Molecular and morphological characterisation of *Ektaphelenchoides ruehmi* sp. n. (Nematoda: Ektaphelenchinae) from southwestern Iran

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Summary. *Ektaphelenchoides ruehmi* sp. n. is described and illustrated based on morphological, morphometic and molecular characters. The new species is characterised by its body length of 355-487 µm in females, a slightly off-set head, lateral field with three lines, total stylet length of 9.0-12.5 µm, excretory pore at 55-70 µm and hemizonid at 59-76 µm distance from anterior end, shape of the tail in females and males and arrangement of papillae in male. The new species comes close in morphology and morphometrics to five known species of the genus, namely *E. attenuata*, *E. musae*, *E. sylvestris*, *E. kelardashtensis* and *E. pini*, mostly by having a long and filiform tail (posterior body). Based on molecular data (the results of the phylogenetic comparisons using sequences of 28S rDNA D2D3 expansion segment), the new species shows more similarity to *E. kelardashtensis* (80.8% similarity in overlapping part of partial sequences of D2D3). Compared with *E. attenuata*, the new species has a shorter body, shorter stylet, lower a and b values, shorter post-uterine sac, more posterior position of vulva, differences in shape of the tail and spicules of male. Compared with *E. musae*, the new species has shorter body, shorter stylet and post-uterine sac, lower b value and presence of male. Compared with *E. sylvestris*, the new species has a shorter stylet, lower a and b values, more anteriorly located vulva, greater distance between vulva-posterior body end and presence of male. Compared with *E. kelardashtensis*, it has a shorter stylet, lower b value and MB, greater value of vulva-posterior body end to body width in vulva ratio, lateral field with three incisures, midventral precloacal papilla (P1), difference in position of P2 and shape of tail and spicules in male. Compared with *E. pini*, the new species has a shorter body and stylet, lower b value and midventral precloacal papilla (P1) and the second pair of subventral papillae (P2). Molecular analyses were performed based on 757 bp partial sequences of 28S rDNA D2D3 segment and showed *E. ruehmi* sp. n. to be unique, but closest to *E. kelardashtensis*. Both species formed a clade with 0.98 Bayesian posterior probability in Bayesian inference and 87% bootstrap value in the Maximum Likelihood method.

Key words: 28S rDNA D2D3, *Ektaphelenchoides*, molecular phylogeny, new species, Khouzestan province, Iran.

The genus *Ektaphelenchoides* Baujard, 1984 was erected by Baujard (1984) and currently contains 11 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, *E. sylvestris* Pedram, Pourjam, Atighi, Ye & Houshmand, 2012, *E. andrassyi* Atighi, Pourjam, Pedram, Ye & Aliramaji, 2013, *E. kelardashtensis* Atighi, Pourjam, Pedram, Ye, Robbins & Namjou, 2013 and *E. poinari* Aliramaji, Pourjam, Atighi, Ye, Roshan-Bakhsh & Pedram, 2014. A review on related hosts and locality of the above mentioned taxa is given in Atighi *et al*. (2012) and Aliramaji *et al*. (2014). Currently, *E. sylvestris* is successfully reared in a co-culture with rhabditid and aphelenchid nematodes and recently the predatory behaviour of *E. spondylis* was observed on *Pseudodiplogasteroides* sp. (Kanzaki, 2014).

During our surveys on plant-parasitic and free-living nematodes in southern Iran, a new species of *Ektaphelenchoides* was recovered from soil samples collected about the rhizosphere of an unidentified weed and is described herein as *E. ruehmi* sp. n. This is the sixth species of the genus described originally from Iran along with *E. hunti*, *E.*

sylvestris, *E. andrassyi*, *E. klardashtensis* and *E. poinari*. The seventh species, *E. compsi* has previously been reported from Iran (Rafiee *et al.*, 2011).

