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Bursaphelenchus anatolius n. sp. (Nematoda: Parasitaphelenchidae), an Associate of Bees in the Genus *Halictus*[1]

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Abstract: Bursaphelenchus anatolius n. sp., a phoretic associate of *Halictus* bees from Ankara, Turkey, is described and illustrated. *Bursaphelenchus anatolius* n. sp. is closest to *B. kevini,* which is phoretically associated with *Halictus* bees from the Pacific Northwest. *Bursaphelenchus anatolius* n. sp. and *B. kevini* appear to be sister taxa based upon several shared morphological features, similar life histories involving phoresy with soil-dwelling *Halictus* bees, and molecular analysis of the near-full-length small subunit rDNA, D2D3 expansion segments of the large subunit rDNA, and partial mitochondrial DNA COI. *Bursaphelenchus anatolius* n. sp. can be differentiated from all other species of *Bursaphelenchus* based upon spicule morphology. The paired spicules are uniquely shaped and ventrally recurved, and both *B. anatolius* n. sp. and *B. kevini* possess extending flaps that open when the spicules are protracted beyond the cloaca. Population growth of *B. anatolius* n. sp. was measured at 23 °C in the laboratory on cultures of the fungus *Monilinia fructicola* grown on lactic acid-treated, 5% glycerol-supplemented potato dextrose agar. Nematode population densities rapidly increased from 110 to about 110,000/9-cm-diam. dish within 21 days.

Key words: Bursaphelenchus anatolius n. sp., cytochrome oxidase subunit I, Halictidae, *Halictus* (*Argalictus*), Hymenoptera, large subunit rRNA, molecular phylogeny, morphology, mycophagy, nematode, Parasitaphelenchidae, phoresy, small subunit rRNA, taxonomy.

A new species of *Bursaphelenchus* was recovered as dauer juveniles (J_{III}) in the median oviduct, ovipositor sac, Dufour's gland, and poison sac of adult females of a soil-dwelling bee of the Hexataenites group in the subgenus *Argalictus* of the genus *Halictus* (Halictidae) from the campus of Hacettepe University, Ankara, Turkey. Bees from this group are found mostly in the Mediterranean region to central Asia, although some are found from as far west as western Europe to as far east as China (Michener, 2000). This nematode is described herein as *Bursaphelenchus anatolius* n. sp. It is only the second described species of *Bursaphelenchus* known to be associated with soil-dwelling bees from the family Halictidae (Giblin et al., 1984; Giblin and Kaya, 1984b) and the fourth species of *Bursaphelenchus* to be described from soil-dwelling bees (Halictidae and Apidae [Anthrophorini]) (Giblin and Kaya, 1983; Giblin-Davis et al., 1990; Ye et al., 2005). Recent molecular work with the small subunit rDNA (SSU), D2D3 expansion segments of the large subunit rDNA (LSU), and partial mitochondrial DNA cytochrome oxidase subunit I (mtCOI) confirm that *B. anatolius* n. sp. is a new species to science (Ye et al., 2005).

The life history of *B. anatolius* n. sp. appears to be similar to that described for *B. kevini* Giblin, Swan & Kaya, where dauers are carried as internal phoretics by their halictid hosts and transferred to a new bee cell in the soil nest during oviposition, cell lining, and(or) provisioning (Giblin and Kaya, 1984b; Giblin-Davis et al., 1990). Once in the new environment, *B. anatolius* n. sp. dauers most likely molt to the propagative phase and feed and develop on fungi that infest the cell walls or provisions. The dauer juvenile stage of *B. anatolius* n. sp. probably becomes more prevalent as the host environment deteriorates and they infest newly emerged female bees as do *B. kevini* (Giblin and Kaya, 1984b).

Bursaphelenchus anatolius n. sp. and *B. kevini* are potentially sister taxa because of similarities in morphology and life history. Recent molecular phylogenetic analysis has provided a meaningful framework for the genus *Bursaphelenchus,* including *B. anatolius* n. sp. (as *B.* sp. ex Halictidae 170 Turkey) (Ye et al., 2005). However, *B. kevini* was not available for sequencing by Ye et al. (2005). Thus, we re-isolated *B. kevini* from *Halictus farinosus* Smith from California for molecular phylogenetic comparisons for this study.

