Molecular and morphological characterization of *Ektaphelenchoides hunti* sp. n. (Nematoda: Ektaphelenchinae) from northern Iran

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Summary. *Ektaphelenchoides hunti* sp. n. is described and illustrated based on morphological and molecular characters. The new species is characterized by its body length of 711-929 μm in the female, offset head region, three incisures in lateral field, total stylet length of 16-23 μm, excretory pore in middle of the metacorpus to slightly posterior to metacorpus base, hemizonid at 95-116 μm distance from the anterior end and males with a body length of 572-809 μm, 12.5-17.5 μm long spicules with rounded condylus, moderately developed short rostrum with blunt end and without a cucullus. The new species most closely resembles *E. compsi* but differs by having three incisures in lateral field *vs* obscure, excretory pore in middle of the metacorpus to slightly posterior to metacorpus base, 75-93 μm from anterior end *vs* posterior to the median bulb 91-113 μm from the anterior end, shorter spicules (12.5-17.5 *vs* 19-24 μm) and basic differences in shape of the posterior body (tail). The comparisons with other species of *Ektaphelenchoides* are also discussed. Molecular analyses were performed based on 793 bp partial ribosomal DNA large subunit D2D3 and showed *E. hunti* sp. n. to be different but closest to *E. compsi* and *E. spondylis*.

Key words: *Ektaphelenchoides compsi, E. pini, E. spondylis*, Kelardasht, new species, taxonomy.

The genus *Ektaphlenchoides* Baujard, 1984 currently contains 6 valid species (Kanzaki *et al*., 2009) namely *E. pini* (Massey, 1966) Baujard, 1984, *E. attenuata* (Massey, 1974) Baujard, 1984, *E. musae* Baujard, 1984, *E. compsi* Baujard, 1984, *E. winteri* Hooper, 1995 and *E. spondylis* Kanzaki, Giblin-Davis & Center 2009. The species *E. pini* was described as *Seinura pini* by Massey (1966) in association with *Dendroctonus adjunctus* in ponderosa pine. In 1974, Massey added another species to *Ektaphelenchoides* as *S. attenuatus* associated with *D. terebrans* in loblolly pine.

Baujard (1984) erected the genus *Ektaphelenchoides* and described two species: *E. compsi* isolated from xylem of *Pinus laricio* Poir and *E. musae* isolated from banana corn tissue from the Ivory Coast. Baujard (1984) also recovered *E. pini* from the xylem of *Pinus laricio* in France. Hooper (1995) described *E. winteri* that was found attaching to larvae of *Xylodiplosis* sp. (Diptera: Cecidomyidae) emerging from logs of oak, *Quercus robur* L., cut from trees at Crickley Hill, Gloucestershire, England. Kanzaki *et al*. (2009) described *E. spondylus*, which was recovered from the body cavity of *Spondylis buprestoides* in Tsukuba, Japan. The last species, *E. sylvestris* Pedram, Pourjam, Atighi, Ye and Houshmand, 2012 was recovered from bark of dead Pinus sylvestris L. in Tehran, Iran.

During nematode surveys in the north of Iran, a new species of *Ektaphelenchoides* was recovered from bark samples of a beech tree (Fagus orientalis Lispky) and described in present paper as *E. hunti* sp. n. This is the third report of *Ektaphelenchoides* from Iran. *Ektaphelenchoides compsi,* was first reported by Rafiee *et al.* (2011) from the rhizosphere of cucumber in a green house in the city of Jiroft.

MATERIALS AND METHODS

Several bark samples were collected from northern cities of Iran during September 2010. The nematodes were recovered from the wood samples by soaking in small amount of water for 48 hours, then handpicked under

stereomicroscope. The nematodeds were heat killed by adding boiling 4% formalin solution and then transferred to anhydrous glycerin and mounted in permanent slides according to De Grisse (1969). Permanent slides were made and examined using a Nikon Eclipse E600 light microscopy. Photographs were taken using an Olympus DP72 digital camera attached to an Olympus BX51 microscope powered with differential interference contrast (DIC). Drawings were made using a drawing tube attached to the microscope and were redrawn using CorelDRAW® software version 12.

