BLAST search was conducted against the GenBank database (Altschul *et al.*, 1990).The sequences were deposited in GenBank with the accession numbers MZ553929 (SSU), MZ542475 (LSU), MZ542474 (ITS).

Sequence alignment and phylogenetic inference. Some published sequences of *Bursaphelenchus* including all from the *eggersi*-group for SSU (13 sequences), D2D3 (21) and ITS (22) from GenBank were included in the phylogenetic analysis. Nematode species and GenBank accession numbers are listed for each taxon in the phylogenetic trees (Figs 4–6). DNA sequences were aligned in ClustalX2 (Larkin *et al*., 2007) using default parameter values. ModelTest 3.04 (Posada & Crandall, 1998) and PAUP*4.0b10 (Swofford, 1998) were used to select the best fitting model using the Akaike Information Criterion (AIC). A Bayesian phylogenetic tree was constructed using MrBayes 3.1.2 (Ronquist & Huelsenbeck, 2003) with four MCMC chains run for 1,100,000 generations. The best fitting model for SSU was GTR + I + G and GTR + G for D2D3 and ITS. Prior distributions were as follows: revmatpr = dirichlet (1,1,1,1,1,1), shapepr = exponential (5), brlenspr = unconstrained: exponential (10). We started the analysis from a random topology using a temperature of 0.2, and a burn-in of 10% of trees. More details of these methods were described in Zhao & Buckley (2009).

Results

Bursaphelenchus hildegardae was found in wood samples (*Pinus radiata*) and *H. ater* beetles collected in the Kaingaroa Forest from March to April 2019. Extraction of 158 wood samples showed 6 to be containing *B. hildegardae* giving an average presence of about 3.8 %, and examination of 50 beetles showed 3 to be carrying *B. hildegardae* giving an average incidence of about 6 %. Subsequently, *B. hildegardae* was also found in beetle samples (*H. ater*) collected from Victoria Forest near Reefton on 28 April 2019; pine forest near Tikokino on 20 May 2020; pine wood samples (*Pinus radiata*) from Golden Downs, Nelson forests on 23 October 2020, and Douglas fir (*Pseudotsuga menziesii*) from Waipori, Dunedin on 11 November 2020, respectively (Table 1).

TABLE 1. Information of *Bursaphelenchus hildegardae* isolated in New Zealand.

Sample Acc. no	*B. hildgardae*	Specimen	Host & collecting date	Locality & Crosby region
T19_02339	Present, ID confirmed by morphology, SSU, LSU & ITS sequences.	Wood discs, living beetles	*Pinus radiata, Hylastes ater* 20 March – 23 April 2019	Taupo. TO. Matea Forest; CPT 0879; Kaingaroa Timberlands. BP.
T19_04309	Present, ID confirmed by LSU & ITS sequences.	Living beetles	*Hylastes ater* 28 April 2019	Victoria Forest near Reefton. BR.
T20_01072	Present, ID confirmed by morphology.	Living beetles	*Hylastes ater* 20 May 2020	Gwavas Forest, Tikokino, RI.
T20_03267	Present, ID confirmed morphology.	Wood discs	*P. radiata* 23 October 2020	Golden Downs Forest, Nelson. NN.
T20_02634	Present, ID confirmed morphology.	Wood discs	*Pseudotsuga menziesii* 11 November 2020	Waipori Forest, Dunedin, DN.

Bursaphelenchus eggersi, which has been reported as established in New Zealand by Dale 1967, has not been found on pine samples or insect vectors received from the delimiting survey carried out during the biosecurity investigation. In addition, *B. eggersi* has not been found from preserved specimens collected by SCION prior to 2002. The morphology of *B. hildegardae*, which is similar to *B. eggersi*, is provided below.

Bursaphelenchus hildegardae
(Figs. 1–6)

Measurements
See Table 2.

TABLE 2. Morphometrics of *Bursaphelenchus hildegardae* isolated from Kaingaroa Forest and Waipori Forest New Zealand. Measurements are in µm and in the form: mean ± SD (range).

