# Molecular and morphological characterisation of *Ektaphelenchoides kelardashtensis* sp. n. (Nematoda: Ektaphelenchinae) from northern Iran

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**Summary.** *Ektaphelenchoides kelardashtensis* sp. n. recovered from bark samples of an unidentified tree in Mazandaran province is described and illustrated based on morphological and molecular characters. The new species is characterised by its body length of 433-577 µm in the females, a slightly off-set head, no apparent lateral field, total stylet length of 13-16 µm, excretory pore at 55-66 µm and hemizonid at 67-78 µm from anterior end, and rare males. The new species comes close in morphology and morphometrics to three known species of the genus, namely *E. attenuata, E. musae* and *E. sylvestris* mostly by having a long and filiform tail (posterior body). Based on molecular data (the results of the phylogenetic comparisons), it shows more similarity to *E. hunti*. Compared with *E. attenuata*, the new species has shorter body, stylet and post-uterine sac and differences in the shape of the male tail and spicules. Compared with *E. musae*, the new species can be separated by its shorter body, stylet and post-uterine sac, greater index a, more anteriorly located excretory pore and hemizonid and the presence of males. Compared with *E. sylvestris*, the new species has a shorter stylet, greater index a, more anteriorly located vulva, hemizonid and excretory pore, difference in shape of posterior body and the presence of males. Compared with *E. hunti*, it has a shorter body, stylet and post-uterine sac, more anteriorly located excretory pore and hemizonid and a different shape of the posterior end. Molecular analyses were performed based on 743 bp partial ribosomal DNA large subunit D2-D3 and showed *E. kelardashtensi*s sp. n. to be unique, but closest to *E. hunti*.

**Key words:** *Ektaphelenchoides attenuata*, *E. hunti, E. musae, E. sylvestris*, Iran, Kelardasht, molecular phylogeny, new species, ribosomal DNA LSU, taxonomy.

The genus *Ektaphelenchoides* was erected by Baujard, 1984 and contains eight valid species, 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, *E. spondylis* Kanzaki, Giblin-Davis & Center, 2009, *E. hunti* Atighi, Pourjam, Pedram, Ye & Robbins, 2012 and *E. sylvestris* Pedram, Pourjam, Atighi, Ye & Houshmand, 2012. During nematode surveys in the northern forests of Iran, a new species of *Ektaphelenchoides* was recovered from bark samples of an unidentified tree with bark beetle galleries and is described herein as *E. kelardashtensis* sp. n. This is the third species of *Ektaphelenchoides* described from Iran along with *E. hunti* and *E. sylvestris*. A fourth species, *E. compsi*, has previously reported from Iran (Rafiee *et al.*, 2011).

## MATERIALS AND METHODS

Several bark samples were collected from northern forests of Iran during May to September 2010. To obtain a cleaner suspension of nematodes, the tray method (Whitehead & Hemming, 1965) was used for the nematode extraction. Nematodes of interest were handpicked under a Nikon SMZ1000 stereomicroscope. The nematodes 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).