In addition to the taxonomic description of *B. anatolius* n. sp., this paper reports molecular phylogenetic comparisons with the re-isolated putative sister species, *B. kevini* from *H. farinosus* from Santa Cruz Island, California, and preliminary population dynamics of *B. anatolius* n. sp. cultured on the fungus *Monilinia fructicola* (Wint.) Honey.

Materials and Methods

A culture of *B. anatolius* n. sp. (designated voucher 170) was initiated from a single fertilized female from a culture that was started from dauer juveniles isolated from an adult female of a soil-dwelling halictid bee of the Hexataenites group in the subgenus *Argalictus* of

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the genus *Halictus* from the Beytepe campus of Hacettepe University, Ankara, Turkey. The nematodes were cultured on *Monilinia fructicola* on 5% (v/v) glycerol-supplemented potato dextrose agar treated with 2 ml of 25% (v/v) lactic acid (LGPDA)/liter after autoclaving but before pouring into petri dishes (Giblin and Kaya, 1984a). All taxonomic and culturing work is based upon this isolate, which has been maintained through sub-culturing on *M. fructicola.*

Adults of *B. anatolius* n. sp. were collected from 14- to 20-day-old cultures on *M. fructicola* and heat-killed for measurements in temporary water mounts. All nematodes were drawn and measured with the aid of a camera lucida and a stage micrometer. Type specimens were from 14- to 20-day-old cultures, fixed in formalin-glycerol for greater than 24 hours, and processed slowly into glycerol before measurement (Southey, 1970). Stylets were measured on selected specimens after squashing to ensure that the conus tip could be seen. Male spicule terminology used is that of Yin et al. (1988). Spicule length is the distance between the condylus and the posterior-most point of the lamina measured in a straight line, unless otherwise stated.

Primers for amplification of the D2D3 expansion segments of the large subunit rRNA (LSU) of *B. kevini,* isolated as dauers from live *H. farinosus* bees collected by R. Thorp in April 2004 from Santa Cruz Island, California, were forward primer D2A (5′ ACAAGTACCGTGAGGGAAAGT 3′) and reverse primer D3B (5′ TGCGAAGGAACCAGCTACTA 3′) (Nunn, 1992). Primers for mitochondrial cytochrome oxidase subunit I (mtCOI) amplification were forward primer COI-F1 (5′ CCTACTATGATTGGTGGTTTTGGTAATTG 3′) and reverse primer COI-R2 (5′ GTAGCAGCAGTAAAATAAGCACG 3′) (Kanzaki and Futai, 2002). Primers for near-full-length small subunit rRNA (SSU) amplification were forward primer 18SF-Burs (5′ ATGCATGTCTAAGTGGAGTATTATA 3′) and reverse primer 18SR-Burs (5′ CTACGGCTACCTTGTTACGACTTTT 3′) (Ye et al., 2005). Methods for amplification and sequencing were as described by Ye et al. (2005). The GenBank accession numbers for the sequences obtained are AY753531 for SSU, AY753532 for D2D3 LSU, and AY753533 for mtCOI. DNA sequences were aligned by Clustal W (http://workbench.sdsc.edu, Bioinformatics and Computational Biology Group, Department of Bioengineering, University of California, San Diego, California) using *Aphelenchoides besseyi* as the outgroup taxon. Phylogenetic analyses were performed in PAUP* 4.0b10 (Swofford, 2002). Neighbor-joining (NJ) analysis (Saitou and Nei, 1987) was conducted using the Tajima-Nei distance option (Tajima and Nei, 1984). Dauers from the same bees used for molecular work were used to establish cultures on *M. fructicola* as described above to confirm identification of *B. kevini.*

Monilinia fructicola was sub-cultured aseptically onto LGPDA in 9-cm-diam. petri dishes and grown for 7 days at 23 °C before inoculation with 110 *B. anatolius* n. sp. in a drop of sterile water. To assess population growth, two replicate cultures were extracted for 4 to 5 hours on a Baermann funnel at 3- to 7-day intervals for 3 weeks after inoculation and measured aliquots of the nematodes were counted using a dissecting microscope.