MATERIALS AND METHODS

Several soil samples were collected from southwestern Iran during 2012-2013. To obtain a cleaner suspension of nematodes, the tray method (Whitehead & Hemming, 1965) was used to extract them. The nematodes were heat killed by adding boiling 4% formalin solution and then transferred to anhydrous glycerin and mounted on permanent slides according to De Grisse (1969). Permanent slides were 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 a small drop of AE buffer (10 mM Tris-Cl, 0.5 mM EDTA; pH 9.0) on a clean slide and squashed using a clean slide cover. The suspension was collected by adding 15 µl AE buffer. DNA samples were stored at -20°C until used as a PCR template. Primers for LSU D2D3 amplification were forward primer D2A (5'-ACAAGTACCGTGAGGGAAAGT-3') and reverse primer D3B (5'-TGCGAAGGAACCAGCTACTA-3') (Nunn, 1992). PCR was carried out in a total volume of 20 µl (13.3 µl distilled water, 2 µl 10× PCR buffer, 0.4 µl dNTP mixture, 0.8 µl 50 mM MgCl2, 1 µl of each primer (10 pmol μl^{-1}), 0.5 µl of Taq polymerase (CinnaGen, Tehran, Iran, *ca* 5 U μl^{-1}), and 1 µl of DNA template). The thermal cycling program was as follows: denaturation at 95°C for 4 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min. A final extension was performed at 72°C for 10 min. PCR product was purified and sequenced directly for both strands using the same primers with an ABI 3730XL sequencer (Bioneer Corporation, South Korea). The newly obtained sequence was submitted to GenBank database under accession number KF509853.

The sequences of D2D3 expansion segments of 28S ribosomal RNA gene (D2D3 LSU) of the new species was compared with those of other nematode species available in GenBank using the BLAST homology search program. The selected sequences were aligned using the ClustalX2 (http://www.clustal.org/) software. The model of base substitution was evaluated using MrModeltest 2 (Nylander, 2004). The Akaike-supported model, a general time reversible model, including among-site rate heterogeneity and estimates of invariant sites (GTR+G+I) was used in phylogenetic analyses. Bayesian analysis was performed to reconstruct phylogenetic tree using MrBayes v 3.1.2 (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003) running the chain for one million generations. After discarding burn-in samples and evaluating convergence, the remaining samples were retained for further analyses. The Markov Chain Monte Carlo (MCMC) method within a Bayesian framework was used to determine equilibrium distribution and help to estimate the posterior probabilities of the phylogenetic trees (Larget & Simon, 1999) using the 50% majority rule. An ML tree was constructed by using RaxmlGUI 1.1 (Silvestro & Michalak, 2011) software using the same nucleotide substitution model as in the BI in 1000 bootstrap replicates. For the both phylogenetic analyses methods (BI and ML), *Poikilolaimus piniperdae* Fuchs, 1930 (DQ059060) was used as the outgroup.

DESCRIPTION

Ektaphelenchoides ruehmi sp. n.

(Figs 1 & 2)

Measurements. Table 1.

Female. Body slightly curved ventrally after heat relaxation. Cuticle finely annulated, annules *ca* 1.0 µm wide. Lateral field with three incisures. Lip region slightly off-set, separated from the rest of body without a distinct constriction. Three lips in lateral view equal in size. Stylet without basal swellings, its conus occupying 40-50% of total stylet length. Procorpus cylindrical, 4.5-6.6 times longer than stylet length, connected to an elongate, rectangular to oval metacorpus, its anterior granular part occupying 28-46% of the total length and the posterior part weakly muscular. Pharyngeal gland lobe dorsally overlapping intestine 0.8-1.3 times the distance from anterior end to the base of median bulb or 4.5-6.7 times body width at median bulb level. Nerve ring surrounding pharynx slightly anterior to excretory pore position. Excretory pore at 55-70 µm distance from anterior end. Hemizonid 3-13 µm posterior to excretory pore. Ovary single, outstretched, oocytes mostly in single file. Spermatheca oval to irregular in shape, having spheroid sperm cells, vaginal perpendicular to body axis, not sclerotised. Post-uterine sac short, less than or equal to half body width at vulva region. Vulva at 258-341 µm from anterior end. Intestine ends in a

blind sac. Anus and rectum indistinct. Distance between vulva and body end 8.8-11.8 times of body width at vulva region. Postvulval body part tapering gradually to a filiform tail tip.