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

| Character | Holotype female | Paratype females | Paratype males |
|---|---|---|---|
| n | - | 20 | 2 |
| L | 468 | 524.0 ± 37.6 (433-577) | 391, 420 |
| a | 33.4 | 38.9 ± 2.5 (34.7-44.4) | 35.5, 32.3 |
| b | 9.2 | 9.4 ± 0.8 (8.0-11.2) | 7.1,7.0 |
| b' | 3.4 | 3.7 ± 0.2 (3.3-4.0) | 3.2, 3.0 |
| c | - | - | 8.3, 9.1 |
| c' | - | - | 5.5, 4.6 |
| V or T | 64.7 | 64.2 ± 1.7 (61.5-68.0) | 27.6, 58.8 |
| Head height | 2.0 | 2.1 ± 0.3 (1.5-2.5) | 2.0, 2.5 |
| Head width | 5.0 | 5.4 ± 0.4 (4.5-6.0) | 4.5, 5.0 |
| Stylet | 14 | 14.5 ± 1.1 (13-16) | 12.5, 15.0 |
| Stylet shaft | 8.0 | 8.7 ± 0.5 (8.0-9.5) | 7.0, 8.0 |
| Conus | 6.0 | 5.8 ± 0.7 (5.0-7.0) | 5.5, 7.0 |
| m[1] | 42.9 | 39.8 ± 2.4 (35.7-43.8) | 45, 47 |
| MB[2] | 84.3 | 84.5 ± 3.6 (79.3-94.0) | 83.6, 86.7 |
| Body width of MB | 10.5 | 10.5 ± 0.6 (8.5-11.0) | 8.5, 10.0 |
| Nerve ring from anterior body | 58 | 61.1 ± 2.6 (54-65) | 61, 64 |
| Median bulb width | 7.5 | 8.1 ± 0.4 (7.5-8.5) | 6.5, 8.5 |
| Median bulb length | 13 | 13.1 ± 0.5 (12.5-14.0) | 10, 11 |
| Median bulb length/diam. ratio | 1.7 | 1.6 ± 0.1 (1.5-1.8) | 1.5, 1.3 |
| Excretory pore | 55 | 59.7 ± 3.2 (55-66) | 58,75 |
| Hemizonid | 65 | 71.5 ± 3.2 (67-78) | 69, 81 |
| Oesophagus | 51 | 55.7 ± 2.9 (50-62) | 55, 60 |
| Overlapping[3] | 86 | 87.7 ± 7.4 (78-114) | 69, 82 |
| Testis or ovary length | 135 | 189.0 ± 29.2 (136-238) | 108, 247 |
| Anal (cloacal) body width | - | - | 8.5, 10.0 |
| Tail | | - | 47, 46 |
| Spicule length (arc line) | - | - | 8, 10 |
| Capitulum | | - | 5, 6 |

[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

Permanent slides were made and examined using a Nikon Eclipse E600 light microscope. Photographs were taken using an Olympus DP72 digital camera attached to an Olympus BX51 microscope 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 hand-picked and transferred to 10 µl AE buffer (10 mM Tris-Cl, 0.5 mM EDTA; pH = 9.0) on a glass microscope slide, macerated with a pipette tip and collected in 50 µl AE buffer. DNA samples were stored at –20°C until used as a PCR template. Primers for LSU D2-D3 amplification were forward primer D2a (5'-ACA-AGT-ACC-GTG-AGG-GAA-AGT-TG-3') and reverse primer D3b (5'-TGC-GAA-GGA-ACC-AGC-TAC-TA-3') (Nunn, 1992). The 25 µl PCR was performed using Apex Taq Red Master Mix DNA polymerase (Genesee Scientific Corporation, San Diego, CA, USA) according to the manufacturer's protocol. The thermal cycler program for PCR was as follows: denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 45 s, and extension at 72°C for 2 min. A final extension was performed at 72°C for 10 min (Ye *et al*., 2007). PCR products were cleaned using ExoSap-IT (Affymetrix, Inc., Santa Clara, CA, USA) according to the manufacturer's protocol and were sequenced by Genomic Sciences Laboratory, North Carolina State University using a sequencing system (Applied Biosystems 3730 XL DNA Analyzer). DNA sequence was deposited in GenBank under the accession number JQ446374 and was compared with other nematode species in