Systematics

Bursaphelenchus anatolius n. sp.
(Figs. 1–3; Table 1)

Measurements listed in Table 1 were made of specimens in temporary water mounts.

Male (n = 25): Body cylindrical, tapered at both ends, J-shaped, tail with strong ventral curl when heat-killed (Fig. 1C), sometimes coiling back upon itself. Cuticle with fine annulation, annules about 0.8 µm wide at mid-body. Lateral field with four incisures, beginning above the metacorpus. Head offset from body. En face view (light microscope) with six lips, inner hexaradiate framework and cephalic annuli indistinct (Fig. 2B). Stylet two-part; conus (5.6 µm) about one third of the stylet length (16.4 µm), shaft (10.7 µm) with basal

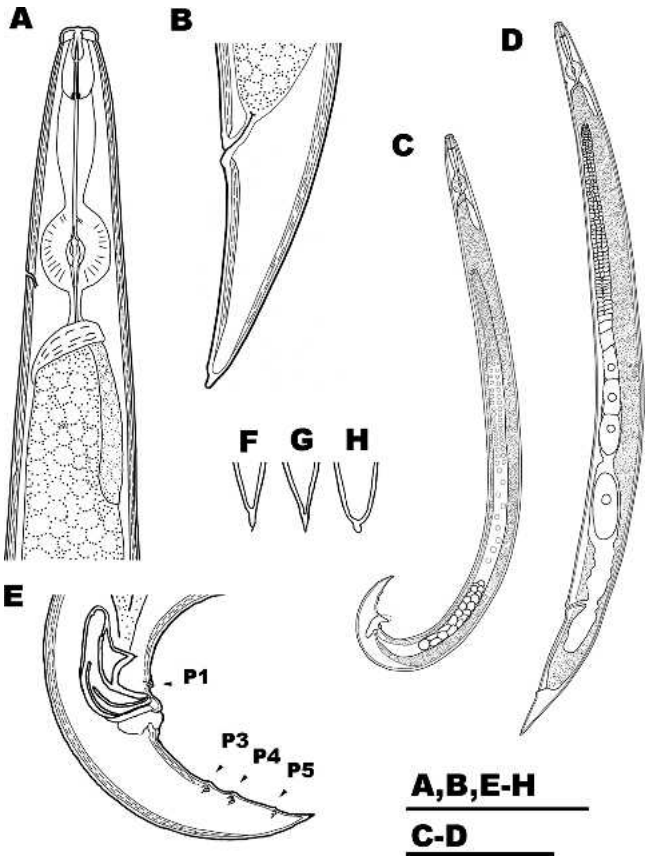

Fig. 1. Drawings of adults of *Bursaphelenchus anatolius* n. sp. in lateral view. A) Anterior region of female. B) Female tail. C) Whole male with spicules protracted. D) Whole female. E) Male tail; P1 = single precloacal papilla, P2 = precloacal pair of papillae not drawn, P3 = postcloacal pair of papillae, P4 = postcloacal pair of papillae, and P5 = caudal pair of papillae. F, G, H) Variations in female tail terminus. (Scale bar A,B,E-H = 50 µm; C-D = 200 µm.)