For molecular study, a single nematode specimen was picked out and transferred to 10µl distilled water on a glass microscope slide, macerated with a pipette tip and collected in 50µl AE buffer (10 mM Tris-Cl, 0.5 mM EDTA; pH 9.0, QIAGEN Inc., Valencia CA, USA). DNA samples were stored at -20°C until used as a PCR template. Primers for 28S D2/D3 amplification were forward primer D2a (5' ACA AGT ACC GTG AGG GAA AGT 3') and reverse primer D3b (5'TGC GAA GGA ACC AGC TAC TA3') (Nunn, 1992). The 25-µl PCR contained 12.5-µl 2X GoTaq DNA polymerase mix (Promega Corporation, Madison, WI, USA), 1 µl each of a 0.4-µM forward and reverse primers solution, and 1 µl of DNA template. The thermal cycling program was as follows: denaturation at 95ºC for 6 min, followed by 35 cycles of denaturation at 94ºC for 30 s, annealing at 55ºC for 30 s, and extension at 72ºC for 1 min. A final extension was performed at 72ºC for 10 min. DNA sequencing was performed using PCR primers for direct sequencing by dideoxynucleotide chain termination using an ABI PRISM BigDye terminator cycle sequencing ready reaction kit (Applied Biosystems, Foster City, CA, USA) in an Applied Biosystems 377 automated sequencer (Applied Biosystems, Foster City, CA, USA) by the Genomic Sciences Laboratory in North Carolina State University (Raleigh, NC 27695, USA). The sequences were deposited into the GenBank database. DNA sequences were aligned by Clustal W (http:// workbench. sdsc.edu, Bioinformatics and Computational Biology group, Dept. Bioengineering, UC San Diego, CA). The molecular sequences of *E. hunti* sp. n. were compared with those of the other nematode species available in GenBank using the BLAST homology search program. The model of base substitution was evaluated using MODELTEST (Posada & Crandall, 1998; Huelsenbeck & Ronquist, 2001). The Akaike-supported model, the base frequencies, the proportion of invariable sites and the gamma distribution shape parameters and substitution rates were used in phylogenetic analyses. Bayesian analysis was performed to confirm the tree topology for each gene separately using MrBayes 3.1.0 (Huelsenbeck & Ronquist, 2001) running the chain for one million generations and setting the "burnin" at 1,000. We used the Markov Chain Monte Carlo (MCMC) method within a Bayesian framework to estimate the posterior probabilities of the phylogenetic trees (Larget & Simon, 1999) using 50% majority rule. The 10001st tree was selected to represent the phylogenetic relationships with branch length and support level.

DESCRIPTION

Ektaphelenchoides hunti sp. n. (Figs 1, 2)

Measurements: See Table 1.

Male. Body slender, J-shaped, ventrally arcuate with the posterior end more ventrally bent after fixation. Cuticle finely annulated. Lateral field with three incisures (not clearly visible in some individuals). Head set off from body contour by a definite constriction, 3.0-4.5 high and 6.0-8.5 µm wide. Stylet 14.5-22.0 µm long without basal knobs, conus *ca* 44.5% of total stylet length. Procorpus cylindrical, connected to a muscular, rectangular metacorpus 9.0-11.5×14-22 µm with the granular part *ca* 30% of its length. Oesophageal glands well-developed, overlapping intestine dorsally *ca* 1.8-2.1 times the distance from anterior end to the base of median bulb and *ca* 8.5-12.5 times body width at median bulb level. Excretory pore with slight variation in position, *i.e.* at the middle of metacorpus to slightly posterior and at 2-8 µm distances from the base of metacorpus. Hemizonid 1.0-1.5 times stylet length posterior to excretory pore and 85-112 µm from anterior body end. Gonad single, outstretched and in some individuals reflexed. Spicules *ca* 2.4 times longer than capitulum width, separate, lamina/calomus smoothly and symmetrically curved, rostrum short, moderately developed with blunt tip. Condylus rounded with round tip and more developed than rostrum. Seven precloacal and postcloacal papillae present with their arrangement as follows: A single midventral precloacal papilla (P1) *ca* 1.2 times cloacal body diam. anterior to

Table 1. Morphometrics of *Ektaphelenchoides hunti* sp. n. All measurements are in μm and in the form: mean ± s.d. (range).