Character	Kaingaroa Forest (*Pinus radiata*)			Waipori Forest (*Pseudotsuga menziesii*)	
	Female	Male	Dauer juvenile	T20-06234 female	T20-06234 male
n	7	7	5	5	3
L	980±170 (807–1250)	942±131 (839–1163)	594±25 (582–635)	1149±102 (1060–1149)	994±64 (929–1059)
a	51.9±3.6 (47.5–58.5)	51.5±5.2 (44.6–60.1)	31.1.8±0.8 (30.3–32.2)	49.1±6.3 (41.0–49.1)	51.0±3.3 (47.3–53.1)
b	11.6±1.4 (9.8–14.5)	11.3±0.9 (10.2–12.7)	10.3±1.9 (7.7–12.5)	14.0±1.4 (12.7–14.0)	12.2±0.3 (11.8–12.4)
c	21.8±2.4 (18.8–25.2)	28.4±3.7 (21.6–32.4)	16.6±1.9 (15.4–16.3)	27.7±3.6 (23.6–27.7)	34.9±3.7 (31.9–39.0)
c’	4.2±0.1 (4.0–4.4)	2.3±0.2 (2.1–2.7)	3.7±0.3 (3.3–3.9)	3.7±0.4 (3.3–3.7)	2.0±0.2 (1.8–2.1)
V/T	73.6% (73.2–74.0) %	49% (44.9–54.8) %	-	73.3% (72.6–73.3) %	71% (69.4–73.1) %
Greatest body diam.	18.8±2.1 (16.9–22.1)	18.3±1.4 (16.8–19.6)	19.1±1.2 (18.1–20.6)	23.5±2.2 (21.7–23.5)	19.5±0.7 (18.7–20.1)
Body diam. at anus	10.7±1.1 (9.2–12.1)	14.4±0.9 (13.5–15.5)	-	11.3±0.8 (10.5–11.3)	14.7±0.8 (14.1–15.5)
Head region height	2.5±3.7 (2.6–3.7)	2.7±0.4 (2.1–3.2)	-	2.7±0.5 (2.4–2.9)	2.8±0.2 (2.5–2.9)
Head region diam.	7.6±0.4 (7.2–8.3)	7.6±0.4 (7.2–8.0)	-	7.2±1.1 (5.4–7.2)	6.5±0.3 (6.3–6.9)
Stylet	13.5±0.8 (12.4–14.4)	13.3±0.9 (12.1–14.5)	-	15.1±0.8 (14.4–15.1)	14.9±0.6 (14.6–15.6)
Stylet cone	6.1±0.6 (5.4–6.4)	5.7±0.8 (4.2–6.2)	-	6.8±0.6 (6.1–6.8)	6.4±0.5 (6.1–6.8)
Ant. end to metacorpus	84.6±10.2 (77.4–107.1)	83.0±4.8 (77.8–91.7)	59.5±10.4 (51.4–74.4)	82.1±3.1 (79.4–82.1)	81.6±3.6 (78.6–85.6)
Metacorpus length	19.6±1.1 (18.8–21.6)	19.1±1.4 (16.8–20.9)	16.9±0.4 (16.4–17.4)	19.2±1.0 (17.4–19.2)	17.3±0.5 (16.8–17.7)
Metacorpus diam.	12.6±0.7 (12.1–13.6)	12.1±1.2 (10.9–14.5)	10.9±1.2 (8.7–11.7)	13.2±0.9 (11.6–13.2)	12.1±0.3 (11.8–12.4)
Metacorpus valve length	5.6±0.5 (5.1–6.0)	5.1±0.9 (4.3–5.9)	3.8 (2.9; 4.7)	5.9±0.5 (5.2–5.9)	5.6±0.2 (5.4–5.7)
Metacorpus valve width	4.0±0.2 (3.9–4.2)	3.8±0.5 (3.3–4.7)	2.8 (2.3; 3.4)	4.1±0.3 (3.7–4.1)	3.5±0.6 (2.9–3.9)
Anteroir end to end of gland lobe	175.6±19.2 (174.0–203.0)	157.5±10.0 (142.1–161.4)	-	172.1±14.6 (153.9–172.1)	168.4±13.3 (153.3–178.3)
Excretory pore from anteroir end	104.1±9.9 (93.3–119.1)	106.6±6.8 (100.1–114.5)	-	114.9±5.0 (109.2–114.9)	102.4±5.3 (96.2–105.6)
Tail length	44.7±3.6 (40.2–50.2)	33.3±3.5 (29.1–36.7)	36.0±41.2 (34.2–40.4)	41.7±2.5 (39.2–41.7)	28.7±3.0 (25.5–31.5)
Postuterine sac	129 ±29.6 (97–183)	-		168 ±30..6 (132–128)	-
Spicule	-	21.5±3.1 (17.8–24.1)		-	21.6±2.0 (20.3–23.9)
Testis	-	447.2±71.4 (377.6–507.6)	-	-	706.4±42.2 (657.9–734.5)