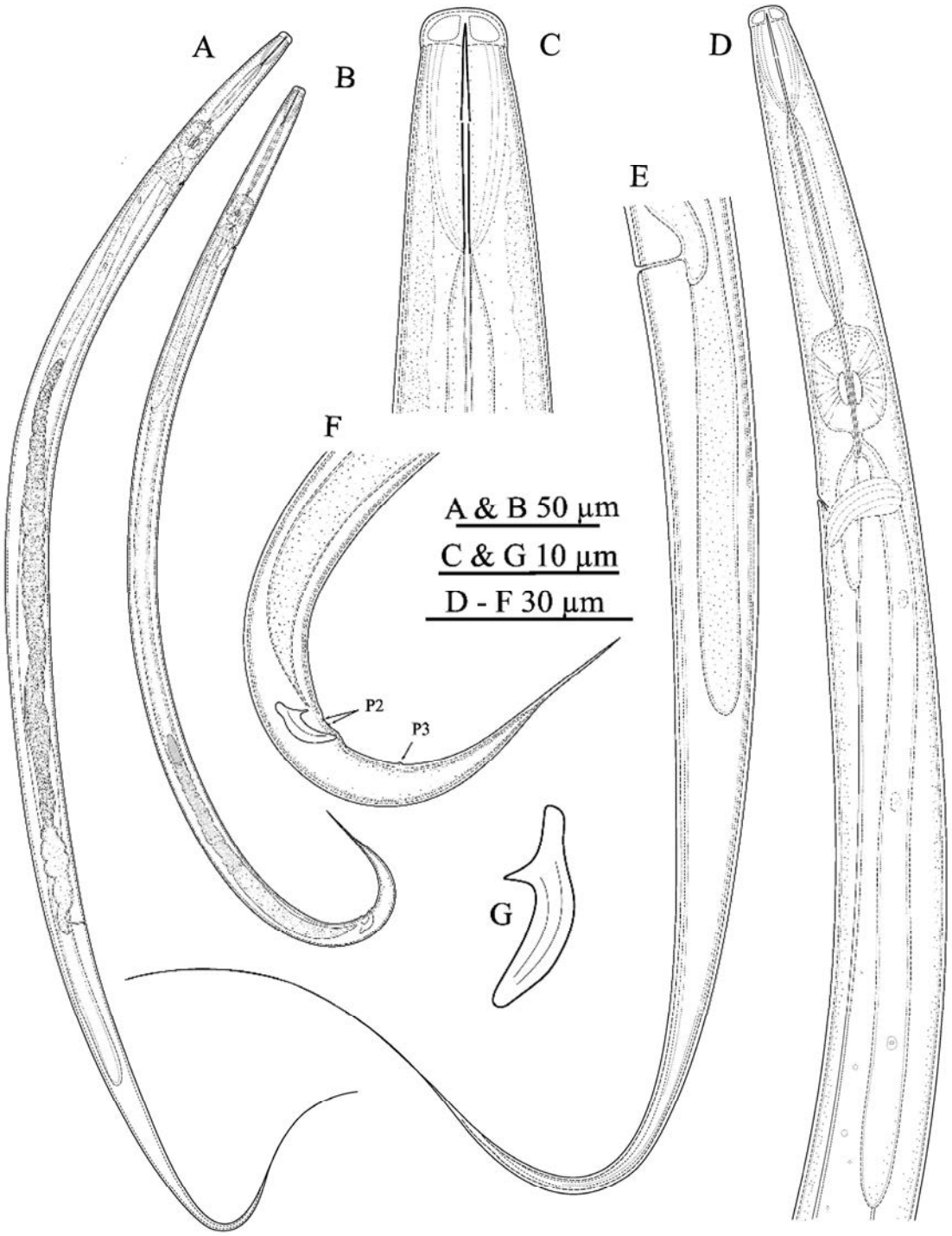


**Fig. 1.** *Ektaphelenchoides kelardashtensis* sp. n. A & B: Entire body of female and male; C: Anterior end of female; D: Pharyngeal region; E: Posterior end of female; F: Posterior end of male; G: Spicules.

GenBank using the BLAST homology search program. The closest sequences were selected for phylogenetic analysis. DNA sequences were aligned by Clustal W (http://workbench.sdsc.edu, Bioinformatics and Computational Biology group, Dept Bioengineering, UC San Diego, CA). 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 using MrBayes 3.1.0 (Huelsenbeck & Ronquist, 2001) running the chain for $1 \times 10^6$ generations and setting the "burn in" at 1,000. MCMC (Markov Chain Monte Carlo) methods was used within a Bayesian framework to estimate the posterior probabilities of the phylogenetic trees (Larget & Simon, 1999) using the 50% majority-rule.