Male. Body slender, J-shaped after heat relaxation, the posterior end more ventrally bent. Anterior region similar to that of female. Testis single, expanded anteriorly. Spicules arcuate, separate, *ca* 2.9 times longer than capitulum width, lamina/calomus complex smoothly and symmetrically curved, condylus well-developed with rounded tip, rostrum pointed, cucullus absent. The midventral precloacal papilla (P1) 9.5 µm anterior to cloacal opening, the second pair of subventral papillae (P2) just posterior to cloacal aperture (1.8 µm distance from cloacal opening) and the third pair (P3) at *ca* 34% of tail length posterior to cloacal aperture, the fourth subventral pair of papillae (P4) absent. Tail conoid, posterior part spicate.

Table1. Morphometrics of *Ektaphelenchoides ruehmi* sp. n. All measurements are in µm and in the form: mean ±s.d. (range).

Characters	*Ektaphelenchoides ruehmi* sp. n.		
	Holotype	Paratype	
	Female	Female	Male
n	–	19.0	2.0
L	411	424.9 ± 39.7 (355-487)	348.5 ± 9.2 (342-355)
a	34.3	34.5 ± 1.9 (31.3-38.0)	33.2 ± 1.4 (32.3-34.2)
b	6.2	6.4 ± 0.4 (5.3-7.0)	5.3 ± 0.2 (5.2-5.5)
b'	3.5	3.4 ± 0.2 (3.0-3.8)	7.7 ± 1.4 (6.7-8.7)
V or T	72.5	69.8 ± 1.9 (67.3-73.3)	–
c	–	–	10.2 ± 1.3 (9.2-11.1)
c'	–	–	4.1 ± 0.8 (3.6-4.6)
Stylet	11	11.0 ± 0.9 (9.0-12.5)	10.0 ± 0.7 (9.5-10.5)
Conus	4.5	5.0 ± 0.6 (4-6)	4.5 ± 0.7 (4-5)
m	41	45.6 ± 2.4 (40.9-50.0)	44.9 ± 3.9 (42.1-47.6)
Anterior end to centre of center of median bulb	47	45.8 ± 4.2 (39-52)	43.5 ± 3.5 (41-46)
MB	71.2	69.5 ± 4.7 (52.2-76.1)	66.4 ± 4.7 (63.1-69.7)
Excretory Pore	64	64.6 ± 5.1 (55-73)	67.0 ± 4.2 (64-70)
Hemizonid	69	69.4 ± 6.2 (59-75)	58.0 ± 9.9 (51-65)
Pharynx	66	66.3 ± 8.5 (57-92)	65.5 ± 0.7 (65-66)
Overlapping	53	59.3 ± 7.5 (56-73)	46.0 ± 7.1 (41-51)
Head-Vulva	298	296.7 ± 28.8 (248-341)	–
Body Width(BW)	12	12.3 ± 1.2 (10-14)	10.5 ± 0.7 (10-11)
PUS	6	4.7 ± 0.9 (3-7)	–
PUS/body width at vulva	0.5	0.4 ± 0.1 (0.3-0.5)	–
Median bulb Length	15	13.2 ± 1.1 (12-15)	12.5 ± 1.4 (11.5-13.5)
Median bulb Width	0.8	7.6 ± 0.6 (7-9)	7.0 ± 0.0 (7-7)
BW in Median bulb	11	10.8 ± 0.5 (10-12)	9.8 ± 0.4 (9.5-10.0)
Vulva-Body end	113	128.2 ± 14.6 (97-151)	–
Anal Body Width	–	–	8.5 ± 0.7 (8-9)
Tail	–	–	34.5 ± 3.5 (32-37)
Spicules length (arc line)	–	–	13 ± 0.0 (13-13)