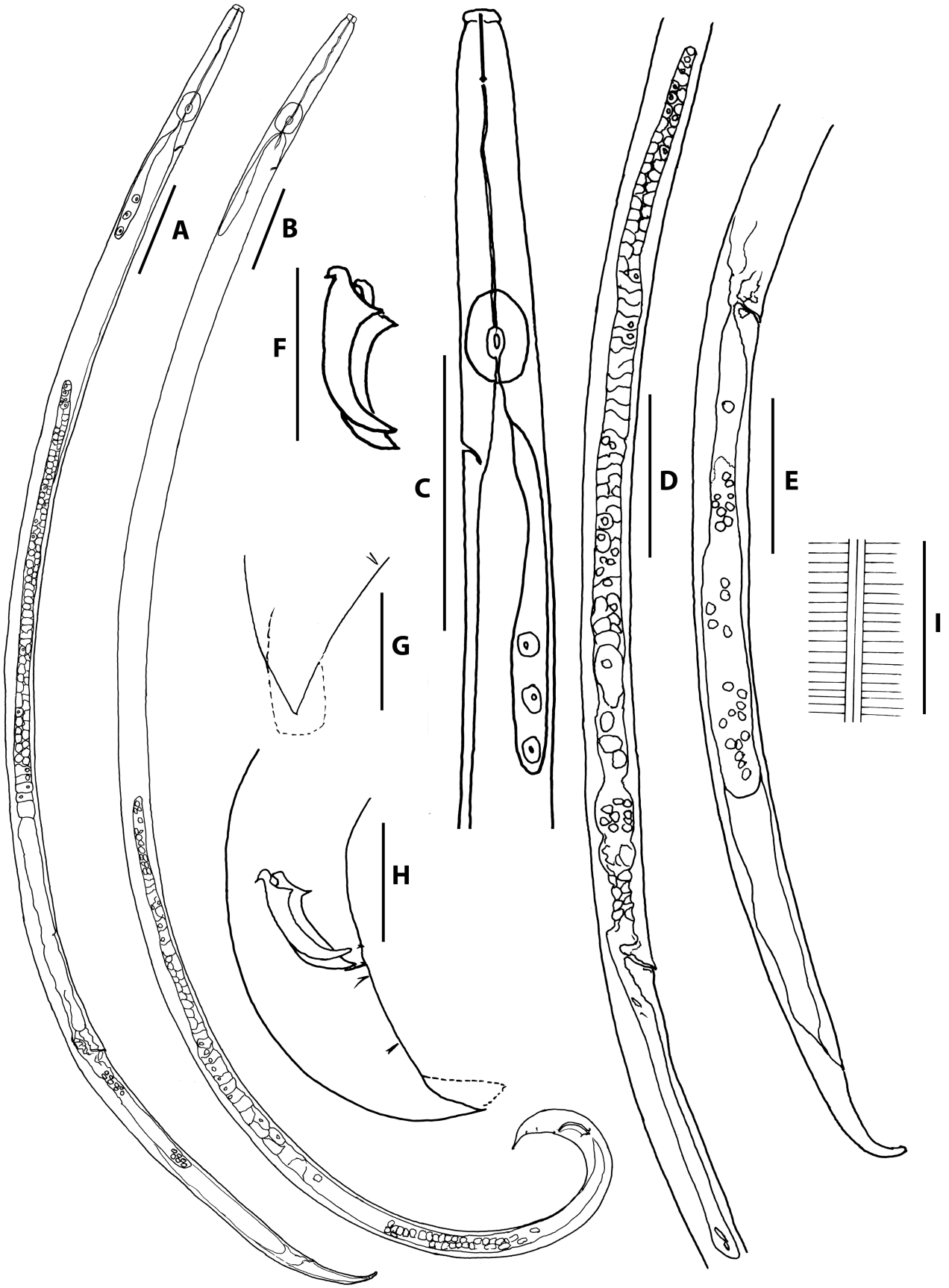

FIG. 1. *Bursaphelenchus hildegardae*. A: Female; B: Male; C: Anterior part of female; D: Reproductive system of female; E: Posterior part of female; F: Spicules; G: Bursa; H: Posterior end of male; I: Lateral lines. (Scale bars: A–C = 50 µm; D–I = 20 µm)

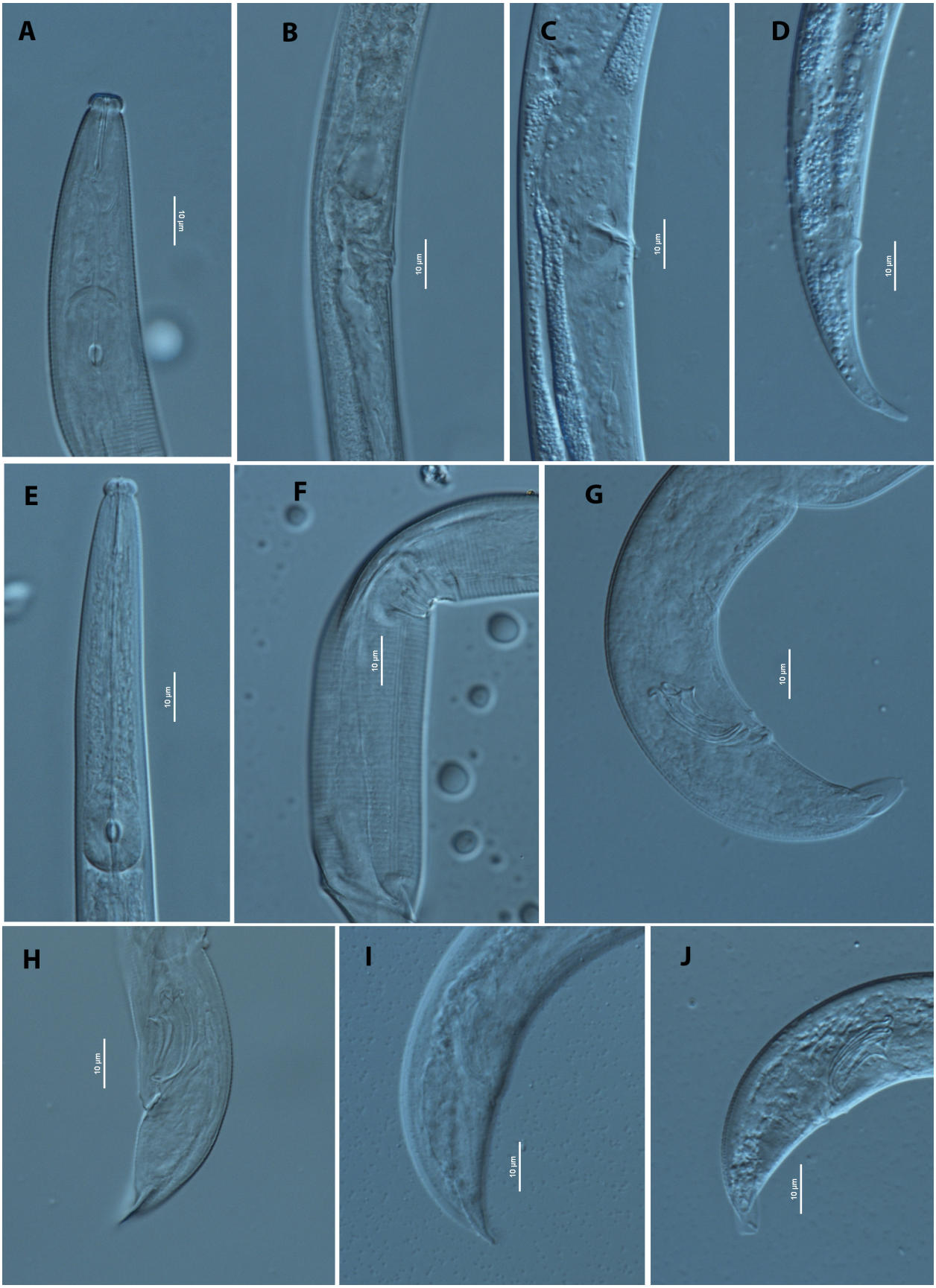

FIG. 2. Light microscope photographs of *Bursaphelenchus hildegardae*. A: Anterior part of female; B–C:Vulva with flap; D: Female tail; E: Anterior part of male; F: Male lateral lines; G–J: Male spicules, busa & Tail. (Scale bars: A–J = 10 µm)