## DESCRIPTION

### *Ektaphelenchoides kelardashtensis* sp. n.
### Figs 1 & 2

**Measurements:** See table 1.

**Female.** Body slightly curved ventrally after heat relaxation. Cuticle finely annulated. Lateral field not observed. Lip region slightly off-set, separated from the rest of the body without a distinct constriction. Stylet without basal knobs, its conus occupying 35.7-43.8% of total stylet length. Procorpus cylindrical, 2.6-3.3 times longer than stylet length, connected to an elongate, rectangular metacorpus, its anterior granular part occupying 25-30% of its total length and the posterior part weakly muscular. Pharyngeal gland lobe dorsally overlapping intestine 1.3-1.6 times the distance from anterior end to the base of median bulb or 7.3-10.8 times body width at median bulb level. Nerve ring surrounding pharyngeal glands and intestine at *ca* 0.5 stylet length posterior to the base of metacorpus. Excretory pore less than one stylet length posterior to base of metacorpus. Hemizonid 7-12 µm posterior to excretory pore position. Vulva at 276-373 µm from anterior end. Reproductive system prodelphic, gonad outstretched, occupying 30-40% of the body length. Oocytes mostly in single file. Spermatheca irregular in shape. Vagina not sclerotised, straight. Post-uterine sac very short, less than half body width at vulva region. Intestine ends in a blind sac. Anus and rectum indistinct. Distance between vulva and body end 12.6-17.4 times body width at vulva region, posterior body end (tail) filiform.

**Male.** Rare. Body slender, arcuate J-shaped after heat relaxation, the posterior end more ventrally bent. Anterior region similar to that in female. Testis single, expanded anteriorly. Spicules arcuate, separate, *ca* 2.2 times longer than capitulum width, lamina/calomus complex smoothly and symmetrically curved, condylus well-developed with rounded tip, rostrum pointed and without cucullus. The midventral precloacal papilla (P1) and the fourth subventral pair of papillae (P4) not observed. The second pair of subventral papillae (P2) just posterior to cloacal aperture (3 µm distance from cloacal opening) and the third pair (P3) at *ca* 34% of tail length posterior to cloacal aperture. Tail conoid with sharply pointed terminus.

**Molecular Characterisation and Phylogeny.** For molecular analysis, the 743 bp ribosomal DNA large subunit (LSU) D2-D3 of *E. kelardashtensis* sp. n. was sequenced. A BlastN search of LSU sequence revealed its sequence is unique and the closest matches are species in *Ektaphelenchoides*, *Devibursaphelenchus* and *Cryptaphelenchus*. Figure 3 presents a phylogenetic tree based on the LSU of many representative aphelenchoidids from a multiple alignment of 823 total characters, in which 214 characters (26%) were constant. The average nucleotide composition was as follows: 17.92% A, 17.61% C, 32.73% G and 31.74% T. Using *Aphelenchus avenae* Bastian, 1865 as the out-group, this tree inferred many highly supported monophyletic groups. The new species, *E. kelardashtensis* sp. n., is in a 98%-supported clade which also includes *E. hunti*, *E. pini*, *Ektaphelenchus obtusus* Massey, 1956, *Ektaphelenchoides compsi*, *E. spondylis*, *Devibursaphelenchus* sp., *D. hunanensis* Yin, Fang & Tarjan, 1988, *D. lini* Braasch, 2004 and *Cryptaphelenchus* sp. The five species of sequenced *Ektaphelenchoides* were not monophyletic and *E. kelardashtensis* is closest to *E. hunti* with 92% support.

**Differential diagnosis and relationships**: The new species is characterised by its body length of 433-577 µm in the females, obscure lateral field, stylet 13-16 µm long, excretory pore at 55-66 µm and hemizonid at 67-78 µm distance from anterior end, respectively. The new species comes close in morphology and morphometrics to three known species of the genus, namely *E. attenuata*, *E. musae* and *E. sylvestris*, mostly by having a long and filiform tail (posterior body). Based on molecular data (the results of the phylogenetic comparisons), it shows more similarity to *E. hunti.*