	Holotype male	Paratype males	Paratype females
n	1	15	20
L	795	702.5 ± 68.7 (572-809)	824 ± 70 (711-929)
a	39.8	46.2 ± 2.3 (39.9-47.2)	40.7 ± 2.3 (35.9-44.5)
b	8.5	8.2 ± 0.4 (7.6-9.2)	8.9 ± 0.7 (7.7-10.0)
b’	3.4	3.1 ± 0.2 (2.9-3.5)	3.3 ± 0.3 (2.9-3.8)
c	16.2	15.5 ± 0.8 (14.0-16.7)	–
c’	4.1	3.8 ± 0.3 (3.3-4.3)	–
V or T	63.8	53.4 ± 8.1 (40.8-66.1)	71.6 ± 0.6 (70.4-72.6)
Head height	3.5	3.7 ± 0.3 (3.0-4.0)	4.1 ± 0.3 (3.5-4.5)
Head width	7.5	6.9 ± 0.4 (6.0-7.5)	7.5 ± 0.5 (6.5-8.5)
Stylet	21	17 ± 2.3 (14.5-22)	19.5 ± 1.6 (16-23)
Stylet shaft	12	9.5 ± 1.5 (7.5-12)	10.8 ± 0.8 (9.0-12)
Conus	9	7.6 ± 1.1 (6.0-10)	8.7 ± 1.0 (7.0-11)
m [1]	43	44.5 ± 3.7 (38.7-51.6)	44.5 ± 2.4 (39-47.8)
MB[2]	87	87.4 ± 2.6 (83.5-95.5)	86 ± 2.0 (81.4-88.5)
Body width of MB	14	14.0 ± 1.6 (11-17)	16.4 ±1.7 (13.0-18.5)
Nerve ring from anterior body	97	88.6 ± 7.0 (75-97)	95.0 ± 5.5 (85-103)
Median bulb width	11	10.1 ± 0.8 (9.0-11.5)	12 ± 1.2 (9.5-14)
Median bulb length	20	19.9 ± 2.0 (14-22)	22.0 ± 1.4 (20-25)
Median bulb length/diam. ratio	1.8	2.0 ± 0.2 (1.6-2.2)	1.9 ± 0.2 (1.6-2.2)
Excretory pore	84	80.1 ± 6.5 (67-91)	84 ± 5 (75-93)
Hemizonid	107	100.3 ± 8.4 (85-112)	107.0 ± 6.2 (95-116)
Oesophagus	93	85.7 ± 6.4 (71-93)	92.0 ± 4.2 (82-99)
Overlapping[3]	138	139.5 ± 16.6 (92-160)	159.5 ± 12 (139-181)
Testis or ovary length	507	376.3 ± 77 (262-535)	341 ± 79.2 (210-453)
Anal (cloacal) body width	12	12 ± 0.9 (10.5-14)	–
Tail	49	45.3 ± 4.6 (35-53)	–
Spicule length (arc line)	15	15.8 ± 1.3 (12.5-17.5)	–
Capitulum	6.5	6.5 ± 0.7 (5.5-7.5)	–

[1] Length of conus as percentage of total stylet length
[2] Distance between anterior end of body and centre of median oesophageal bulb as percentage of oesophageal length
[3] Distance from oesophagus-intestine junction to end of dorsal gland tip

cloacal aperture, one pair subventral papillae (P2) at level of or just posterior to cloacal aperture, one subventral pair of postcloacal papillae (P3) at *ca* 47% of tail length posterior to cloacal aperture and one small subventral pair of papillae (P4) at *ca* 54% of tail length posterior to cloacal aperture. Tail dorsally convex, ventrally concave, about 2.5 times cloacal body width long with pointed tip.