Description

Adults

Body length ranging from 839–1163 and 807–1250 µm for male and female, respectively. Body cylindrical, moderate to slender, i.e., a = 44.6–60.1 and 41.0–58.5 for male and female, respectively. Cuticle thin, annulated, lateral field bearing three lines, i.e., two ridges. Head distinctly offset from body, separated by a clear constriction (Figs. 1A, B & C; 2A & E). Stylet with narrow lumen comprising a short cone ca 39% – 45% of total stylet length and a shaft with slightly clear basal swelling. Procorpus cylindrical, ending in well-developed median bulb, i.e., 19.6 µm in length x 12.6 µm in width for female and 19.1 µm in length x 12.1 µm in width for male. Metacorpal valve clearly observed, i.e., 5.1–6.0 µm in length x 3.9–4.3 µm in width for female and 4.2–5.9 µm in length x 3.3–4.7 µm in width for male, located at middle of, or slightly posterior to, centre of median bulb. Dorsal esophageal gland orifice opening into lumen of metacorpus mid-way between anterior end of metacorpal valve and anterior end of metacorpus. Pharyngo-intestinal junction ca 3.5–6.0 µm posterior to median bulb. Dorsal pharyngeal glands overlapping intestine dorsally, extending ca 154–203 µm long for female and 142–178 µm long for male, posterior to median bulb. Position of excretory pore posterior to median bulb ca 93–119 µm long for female and 96–115 µm long for male from anterior head end, respectively. Nerve ring surrounding esophageal glands and intestine slightly posterior to pharyngo-intestinal junction. Hemizonid at ca 7.5–10.0 µm posterior to excretory pore for female and 8.0–13.0 µm posterior to excretory pore for male.

Female

Body smoothly ventrally arcuate when killed by heat (Fig. 1B). Cuticle marked by fine transverse striations, ca. 1.0 µm wide. Lateral field with three lines, ca. 1.8 µm wide in midbody (Fig. 1I). Head set off by a distinct constriction, ca. 3 µm high, 8 µm wide (Fig. 2A). Stylet slender, almost without basal swellings, shaft forming about 2/3 of total stylet length. Procopus cylindrical. Median bulb elongated oval with conspicuous centrally placed valve plates. Oesophageal gland lobe extending dorsally for about five body widths long, down the body. Nerve ring located closely posterior to metacorpus. Ovary anteriorly outstretched. Developing oocytes in multiple rows in ovary. Oviduct tube-like. Spermatheca oval shaped, filled with well-developed sperm. Crustaformeria quadricolumella form, conspicuous. Uterus irregularly rounded. Vagina perpendicular to body surface, slightly inclined anteriorly. Vulval opening with distinctive short vulval flap (Figs. 1D & E; 2B & C). Post-uterine sac (PUC) long and conspicuous, i.e., 6–7 vulval body diam. long, often containing sperm. Rectum and anus present, functional. Tail bent ventrally, elongate, conoid shape. Tail tip simple and blunted, no indentation observed before the end.

Male

Anterior body part and cuticle similar to those of female (Fig. 2E). Tail region strongly ventrally arcuate when killed by heat (Figs. 1B; 2G). Gonad outstretched, sometimes reflexed backwards, occupying 45–55% of total body length. Spermatocytes and spermatozoa arranged in multiple rows, tightly packed in testis. Tail appearing distinctly claw-like at terminus in lateral view. Lips of cloacal aperture slightly protruding. Spicules paired, relatively straight, rosethorn-shaped. Capitulum of spicule well developed, short condylus with a relatively thin dorsally hooked end (ca. 2 µm long) and a pointed short triangular rostrum (ca. 2 µm long) with pointed tip. Spicule blade ventrally curved consisting of smoothly and clearly ventrally curved and well cuticularised dorsal limb (Figs. 1F & H; 2 G & H). Distal tip of spicule thin, truncate, without cucullus. Gubernaculum absent. Bursal flap present, starting from level of posteriormost genital papillae (P4). Seven genital papillae, i.e., one ventral papilla (P1) and three subventral paired papillae (P2, P3, P4) present: precloacal P1, ventral, 2–3 µm anterior to cloacal opening (CO); P2 on subventral body adanal or slightly anterior to CO; P3 on ventro-subventral body, located mid-way between CO and tail tip; P4 on ventro-subventral body, located at tip of bursal flap or slightly posterior. P4 slightly smaller than P1, P2 and P3. Bursal flap roundish-rectangular to oval in shape, surrounding tail terminus, not indented (Figs. 1G & H; 2J)

Dauer Juvenile

Dauer juvenile found under the elytra of *H. ater*. Body slender, 569–635 µm long. Cuticle with smooth surface, fine annulation was observed clearly, lateral lines not seen. Median bulb oval, well-developed, 17.0 µm in length x 10.9 µm in width (Figs. A & B). Metacorpal valve clearly observed, 3.8 µm in length x 2.9 µm in width, present at middle of, or slightly posterior to, centre of median bulb. Excretory pore not conspicuous. Hemizonid not observed. Cephalic region dome-shaped (Figs. A & B). Stylet observed with some examined specimens. Pharyngo-intestinal

junction not conspicuous. Pharyngeal gland lobe not observed. Anus present, conspicuous (Fig. 3D). Tail conical, bluntly pointed (Figs. 3C & D).