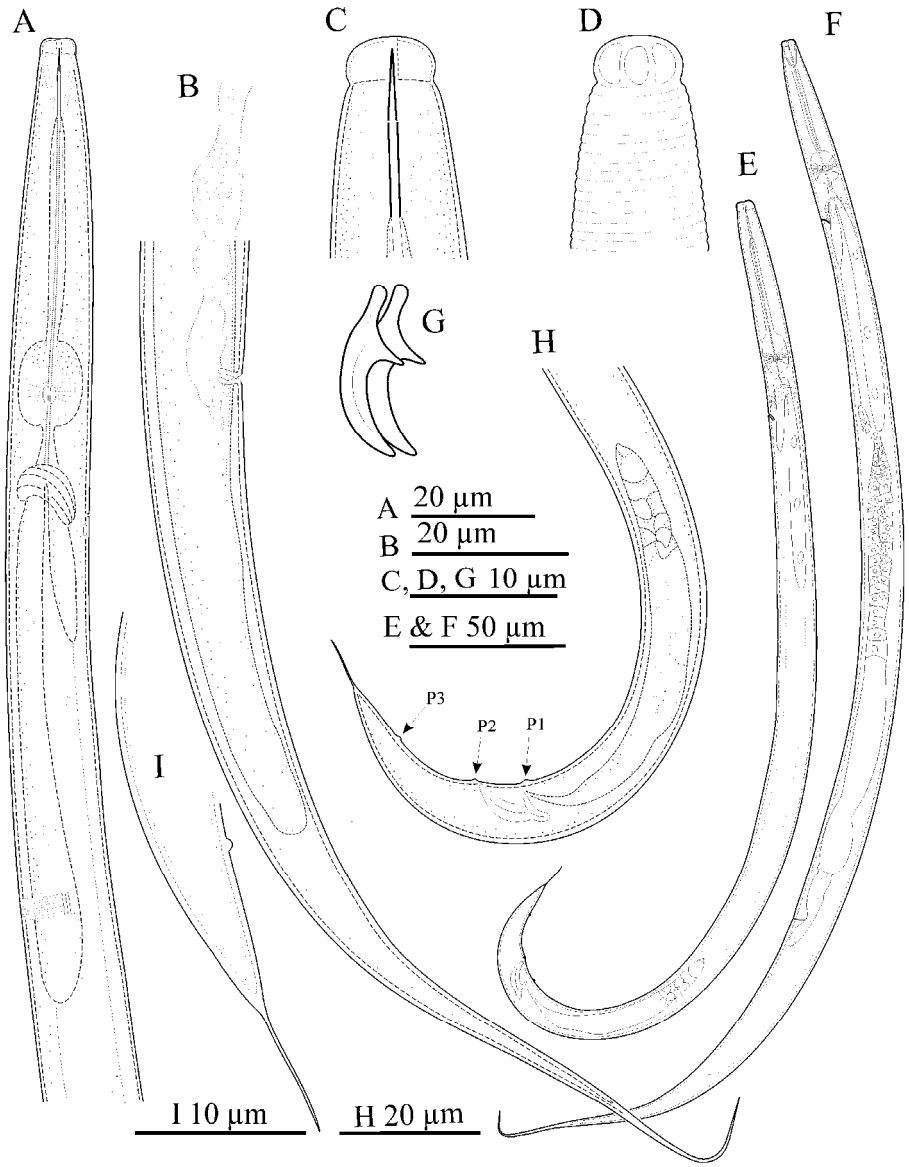

Fig. 1. Line drawing of *Ektaphelenchoides ruehmi* sp. n. A: Pharynx in detail; B: Female posterior body region and part of its reproductive system showing spermatheca having sperm cells; C: Anterior end and stylet; D: Lips and body annules; E & F: Entire body of male and female; G: Spicules; H: Male posterior end showing papillae; I: Male tail tip.