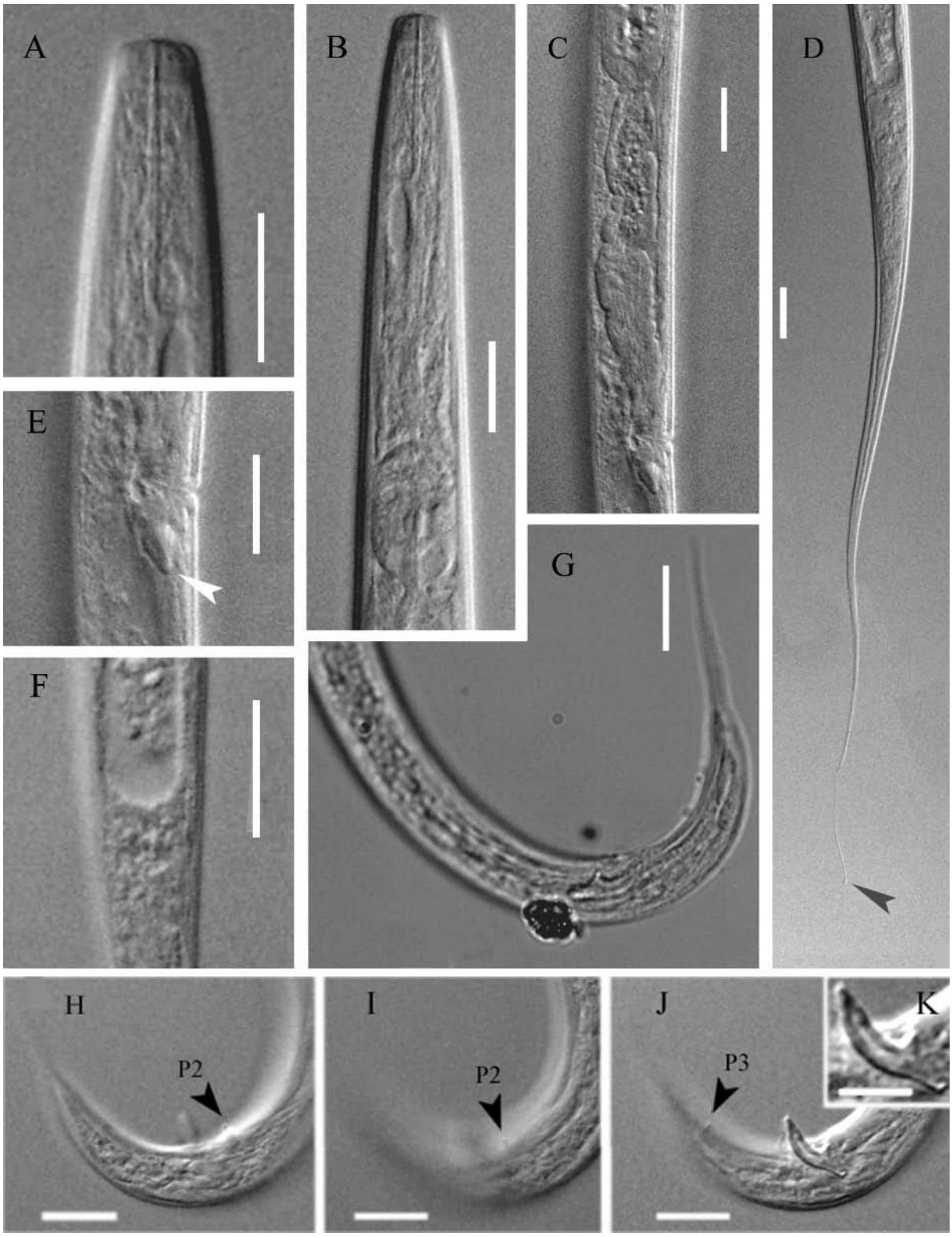


**Fig. 2.** *Ektaphelenchoides kelardashtensis* sp. n. A & B: Anterior end in detail; C: Part of female reproductive system; D: Posterior end of female showing tail end (arrow); E: Post-uterine sac; F: Blind end of intestine; G: Male posterior end; H-J: Second and third pair of male papillae (H and I: P2, J: P3); K: Spicule morphology in detail (All scale bars = 10 µm, except K = 5 µm).