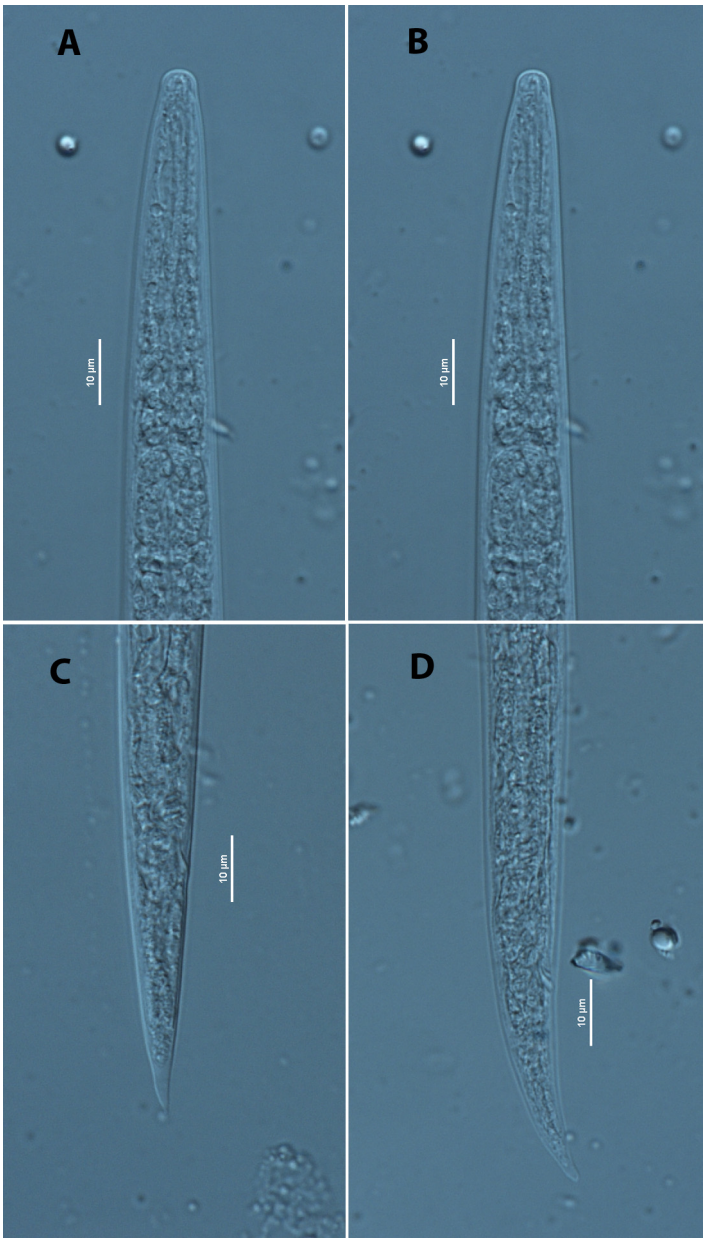

FIG. 3. Light microscope photographs of *Bursaphelenchus hildegardae*. Dauer juvenile. A–B: Anterior part; C–D: Posterior part. (Scale bars: A–D = 10 µm)

HOST AND LOCALITY

Bursaphelenchus hildegardae, extracted from pine wood (*Pinus radiata*) and bark beetle (*H. ater*) samples collected from Kaingaroa Forest in the central North Island of New Zealand (NZ) (38° 24' 36.394"S; 176° 33' 44.989" E) in March and April 2019; and from Douglas fir (*Pseudotsuga menziesii*) from Waipori, Dunedin in the South Island of NZ (45° 56' 29.222" S; 170° 5' 29.27" E) on 11 November 2020.

MATERIAL EXAMINED

Seven females (slide nos NNCNZ 3350–3352), seven males (slide nos NNCNZ 3353–3354) and five dauer juveniles (slide nos NNCNZ 3355) from Kaingaroa Forest; five females (slide nos NNCNZ 3356–3358) and three males (slide nos NNCNZ 3359–3362) from Waipori deposited at the National Nematode Collection, New Zealand (NNCNZ).

TABLE 3. Comparative morphology of *Bursaphelenchus* spp. in the *eggseri*-group (Braasch *et al*. 2009).

Species	L of female (μm)	Incisures in lateral field	L uterine sac (μm)	Tail shape	Vulva flap	Spicule L (μm)	Caudal papillae (pairs)	Plant host	Insect vectors	Reference
B. eggersi	(990–1122)	3	152–160		Absent	18–24	Unknown	*Pinus silvestris*	*Hylurgops palliatus*	Rühm 1956
B. glochis	1100±130 (860–1300)	3	88–249	Rouned, small hook	Present (1μm)	19.6±1.3 (18–22)	1 single, 3 pairs	*Pinus silvestris*	Unknown	Brzeski & Baujard 1997
B. hildegardae (Gemany)	1032±123 (855–1335)	3	144±21 (110–187)	Rouned, intentated	Present	26±1.5 (23–28)	1 single, 3 pairs	*Pinus silvestris*	*Hylurgops palliatus*	Braasch *et al*. 2006
B. tusciae	820.8±51.9 (742.4–1040.6)	3	121.9±18.7 (92.0–164.6)	Rounded, indented or digitate	Present	20.8±0.7 (19.4–21.8)	1 single, 3 pairs	*Pinus radiata*	Unknown	Ambrogioni & Marinari Palmisano, 1998
B. hildegardae (NZ)	980±170 (807–1250)	3	129 ±29.6 (97–183)	Rouned	Present	21.5±3.1 (17.8–24.1)	1 single, 3 pairs	Red pine, white pine	*Hylastes ater*	This study
B. elytrus	890–960	unknown	178–187	Rounded, acute	Absent	Unknown	3 pairs	Ponderosa pine	*Hylurgops pinifex*	Massey 1971
B. newmexicanus	1500	unknown	222–259	Rounded, conoid	Present	Unknown	2 pairs		*Hylurgops* sp.	Massey 1974

MORPHOLOGICAL DIAGNOSIS AND RELATIONSHIPS

The New Zealand isolate of *B. hildegardae* is morphologically closest to the original description of *B. hildegardae* (Braasch *et al*., 2006). However, it varies from the original description in the male with bursa shape, spicule size and b index. In the original description, it states that the male has a distinct V-shaped dorso-ventrally visible terminal bursa, but in the New Zealand specimens of *B. hildegardae* it has not been observed (Figs. 1G; 2G & J). The spicule sizes were 23–28 μm vs 18–24 μm; and the b values were 8.6–10.2 vs 10.2–12.7 in the German and New Zealand isolates respectively.

The New Zealand isolate of *B. hildegardae* is also morphologically similar to *B. eggersi*, *B. elytrus* Massey, 1971*, B. glochis* Brzeski & Baujard, 1997*, B. newmexicanus* Massey, 1974 and *B. tusciae* Ambrogioni & Palmisano, 1998. However, it can be differentiated from them by spicule shape, particularly by the thin hook-like condylus (Table 3).