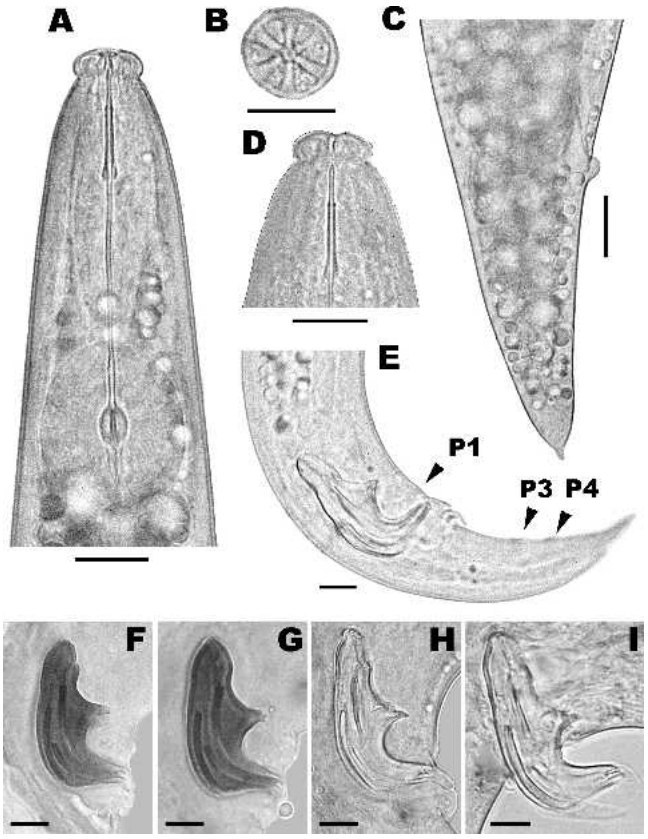

FIG. 2. Photomicrographs of adults of *Bursaphelenchus anatolius* n. sp. A) Male anterior region (lateral view). B) En face view. C) Female tail (lateral view). D) Female head and stylet (lateral view). E) Male tail (lateral view); P1 = single precloacal papilla, P3 = postcloacal pair of papillae, and P4 = postcloacal pair of papillae. F,G) Spicules stained with 1% acetic orcein, same male specimen but different focal planes (lateral view). H) Spicules (lateral view). I) Protracted spicules showing extended and expanded distal flap. (Scale bar = 10 µm.)

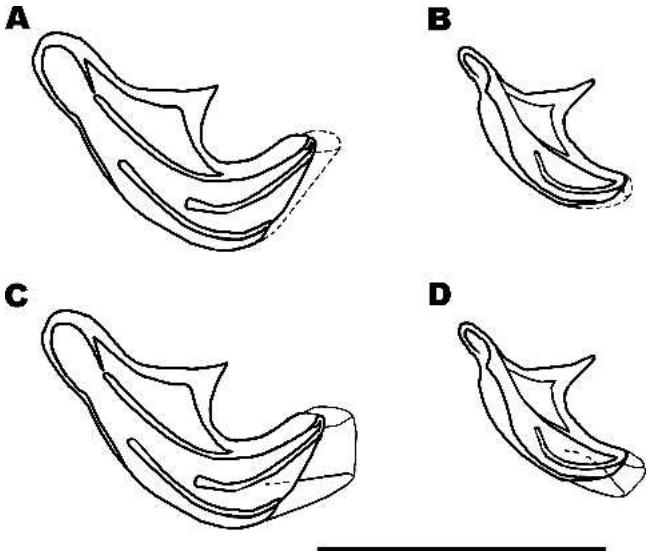

FIG. 3. Drawings of *Bursaphelenchus* spicules in lateral view. A) *B. anatolius* n. sp. spicules retracted within body. B) *B. kevini* spicules retracted within body. C) *B. anatolius* n. sp. spicule tips protracted through the cloaca showing the extended and expanded flap at distal ends. D) *B. kevini* spicule tips protracted through the cloaca showing the extended and expanded flap at distal ends. (Scale bar = 30 µm.)

thickenings (Table 1; Fig. 2A,D). Procorpus about two stylet lengths long, ending in a well-developed metacorpus (Figs. 1A; 2A). Dorsal esophageal gland orifice opens into lumen of metacorpus about 1 metacorpal valve length above metacorpal valve. Esophagointestinal junction about 1 metacorpal valve length behind metacorpus (difficult to see in most specimens). Postcorpus glandular. The excretory pore is usually located between the base and the middle of the metacorpus. Hemizonid obscure, when visible, about 40 to 45 µm below base of metacorpus. Gonad outstretched, sperm amoeboid. Tail ventrally curled; 2.4 anal body widths long. Bursal flap present. Nine ventral preanal and postanal papillae: one preanal papilla (P1) in ventral midline at about 8 µm above cloaca; one pair of preanal subventral papillae (P2) at 3.5 µm above cloaca; one pair of subventral postanal papillae (P3) at 47% of tail length from cloaca; another pair of subventral postanal papillae (P4) posterior to P3 at 63% of tail length behind cloaca; one ventral pair of papillae (P5) about 8.4 µm above tail tip, obscure in most specimens (Figs. 1E; 2E). Spicules separate; spicule length measured along its arc/capitulum width (distance between condylus and rostrum) ratio is about 2.8 (range 2.2–2.9); rostrum sharply conical; junction of rostrum and calomus smoothly curved; lamina complex with midpoint widened and rounded; dorsal and ventral aspects of blade appear separate; lamina dorsal line angular in ventral third; spicular tip bluntly rounded; cucullus absent (Figs. 1E; 2E,F,G,H,I; 3A,C). Spicules are recurved so that a line drawn through the anterior-most points of the condylus and rostrum (along the capitulum) and projected ventrally will intersect a line that ventrally extends the uniquely angular dorsal third of the lamina at about 80°. Non-staining flaps (Fig. 3A) open and expand the laminar tips when the spicules are extruded beyond the cloacal opening (Fig. 3C).