Female. Body slightly curved ventrally when heat-killed. Anterior region similar to that in male. Ovary single and outstretched. Oocytes mostly in single file. Spermathecae oval to irregular in shape and most filled with sperm. Uterus thick-walled, usually containing an egg. Crustaformeria visible in some individuals. Vagina not sclerotised, straight and directed somewhat anteriorly. Post-uterine sac *ca* 2.8

times vulva body diameter long and sometimes filled with large sperm. Intestine appears to end in a blind sac at about two thirds the distance from the vulva to posterior end; no rectum or anus seen. Distance of vulva to posterior end *ca* 12.5 times vulval-body width, posterior end (tail) dorsally convex, with a conical posteriorly bent pointed tip.

Molecular characterization and phylogeny of *Ektaphelenchoides hunti* sp. n. For molecular analysis, the 793-bp ribosomal DNA large subunit (LSU) D2D3 (JN714466) was sequenced. A Blastn search of LSU sequence revealed the highest match as to *E. compsi* and *E. spondylis.* Sequence alignment of *E. hunti* sp. n. and *E. compsi* yielded 817 total characters with 613 constant nucleotides (75.0% identity) and 27 insertions/deletions. Sequence alignment of *E. hunti* sp. n. and *E. spondylis* yielded 768 total characters with 569 constant nucleotides (74.1% identity) and 23 insertions/deletions. Sequence alignment of *E. hunti* sp. n. and *E. pini* yielded 802 total characters with 576 constant nucleotides (71.8% identity) and 77 insertions/deletions. The large sequence disparity in LSU supported *E. hunti* sp. n. as a distinct unique species when compared with all available nematode species in GenBank.

Fig. 3 presents a phylogenetic tree based on the LSU of many representative Aphelenchids from a multiple alignment of 817 total characters, in which, 186 characters (22.8%) were constant. The average nucleotide composition was as follows: 18.71% A, 17.59% C, 31.91% G and 31.79% T. Using *Aphelenchus avenae* Bastian, 1865 as the outgroup, this tree inferred many highly supported monophyletic groups. *Ektaphelenchoides hunti* sp. n. is in a 100%-supported monophyletic clade which also includes *E. compsi*, *E. spondylis, Aphelenchoides stammeri* Körner, 1954, *Deviburs*a*phelenchus* sp., *Deviburs*a*phelenchus hunanensis* Yin, Fang & Tarjan, 1988, *D. lini* Braasch, 2004, *Ektaphelenchoides pini, Ektaphelenchus obtusus* Thorne & Malek, 1968 and *Cryptaphelenchus* sp. The four species of sequenced *Ektaphelenchoides* were not monophyletic and *E. hunti* sp. n. was basal to *E. compsi* and *E. spondylis*. The LSU data set in this study was very variable with a low number of constant characters. The identity between *E. hunti* sp. n. and the closest putative relatives of *E. compsi* and *E. spondylis* had only about 75% identity. The large sequence divergence results in many ambiguous sites in multiple alignments making phylogenetic analysis challenging with the current available sequences of aphelenchids from GenBank.

Differential diagnosis and relationships. *Ektaphelenchoides hunti* sp. n. is characterized by its distinctly offset head, three incisures in lateral field, a total stylet length of 16-23 µm, position of excretory pore at the level of the middle of the metacorpus to slightly posterior to the metacorpus base and at 2-6 µm distance from base of metacorpus. Position of hemizonid 95-116 µm from anterior end. The new species is typologically closest to *E. compsi*, *E. pini* and *E. spondylis*.

The new species differs from *E. compsi* by having three incisures in lateral field *vs* lateral field obscure, position of excretory pore at level of the middle of the metacorpus to slightly posterior to metacorpus base 75-93 µm from anterior end *vs* posterior to median bulb and at 91-113 µm, shorter spicules (12.5-17.5 *vs* 19-24 µm), different shape of posterior body (tail) in females and males (dorsally convex, with a conical slightly posteriorly bent pointed tip *vs* ventrally bent, regularly conical and dorsally convex, ventrally concave with pointed tip *vs* regularly conical with strongly ventrally bent end, respectively).