Molecular characterisation and phylogeny. Sequencing of D2D3 segment of 28S rRNA gene of the new species yielded 757 base pairs. A BlastN search using this sequence, revealed it as a unique sequence and the closest matches were species of genera *Ektaphelenchoides*, *Devibursaphelenchus* Kakulia, 1967 and *Cryptaphelenchus* Fuchs, 1937. Thirty-nine aphelenchid species were selected for reconstructing the phylogenetic tree using *Poikilolaimus piniperdae* as outgroup, yielding in 821 total characters from which 189 characters were conserved, 611 characters variable and 521 characters parsimony informative. The average nucleotide composition of this dataset was as follows: 22.9% A, 19.3% C, 30.5% G and 27.3% T. Figure 3 presents a phylogenetic tree inferred from BI. The BPP and ML BS values exceeding 50% are given for the appropriate clades in the shape BPP/ML BS. The new species, *E. ruehmi* sp. n. is in a highly supported clade with *E. kelardashtensis* in both BI and ML analyses. This could be explained with their close morphology too. All species of *Ektaphelenchoides* form a fully supported group with *Ektaphelenchus obtusus* Massey, 1956, *Devibursaphelenchus wangi* Gu, Wang & Zheng, 2010, *D. hunanensis* Yin, Fang & Tarjan, 1988, *D. lini* Braasch, 2004 and *Cryptaphelenchus* sp. in BI. This group was not supported in ML method. Based