MOLECULAR PHYLOGENETIC RELATIONSHIPS

Sequences for partial SSU, D2/D3 and ITS were amplified from a female, male, juvenile and dauer juvenile respectively. These PCR products were subjected to direct sequencing and aligned with those sequences from published *B. hildegardae* data*.* Comparisons of trees inferred from Bayesian analyses are shown in Figs 4–6. Molecular phylogeny of near full length SSU, D2/D3 expansion segments of LSU and ITS region indicate that the specimen collected in New Zealand is phylogenetically close to *B. hildegardae* (Figs 4–6). Molecular analyses of an individual female, male, juvenile and dauer juvenile of the nematode confirmed they are the same species. All three trees derived from SSU, D2/D3 and ITS sequences, grouped the New Zealand isolate of *B. hildegardae* together with the isolates of *B. hildegardae* from Germany.

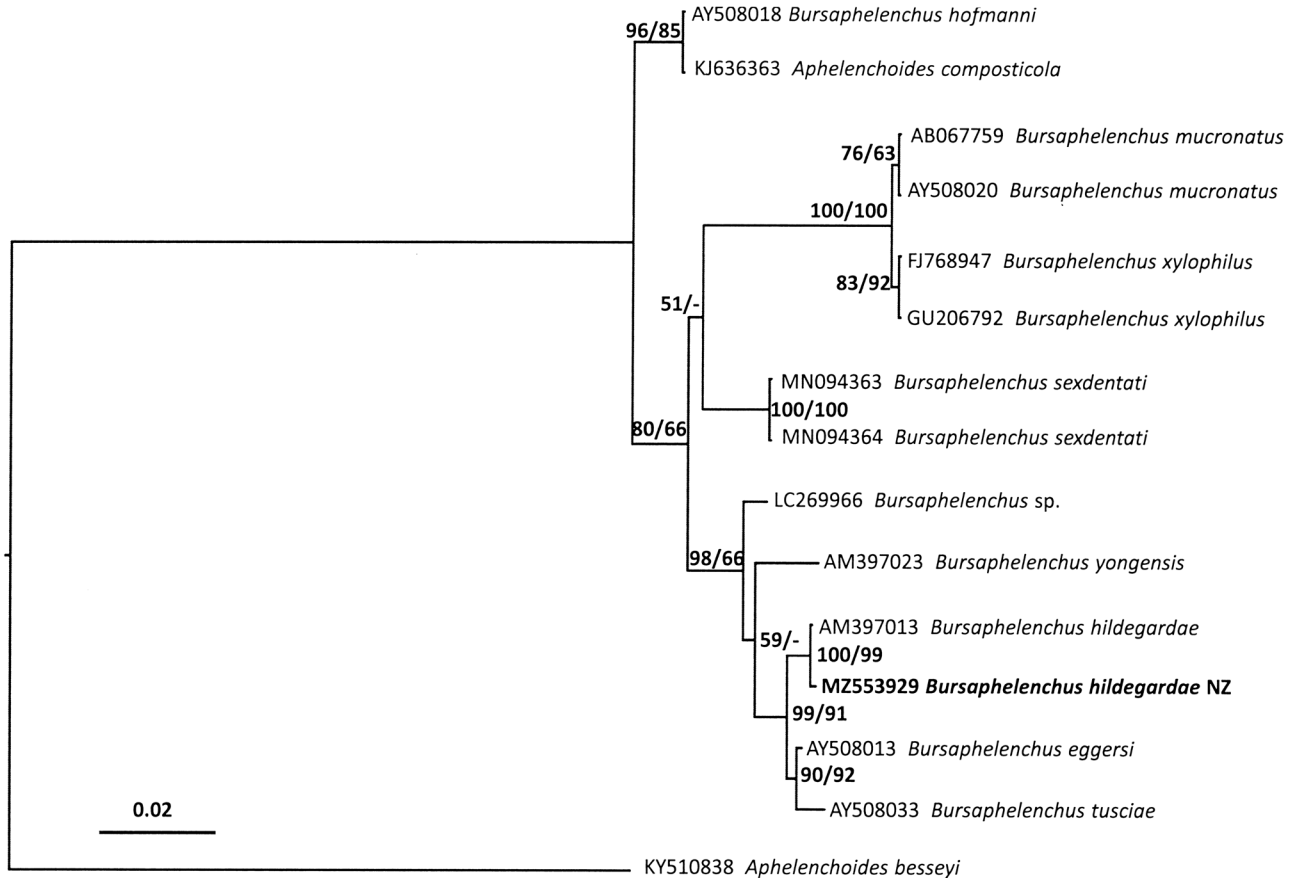

FIG. 4. Bayesian phylogenetic tree inferred from SSU gene DNA sequences of *Bursaphelenchus hildegardae*. Posterior probabilities greater than 50% are given on appropriate clades. Nematode species, GenBank accession numbers and locations are listed for each taxon, if known.

The consensus tree inferred from SSU (Fig. 4) shows that *B. hildegardae* is clustered with the only SSU sequences of *B. hildegardae* (AM397013) available in GenBank, with a posterior probability of 100% and a bootstrap of 100% support, respectively. The results of a BLAST search also showed that New Zealand *B. hildegardae* is nearly identical to the *B. hildegardae* sequences (AM397013) having 99.9% with one bp difference, and close to *B. eggersi* (AY508013) having 99.4% with nine bp difference and *B. tusciae* (AY08033) having 98.9% with 16 bp difference for the 18S sequences.

The consensus tree inferred from LSU (Fig. 5) shows that the New Zealand isolate of *B. hildegardae* is grouped with the only LSU sequences of *B. hildegardae* (AM396569) available in GenBank, with a posterior probability of 100% and a bootstrap of 100% support respectively. From the results of a BLAST search, the sequences of 28S from the New Zealand isolate were almost identical to the only *B. hildegardae* sequences (AM396569) having 99.7% with two bp difference. It is also close to two *B. eggersi* sequences (AY508078 and MW258275) having 95.9% and 95.8%, and two *B. tusciae* sequences (AY508104 and MW358271) having 96.4% and 96.5% identity for the 28S sequences, respectively.