Compared to *E. pini*, the new species has a shorter stylet (16-23 *vs* 26 µm), position of excretory pore is at the level of the middle of the metacorpus to slightly posterior to metacorpus base, 75-93 µm from anterior end *vs* 1/3 body width posterior to median bulb, hemizonid 23 µm from excretory pore *vs* immediately posterior to excretory pore, longer post-uterine sac (1.4-4.0 times body width *vs* 1.0), shorter spicules (12.5-17.5 *vs* 24 µm) and different shape of tail in females.

Compared with *E. spondylis*, *E. hunti* sp. n. has a distinctly offset head *vs* without a sharp constriction, three incisures in lateral field *vs* obscure, position of excretory pore at level of the middle of the metacorpus to slightly posterior to metacorpus base 75-93 µm from anterior end *vs* one metacorpal length posterior to metacorpus and 85-99 µm from the anterior end and shorter spicules (12.5-17.5 *vs* 23-26) without cucullus *vs* having plate-like cucullus.

Taxonomic remark. Even though this species may fit typologically into *Ektaphelenchoides*, the molecular distances suggest that this is a tentative assignment requiring further comparisons with other more closely related nematodes.

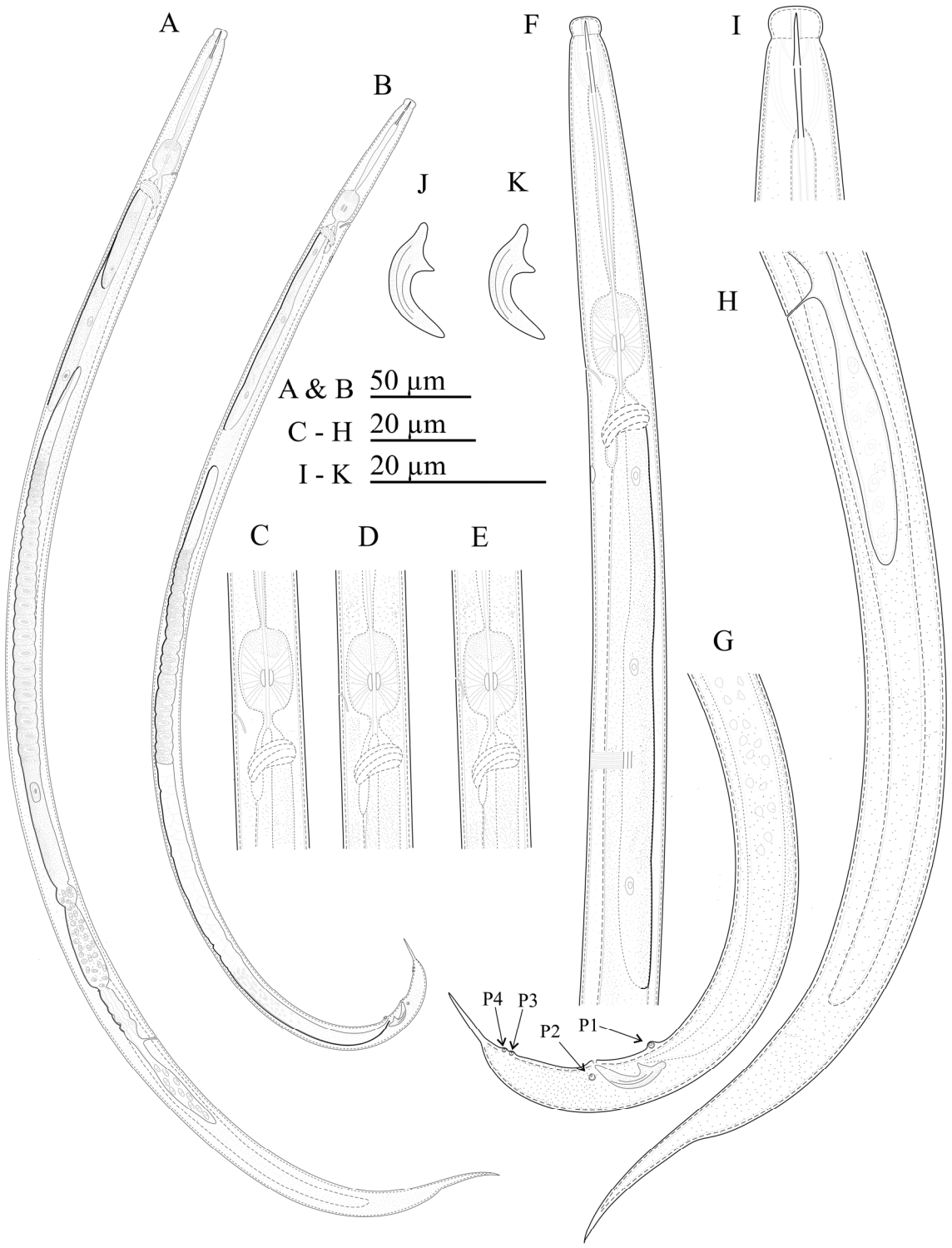

Fig. 1. *Ektaphelenchoides hunti* sp. n. A & B: Entire body of male and female; C-E: Variation in excretory pore position; F: Pharyngeal region; G: Male caudal region; H: Female posterior end; I: Anterior end in detail; J & k: Spicules.