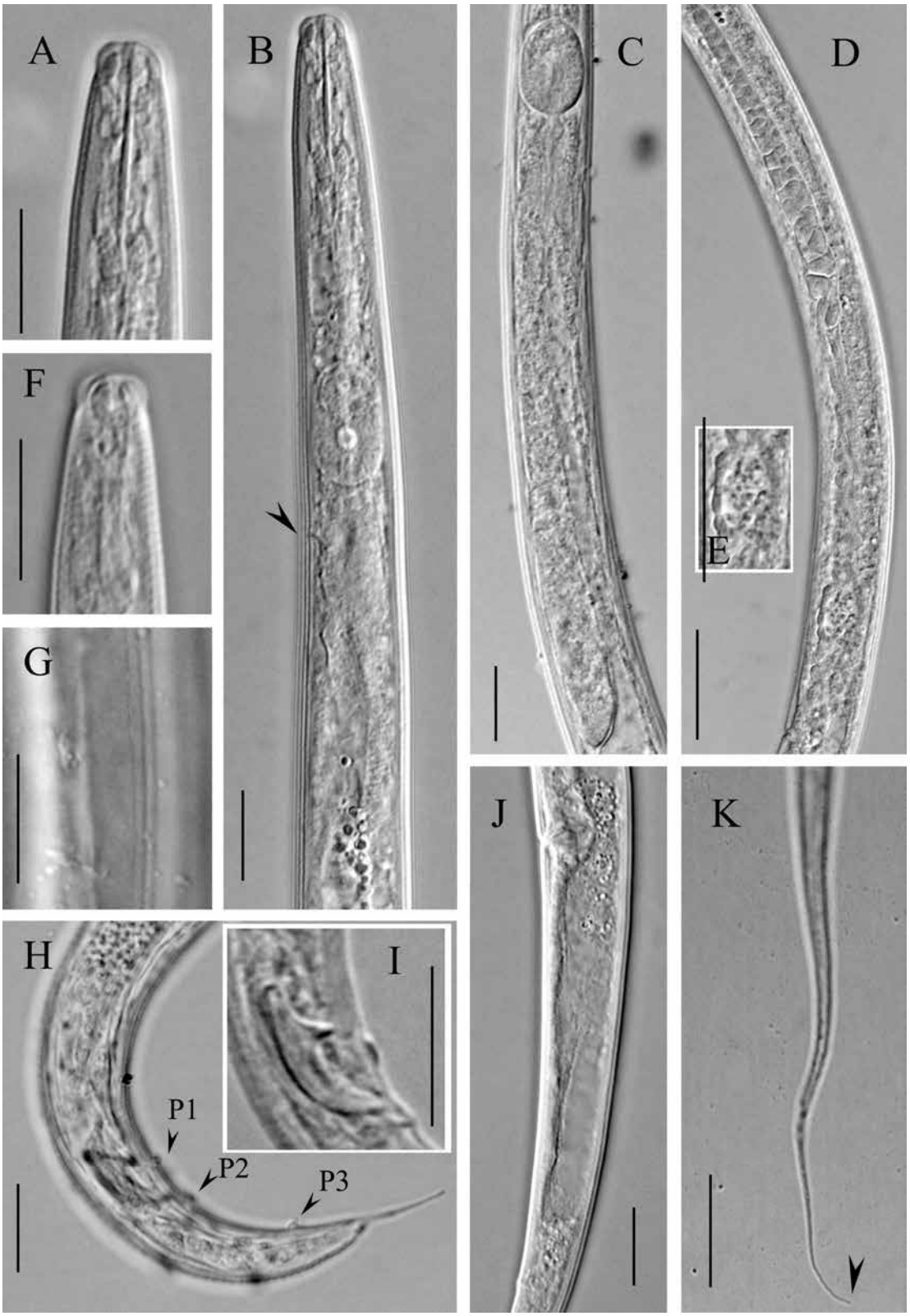

Fig. 2. Microphotographs of *Ektaphelenchoides ruehmi* sp. n. A: Anterior end and stylet; B: Pharynx and the position of excretory pore (arrow); C: Part of pharynx showing overlapping; D: Part of female genital tract; E: Spermatheca in detail, F: Lips and body annules, G: Three lateral lines; H: Male posterior end and papillae; I: Spicules in detail; J: Blind end of intestine (female); J: Female tail tip. (All scale bars = 10 μm).