Female (n = 25): Adult female cuticular features and cephalic regions (exterior and interior) similar to males. Ventrally arcuate when killed with heat (Fig. 1D). Ovary single and outstretched (Fig. 1D). Vulva a transverse slit; vulval lips protrude slightly. Vulval position at 80% of body length (Table I). Vagina vera cuticularized. Thin, paired, pronged structures at junction of uterus and post-uterine sac similar to structures in *B. seani* and *B. kevini* but difficult to see. Length of post-uterine sac (PUS) short (56.5 µm), PUS/body width at vulva about 1.4, and PUS length about 50% the distance from vulva to anus (Table I). PUS sometimes with egg or amoeboid sperm. Lateral field extends from just above the metacorpus to within 12 µm of tail tip. Anus dome-shaped slit. Tail is ca. 2.6 anal body widths long (range 2.2 to 3.0), uniformly conoid with pointed or digitate tip, occasionally mucronate (Fig. 1B,F,G,H).

Diagnosis: Bursaphelenchus anatolius n. sp. is distinguished from all other described species of *Bursaphel-*

TABLE 1. Morphometrics of male holotype and female allotype in glycerol and 25 male and female specimens each of *Bursaphelenchus anatolius* n. sp. in temporary water mounts (measurements in µm).

Measurement or ratio	Holotype (male)	Water mounts (males)	Allotype (female)	Water mounts (females)
n	1	25	1	25
Length (mean ± S.D.; Range)	580	849 ± 103 (667–1037)	780	888 ± 97 (684–1052)
Body width (at vulva for females) (males = GBW; females = VBW)	26.0	38.6 ± 6.6 (28.8–50.0)	34.0	38.3 ± 4.4 (30.4–47.7)
Stylet length	16.1	16.4 ± 0.5 (15.5–17.0)	16.0	16.3 ± 0.4 (15.8–17.3)
Stylet (conus length)	5.5	5.6 ± 0.2 (5.2–5.8)	5.5	5.6 ± 0.1 (5.5–5.8)
Stylet (shaft length)	10.6	10.7 ± 0.4 (10.4–11.5)	10.5	10.6 ± 0.4 (10.4–11.8)
Esophagus length (Head to metacorpus base)	55.0	71.3 ± 3.3 (66.7–78.2)	69.0	71.4 ± 3.4 (63.3–77.1)
Spicule length	47.0	34.2 ± 1.7 (29.9–36.2)	—	—
Vulva-to-anus distance (VA)	—	—	92.0	119.1 ± 12.3 (89.7–142.5)
Post-uterine sac length (PUS)	—	—	46.0	56.5 ± 11.5 (43.1–86.8)
Anal body width (ABW)	20.5	30.4 ± 3.8 (23.6–35.7)	21.0	22.3 ± 1.8 (18.4–25.9)
Tail length	53.0	70.7 ± 6.6 (56.4–85.7)	48.0	98.6 ± 4.1 (50.0–65.0)
a	22.3	22.2 ± 1.7 (17.9–24.8)	22.9	23.2 ± 1.5 (20.9–26.4)
b_1	10.5	11.9 ± 1.5 (9.7–14.5)	11.3	12.4 ± 1.3 (9.8–15.0)
c	10.9	11.9 ± 1.1 (10.1–13.9)	16.3	15.5 ± 1.4 (13.2–17.9)
c′	2.6	2.2 ± 0.1 (2.1–2.6)	2.3	2.6 ± 0.2 (2.2–3.0)
V	—	—	82.1	80.1 ± 1.2 (78.4–82.4)
PUS/VBW	—	—	1.4	1.4 ± 0.2 (1.2–2.1)
PUS/VA	—	—	0.5	0.5 ± 0.1 (0.4–0.7)

enchus by the unique morphology of the spicules in males and by sequence differences in SSU, D2D3 LSU, and mtCOI.