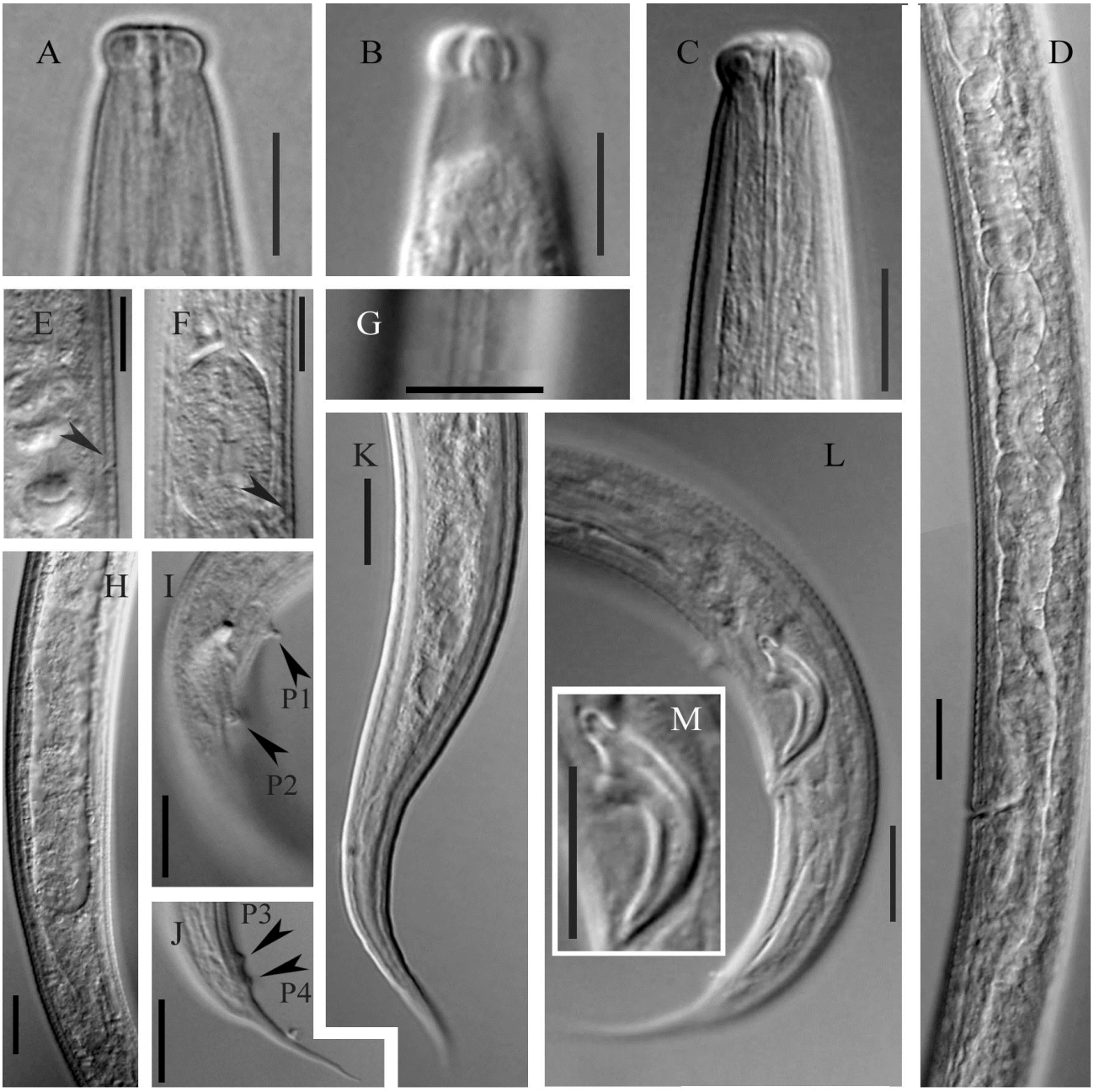

Fig. 2. A-C: *Ektaphelenchoides hunti* sp. n. Anterior end, A: Offset head, B: Lip morphology, C: Stylet; D: Part of female genital tract; E & F: Variation in excretory pore position (arrows); G: Three lateral lines; H: Blind end of the intestine; I & J: Male papillae (arrows), I: P1 and P2, J: P3 and P4; K: Female posterior end; L: Male posterior end, M: Spicule in detail. (All scale bars = 10 µm).

Type habitat and locality. Recovered from bark samples of a beech tree (*Fagus orientalis* Lipsky) with bark beetle galleries in Kelardasht, Mazandaran Province, northern Iran.

Type material. Holotype male, 5 paratype males and females deposited at Nematode Collection of the Faculty of Agriculture, Tarbiat Modares University, Tehran, Iran. Three female and three male paratypes deposited at each of the following collections: CABI Europe-UK, Egham, Surrey, UK; USDA Nematode Collection, Beltsville, MD, USA and Department of Nematology, Agricultural University, Wageningen, the Netherlands.

Etymology. The new species is named in honor of Prof. David J. Hunt, a pioneering scientist in the systematics of aphelenchids.