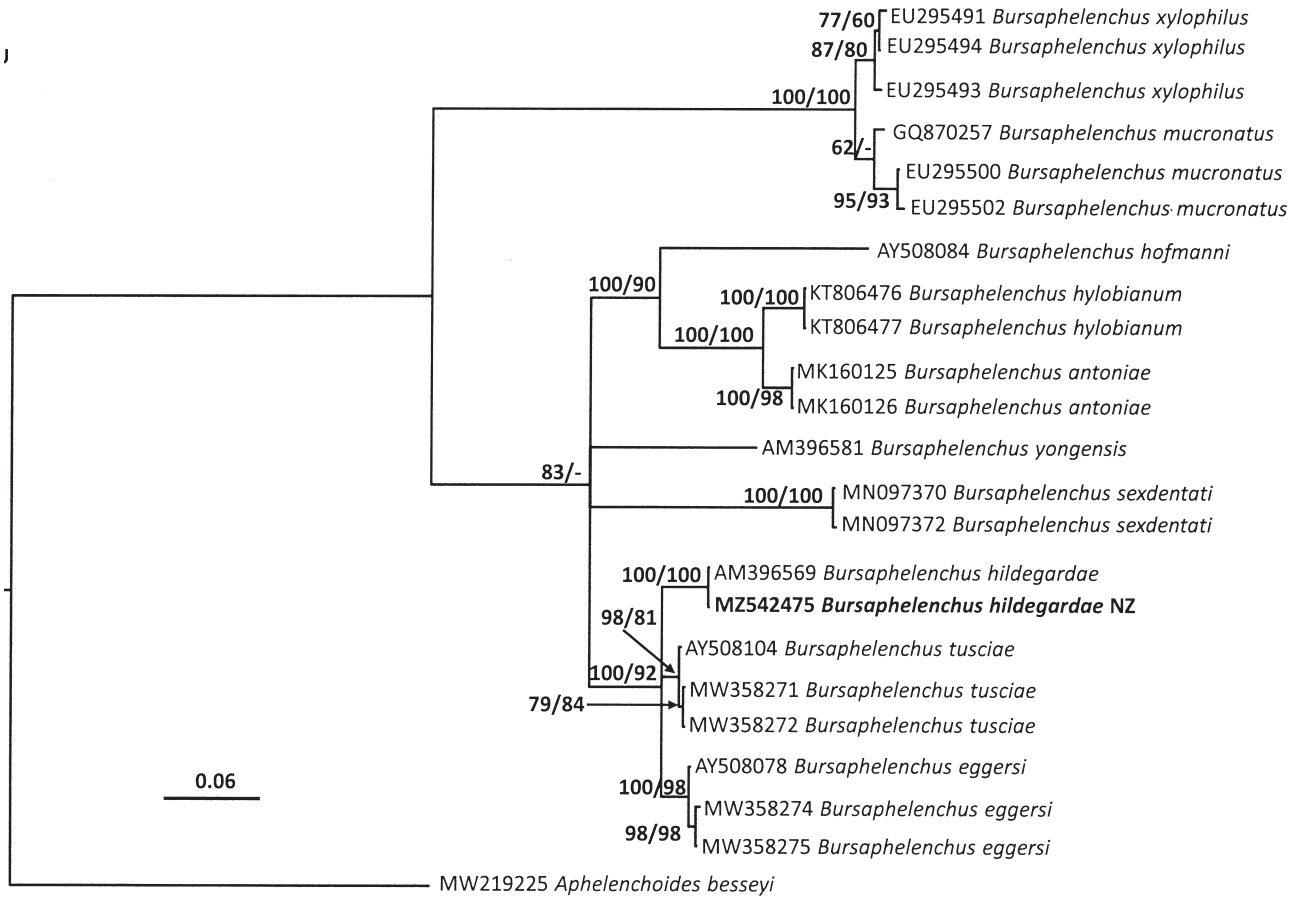

FIG. 5. Bayesian phylogenetic tree inferred from D2D3 gene DNA sequences of *Bursaphelenchus hildegardae*. Posterior probabilities greater than 50% are given on appropriate clades. Nematode species, GenBank accession numbers and locations are listed for each taxon, if known.

The consensus tree inferred from ITS (Fig. 6) shows that the New Zealand isolate of *B. hildegardae* is monophyletic with a posterior probability of 100% (a bootstrap of 100%) when two ITS sequences of *B. hildegardae* (AM269736 and HQ197354) available in GenBank are included. The results of a BLAST search showed that the sequences of ITS from the New Zealand isolate were 100% identical to the *B. hildegardae* sequences (AM269736) and close to a sequences from Spain (HQ197354) having 99.48% identity for the ITS sequences, respectively.