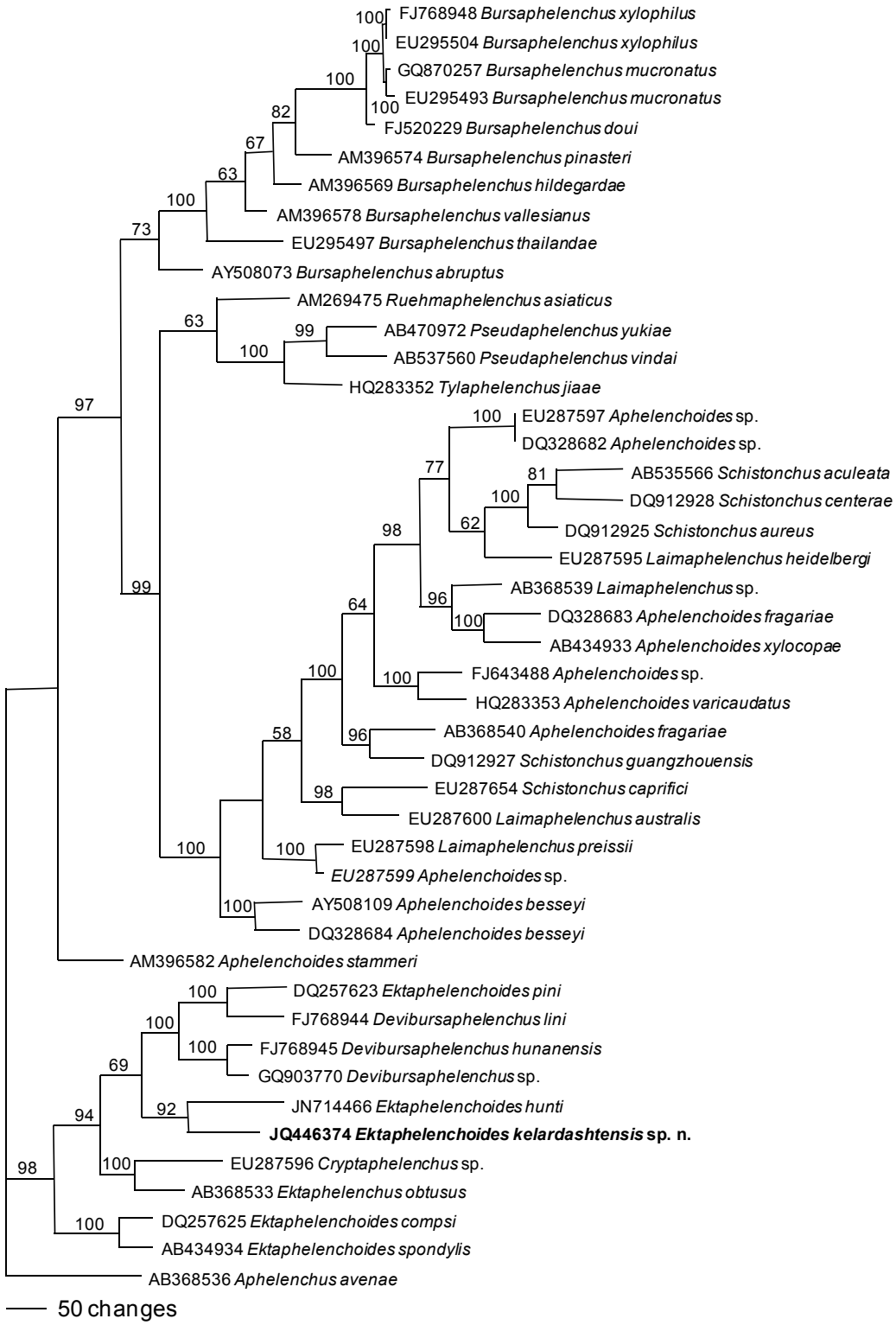


**Fig. 3.** Bayesian 10001st tree inferred from the rDNA 28S D2/D3 under GTR+I+G model (lnL= –18125.4434; AIC = 36270.8867; freqA = 0.1792; freqC = 0.1761; freqG = 0.3273; freqT = 0.3174; R(a) = 1.0525; R(b) = 3.4288; R(c) = 1.1684; R(d) = 0.6526; R(e) = 4.4772; R(f) = 1; Pinvar = 0.1876; Shape = 0.8104). Posterior probability values exceeding 50% are given on appropriate clades.