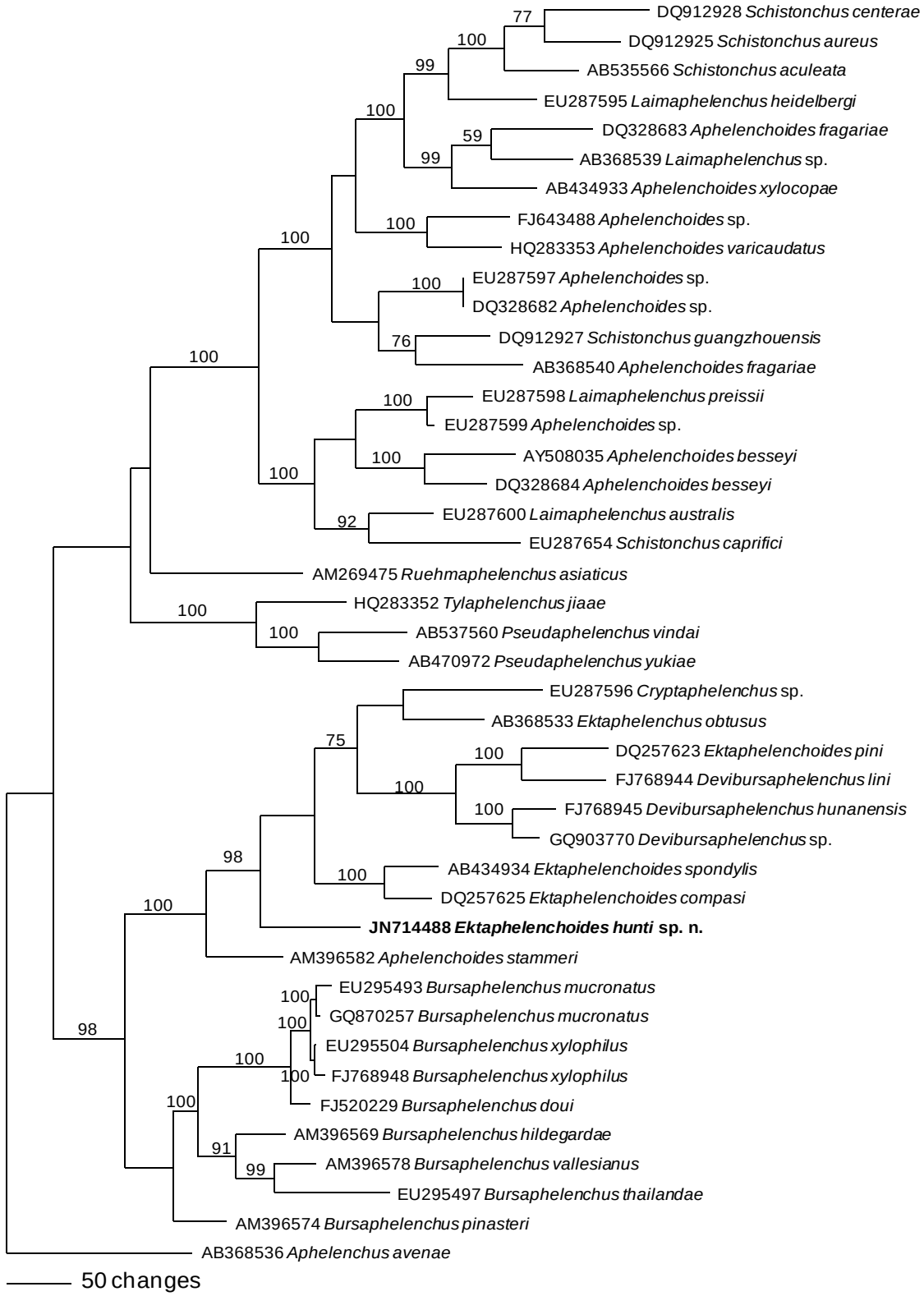

Fig. 3. *Ektaphelenchoides hunti* sp. n. The 10001st Bayesian tree inferred from LSU D2/D3 under GTR+I+G model (lnL=16806.2656; freqA=0.1871; freqC=0.1759; freqG=0.3191; freqT=0.3179; R(a)=1.287; R(b)=3.2662; R(c)=1.4121; R(d)=0.8576; R(e)=4.1178; R(f)=1; Pinvar=0.1661; Shape=0.9103). Posterior probability values exceeding 50% are given on appropriate clades.

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M. R. Atighi, E. Pourjam, M. Pedram, Weimin Ye, R. T. Robbins. Молекулярная и морфологическая характеристика *Ektaphelenchoides hunti* sp. n. (Nematoda: Ektaphelenchinae) из северного Ирана.

Резюме. Дано молекулярное и морфологическое описание *Ektaphelenchoides hunti* sp. n. Новый вид характеризуется длиной тела 711-929 мкм у самок, обособленным головным концом, тремя инцизурами латерального поля, общей длиной стилета 16-23 мкм, экскреторной порой на уровне середины метакорпуса, гемизонидом на расстоянии 95-116 мкм от головного конца, длиной тела самцов 572-809 мкм и спикулами длиной 12,5-17,5 мкм с округленным кондилюсом, умеренно развитым коротким рострумом с притупленым концом и без кукулюса. Новый вид близок к *E. compsi*, но отличается наличием трех четких инцизур латерального поля (в отличие от плохо различимых), экскреторной порой на уровне середины метакорпуса (в отличие от ее положения сзади от основания метакорпуса у *E. compsi*) – т.е. в 75-93 мкм от головного конца, а не сзади от медианного бульбуса в 91-113 мкм от головного конца, более короткими спикулами (12,5-17,5 *vs* 19-24 мкм) и явными отличиями в форме задней части тела (хвостового конца). Обсуждаются отличия от других видов рода *Ektaphelenchoides*. Основанный на частичной последовательности D2D3-участка рибосомальной ДНК анализ показал, что *E. hunti* sp. n. отличается от ближайших видов *E. compsi* и *E. spondylis.*