Relationships: Based upon morphology and host associations, *B. anatolius* n. sp. is closest to *B. kevini*. Both nematodes are internally carried as J_{III} dauer juveniles in adult bees of the genus *Halictus* and propagate on fungi in soil nests of their hosts. Both are stout (a ratios <36). Males of these species have spicules resembling those of the *B. hunti* grouping (Giblin and Kaya, 1983). Spicules from this group have a broad blade, complex lamina, and are blunt at the tip. Both *B. anatolius* n. sp. and *B. kevini* possess spicules with the synapomorphy of non-staining flaps that extend and expand when the spicules are protracted beyond the cloaca (Figs. 1C; 2I; 3C,D). Males of both species have nine caudal papillae that occur in the same relative locations. In addition, males and females of both species have thickenings at the base of their stylets, excretory pores at the level of their metacorpuses, and four incisures in their lateral fields. The dimensions of the PUS are similar in females of both species. Females of *B. kevini* possess an easily visualized pair of pronged structures at the junction of the uterus and PUS, whereas these structures in *B. anatolius* n. sp. are present but not well defined.

Males of *B. anatolius* n. sp. and *B. kevini* can be separated by spicule length; *B. anatolius* n. sp. has larger spicules 34 µm (range 30–36, *n* = 24) than *B. kevini* 25 µm (range 18–30, *n* = 56) when measured in a straight line from the anterior-most point of the condylus to the distal end of the spicule. The dorsal line of the spicular lamina in *B. kevini* is smoothly and symmetrically curved, whereas this line in *B. anatolius* n. sp. is angular in the last third of the spicules (Figs. 1E; 2E,F,G,H,I; 3A,C). Due to this recurvature, a line drawn through the anterior-most points of the condylus and rostrum (along the capitulum) and ventrally projected will intersect a line that ventrally extends the dorsal third of the lamina at about an 80° angle versus about 35° for *B. kevini*. Another way of quantifying this recurvature of the spicules involves the ratio of the capitulum width (distance between condylus and rostrum) divided by

the shortest distance from the distal tips of the lamina to a line that ventrally extends along the capitulum, being >3 in spicules of *B. anatolius* n. sp. and <2 in *B. kevini*. The ratio of the spicule length measured along its arc divided by the capitulum width also distinguishes *B. anatolius* n. sp. with a ratio of 2.8 (range 2.2–2.9, n = 24) from *B. kevini* with a ratio of 1.9 (range= 1.8–2.1, n = 11). The spicular rostrum of *B. anatolius* n. sp. is sharply conical, whereas *B. kevini* is more thorn-like.

Molecular Phylogenetic Relationships: The SSU sequence from *B. anatolius* n. sp. (AY508025; 1730 bp) shared 94.9% identity (1647 identical bases/1736 aligned bases) with the sequence from *B. kevini* (AY753531; 1695 bp). The tree produced with NJ analysis of the SSU sequence from *B. kevini* with other *Bursaphelenchus* sequences available from Ye et al. (2005), including *B. anatolius* n. sp. (Fig. 4A), gives 100% bootstrap support for a clade including these two as sister taxa. This supports the morphological and host association data, and the level of 5.1% dissimilarity strongly supports the assertion that these species are separate. For example, well-documented nominal species such as *B. xylophilus* and *B. mucronatus* share 99% identity at SSU (Ye et al., 2005). In addition, mating attempts between the two species have all been negative, supporting species status using the biological species concept.

There is good bootstrap support (94%) for including the *B. anatolius* n. sp. and *B. kevini* clade within a subclade including the *B. xylophilus* group (*B. xylophilus, B. mucronatus,* and *B. fradulentus*), the *B. hunti* group (including *B. fungivorus* and *B. seani*), and *B. cocophilus* and *B.* sp. ex *Carpophilus humeralis* (Fig. 4A). Based upon spicule morphology, *B. anatolius* n. sp. is similar to members of the *B. fungivorus* group, *B. cocophilus,* and *B.* sp. ex *Carpophilus humeralis* but not the *B. xylophilus* group.