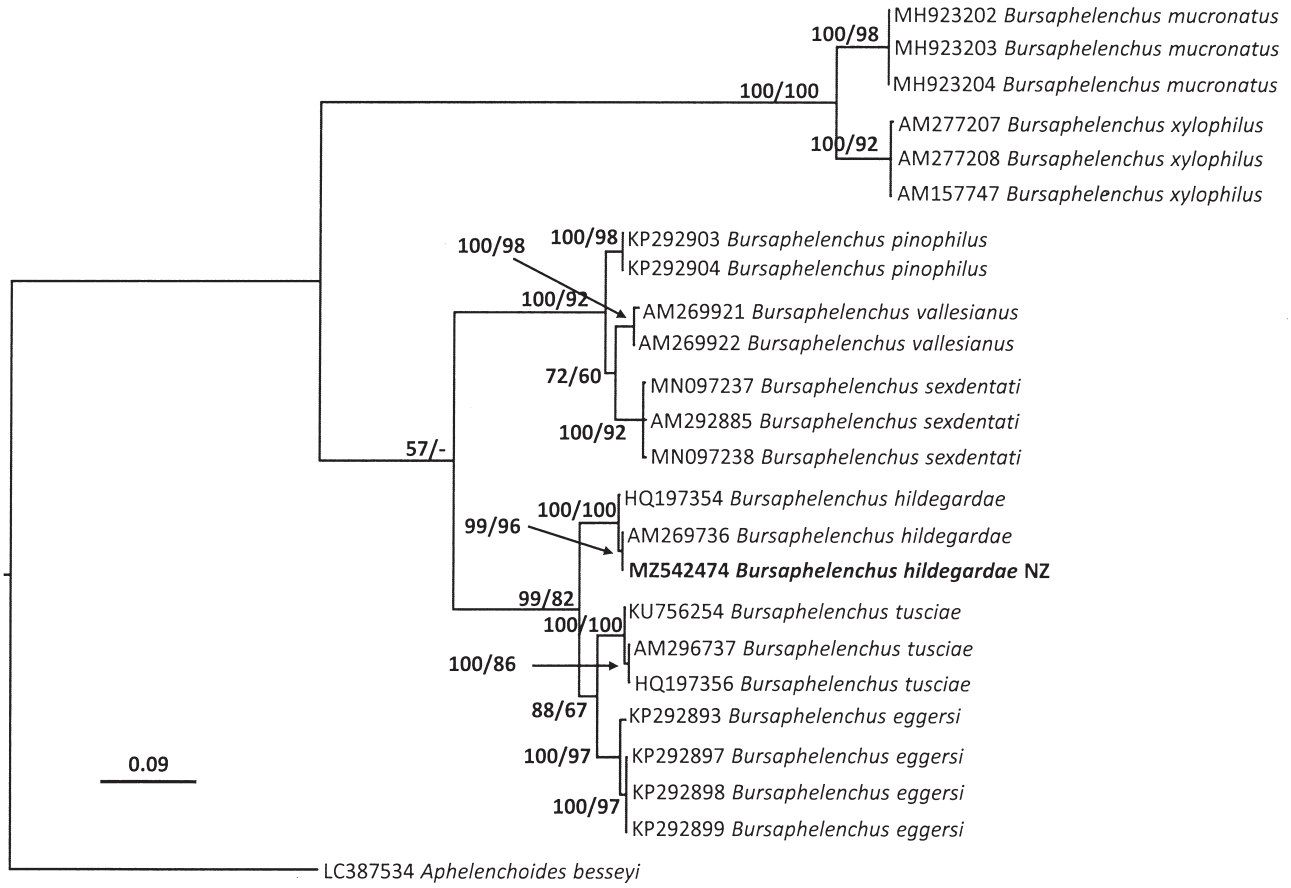

FIG. 6. Bayesian phylogenetic tree inferred from ITS gene region DNA sequences of *Bursaphelenchus hildegardae*. Posterior probabilities greater than 50% are given on appropriate clades. Nematode species, GenBank accession numbers and locations are listed for each taxon, if known.

Discussion

Bursaphelenchus hildegardae was described from Germany from *Pinus silvestris* and the dauer juvenile was found under the elytra of a beetle, *Hylurgops palliatus* (Gyllenhal, 1813) (Braasch *et al.,* 2006). Since then it has been isolated in Spain from congeneric pine trees (*P. nigra, P. pinaster* and *P. silvestris*) in 2010, 2012, 2018 and 2019 (A.A. Argibay, pers. comm; Zamora *et al.* 2016). Later on, it was detected from pine trees in Australia in 2016 (Carnegie & Nahrung, 2019). No further record is known anywhere in the world.

This is the first record of *B. hildegardae* in New Zealand and the second report from the southern hemisphere in addition to Australia. However, this species may have been present in the country for a long time. *B. hildegardae* is morphologically similar to *B. eggersi* and both species belong to the *eggersi*-group (Braasch, 2001; Braasch *et al.,* 2006). As *B. hildegardae* was described in 2006, it is likely that Dale had isolated *B. hildegardae* in 1967 but identified this nematode as the closest organism known at the time - *B. eggersi*. Dale (1967) reported that he isolated *B. eggersi* from the bark beetle *H. aster* in eight of 14 sites across the North and South Islands of New Zealand. In the current study, *B. eggersi* was not found from any of the pine samples or *H. ater* specimens. This is further evidence, suggesting that *B. hildegardae* (but not *B. eggersi*) may have been well established in New Zealand for a long time but previously misidentified. So far, the nematode has not been recorded to cause problems to pine trees.

A range of information was used to assess the potential economic impact of *B. hildgardae* in New Zealand, which included searching the literature and consulting experts of this group of nematodes. Based on current limited literature on the overall effects for *B. hildegardae*, it was concluded that: 1) further information about the status of this nematode in New Zealand is likely to emerge as a result of any ongoing surveillance and diagnostic work that might be associated with managing pine forest problems; and 2) pathogenicity of *B. hildegardae* needs to be further studied.

Bursaphelenchus hildegardae belongs to the *eggersi*-group with five other members, viz., *B. eggersi*, *B. elytrus*, *B. glochis*, *B. newmexicanus* and *B. tusciae*. The key below to the known species of *eggersi*-group was designed.

Key to *B. hildegardae* and nematodes in the *eggersi*-group

1. Male caudal papillae 2 pairs; longer body (1500 µm for female, 1250 µm for male) . . . . . *B. newmexicans*
-. Male caudal papillae more than 2 pairs; female body less than 1500 µm 2

2. Vulva flap absent 3
-. Vulva flap present. 4

3. Male caudal papillae 3 pairs; female body 890–960 µm.. . . . . *B. elytrus*
- Male caudal papillae 1 single and 3 pairs; female body 990–1122 µm . . . . . *B. eggersi*

4. With a hook or knob-like spicules 5
- Without a hook or knob-like spicules. . . . . *B. glochis*

5. Condylus small, constantly recurved, ending as a thin hook. . . . . *B. hildegardae*
- Condylus variably recurved, formed with a depressed centre, a thicker, sometimes knob-like end . . . . . *B. tusciae*

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