Compared with *E. attenuata*, the new species has a shorter stylet (13-16 *vs* 20 µm), shorter body (433-577 *vs* 950 µm), slightly *vs* clearly set off lip region, shorter post-uterine sac *ca* 0.2-0.5 body width *vs* 1.5, spicules with pointed rostrum *vs* rounded and male tail sharply pointed *vs* filiform.

Compared to *E. musae*, the new species has shorter stylet (13-16 *vs* 18.5-22.0 µm), shorter body (433-577 *vs* 500-710 µm), greater a index (35-44.5 *vs* 28-33), shorter post-uterine sac (3-6 *vs* 9-19 µm), more anteriorly located excretory pore and hemizonid (55-66 *vs* 60-74 µm and 67-78 *vs* 78-89 µm, respectively).

Compared with *E. sylvestris*, the new species has shorter stylet (13-16 *vs* 18-23 µm), greater a index (35-44.5 *vs* 25-32), more anteriorly located vulva (V = 61.4-68.5 *vs* 75.0-76.5), more anteriorly located hemizonid (67-78 *vs* 94-111 µm) and excretory pore (55-66 *vs* 72-85 µm) and difference in shape of posterior body (filiform *vs* conical).

Compared with *E. hunti*, it has a shorter body (433-577 *vs* 711-929 µm), shorter stylet (13-16 *vs* 16-23 µm), more anteriorly located excretory pore (55-66 *vs* 75-93 µm) and hemizonid (67-78 *vs* 85-112 µm), difference in shape of posterior body (filiform and straight *vs* conical and posteriorly bent) and shorter post-uterine sac (0.2-0.5 *vs* 1.5-4.0 times body width).

**Type habitat and locality:** Recovered from bark samples of an unidentified tree with bark beetle galleries, in Kelardasht forests, Mazandaran province, northern Iran.

**Type material:** Holotype female, five paratype females and one male deposited at Nematode Collection at the Faculty of Agriculture, Tarbiat Modares University, Tehran, Iran. Three female 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 species epithet refers to the name of the region (Kelardasht forests) from where the new species was recovered.

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

**Резюме.** Представители нового вида *Ektaphelenchoides kelardashtensis* sp. n. были извлечены из коры дерева не определенного вида в провинции Мазандаран. Приводятся морфологические и молекулярно-таксономические данные по новому виду. Новый вид характеризуется длиной тела самок в пределах 433-577 мкм, слегка обособленным перетяжкой головным концом, неразличимыми латеральными полями, общей длиной стилета 13-16 мкм, экскреторной порой и гемизонидом, отстоящими от переднего конца тела на 55-66 мкм и 67-78 мкм, соответственно. Самцы редки. Новый вид близок по морфологии и морфометрии к трем известным видам этого рода: *E. attenuata, E. musae* и *E. sylvestris*, в основном из-за длинного нитевидного хвоста (задней части тела) всех четырех видов. По результатам филогенетического анализа, новый вид обнаруживает больше сходства с *E. hunti*. От *E. attenuata* новый вид отличается меньшей длиной тела, стилета и поствульварного мешка (рудимента задней матки), а также несколько иным строением хвостового конца и спикул самца. От *E. musae* новый вид отличается меньшей длиной тела, стилета и поствульварного мешка, более высоким значением индекса ‘a’, расположенными ближе к головному концу экскреторной порой и гемизонидом, а также наличием самцов. В отличие от *E. sylvestris*, новый вид имеет более короткий стилет, большее значение индекса ‘a’ и расположенные ближе к головному концу вульву, экскреторную пору и гемизонид, иную форму задней части тела и присутствие самцов в популяции. По сравнению с *E. hunti* новый вид имеет меньшую длину тела, стилета и поствульварного мешка, расположенные ближе к головному концу экскреторную пору и гемизонид и иную форму задней части тела. Нуклеотидная последовательность D2-D3 сегмента рибосомального гена большой субъединицы рибосомы нового вида (длина 743 bp) уникальна, но показывает признаки родства с *E. hunti*.