The LSU sequence from *B. anatolius* n. sp. (AY508093; 759 bp) shared 89% identity (695 identical bases/781 aligned bases) with the sequence from *B. kevini* (AY753532; 779 bp). Although there was strong (100%) bootstrap support for the *B. anatolius* n. sp. and *B. kevini* clade, there was no resolution of deeper branches (Fig. 4B).

The mtCOI sequence from *B. anatolius* n. sp. (AY508056; 627 bp) shared 86.8% identity (544 identical bases/627 aligned bases) with the sequence from *B. kevini* (AY753533; 627 bp). Although *B. anatolius* n. sp. and *B. kevini* are sister taxa in the same clade, bootstrap support was poor (54%) (Fig. 4C).

Neighbor-joining analysis of combined SSU, LSU, and mtCOI 0-fold sequence data yielded a tree with strong bootstrap support (100%) for the *B. anatolius* n. sp. and *B. kevini* clade and good support (83%) for a shared common ancestor inclusive of the *B. xylophilus* group (Fig. 4D). Support for a shared common ancestor with the *B. fungivorus* group or *B. cocophilus* and *B.* sp. ex *C. humeralis* was only 70% (Fig. 4D). The biology of *B. anatolius* n. sp. and *B. kevini* differs from that of the *B. xylophilus* group in that they are carried in the reproductive tracts of their respective *Halictus* bee hosts and propagate on fungi in the subterranean cells of their host's progeny. In contrast, the members of the *B. xylophilus* group propagate in the sapwood of trees, e.g., gymnosperms (*B. xylophilus* and *B. mucronatus*) or angiosperms (*B. fraudulentus*), and are associated or suspected to be associated with wood-boring longhorn beetles (Cerambycidae) (Giblin-Davis, 1993). The species of the *B. xylophilus* group are either facultative plant and fungal parasites or mycophagous only.

That *B. anatolius* n. sp. and *B. kevini* share similar morphologies and associations with different bees from the genus *Halictus* from different geographical regions suggests possible evolution via association by decent. In contrast, the two other *Bursaphelenchus* associates of bees from *Anthophora* species in the western (*B. seani* Giblin and Kaya/*A. bomboides stanfordiana* Cockerell) and eastern United States (*B. abruptus* Giblin-Davis, Mundo-Ocampo, Baldwin, Norden & Batra / *A. abrupta* Say) are very divergent in terms of morphology (Giblin and Kaya, 1983; Giblin-Davis et al., 1993) and molecular phylogeny (Ye et al., 2005; this study). This might suggest a more recent independent sampling and association of these bees, with mycophagous and soil-inhabiting *Bursaphelenchus* species capable of associating with insects and less time for association by decent.

Type host and locality: Holotype male and allotype female were from a 20-day-old culture on *M. fructicola.* The culture was started from dauer juveniles of *B. anatolius* n. sp. isolated from the reproductive tract of an adult female of *Halictus* sp. caught with a collecting net at Hacettepe University in Ankara, Turkey, on 8 June 2001. The bee was collected from *Onopordum turcicum* Danin (Asteraceae).

Type designations: Holotype male and allotype female and additional material deposited at the University of California-Riverside Nematode Collection. Paratypes (males and females same data as holotype) deposited at the University of California, Davis; USDA Nematode Collection, Beltsville, Maryland; and the Nematology Department, Rothamsted Experiment Station, Harpenden Herts., England.

Etymology: This species name is derived from the word Anatolia which, in ancient times, encompassed the Asiatic region of contemporary Turkey where this nematode was discovered.

Laboratory culture: Destructive harvests of *B. anatolius* n. sp. on *M. fructicola* on LGPDA after 7, 14, and 21 days post-inoculation with 110 nematodes yielded averages of 1,260, 15,094, and 107,240 nematodes, respectively. This preliminary assessment of *B. anatolius* n. sp. culture suggests similar dynamics to what was reported for *B. seani* on *M. fructicola* on lactic acid supplemented PDA (Giblin and Kaya, 1984a). The relative proportion of nematode eggs was highest in cultures at 14 days

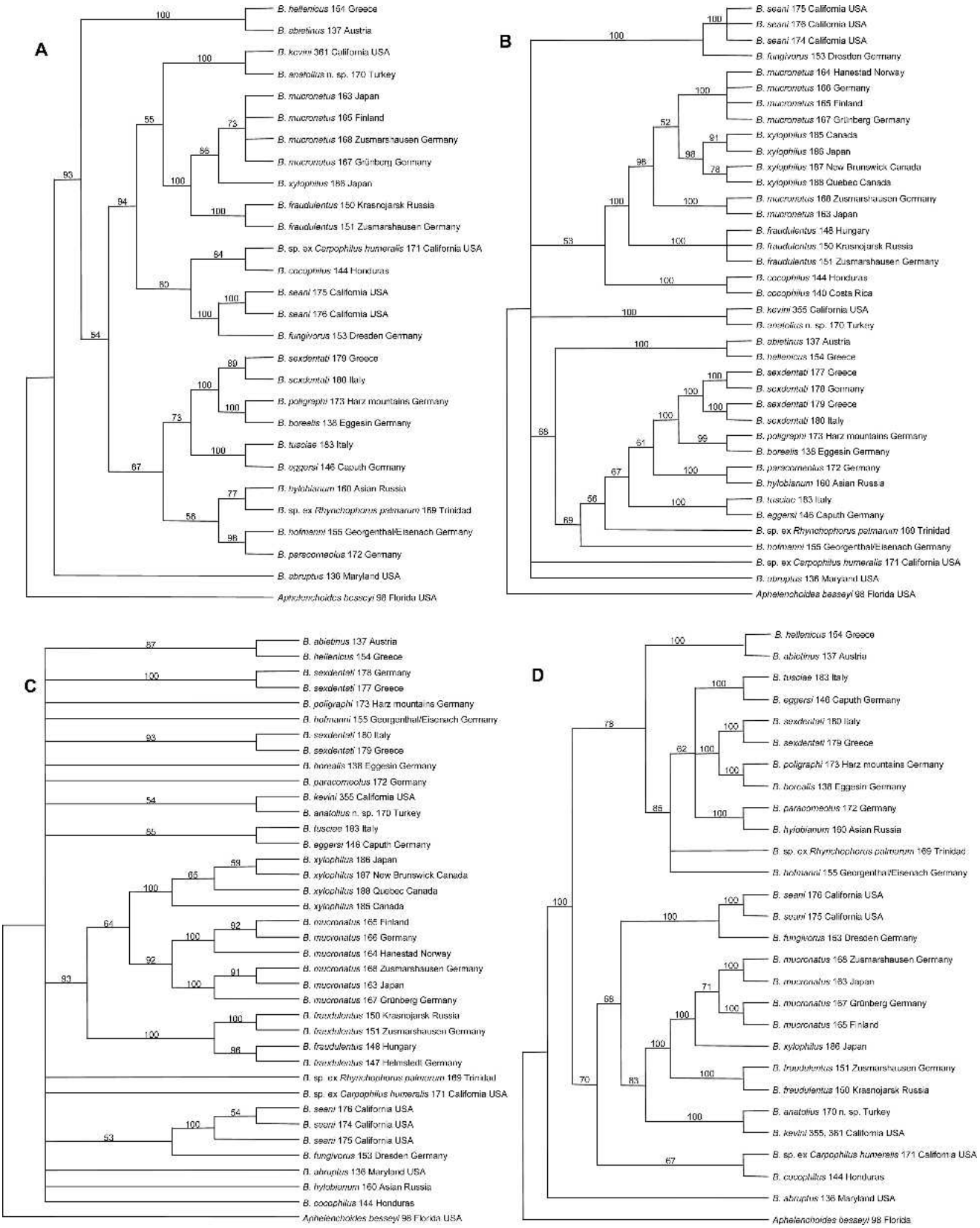

FIG. 4. Neighbor-joining trees inferred from A) SSU sequences, B) LSU sequences, C) mtCOI, D) combined SSU, LSU, mtCOI 0-fold degenerate sequences. All bootstrap values are based on 1,000 replicates.

(13%). These data confirm that *B. anatolius* n. sp. is mycophagous and that *M. fructicola* is a good host.

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