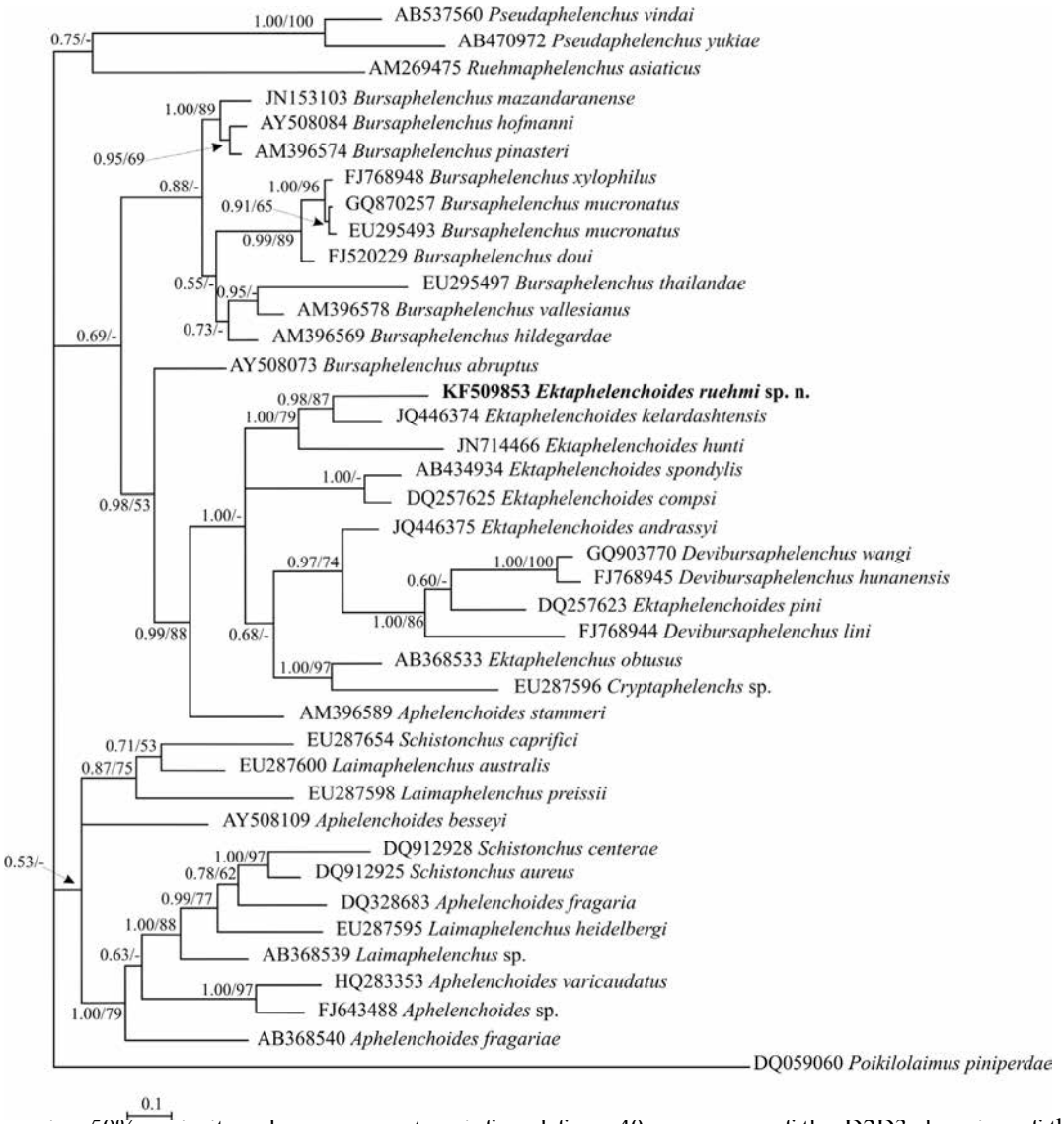

Fig. 3. Bayesian 50% majority rule consensus tree inferred from 40 sequences of the D2D3 domains of the 28S rDNA under the GTR+I+G model. BPP and ML BS values are given for each appropriate clade in the shape BPP/ML BS. The newly sequenced species of *Ektaphelenchoides* is in bold.

on the given phylogenetic tree, the six sequenced species of *Ektaphelenchoides* with the one another species presented in this study are not monophyletic.

Differential diagnosis and relationships. The new species is characterised by its body length of 355-487 µm in females, lateral field with three incisures, stylet 9.0-12.5 µm long, excretory pore at 55-70 µm and hemizonid at 59-76 µm distance from anterior end, post-uterine sac short, less than or equal to half body width at vulva region, distance between vulva and body end 8.8-11.8 times body width at vulva region, posterior body end (tail) filiform, having rare males in population characterised by arcuate spicules, *ca* 2.9 times longer than capitulum width with well-developed condylus having rounded tip, pointed rostrum, lacking cucullus, midventral precloacal papilla (P1) 9.5 µm anterior to cloacal opening, the second pair of subventral papillae (P2) just posterior to cloacal aperture, the third pair (P3) at *ca* 34% of tail length posterior to cloacal aperture and lacking fourth subventral pair of papillae (P4). The new species comes close in morphology and morphometrics to five known species of the genus namely *E. attenuata*, *E. musae*, *E. sylvestris*, *E. kelardashtensis* and *E. pini* mostly by having a long and filiform tail (posterior body). Based on molecular data (the results of the phylogenetic comparisons using sequences of 28S rDNA D2D3 expansion segment), it shows more similarity to *E. kelardashtensis*.

Compared with *E. attenuata*, the new species has a shorter stylet (9.0-12.5 *vs* 20 µm), shorter body

(355-487 *vs* 870-880 µm), lower a ratio (31-38 *vs* 34-42), lower b ratio (5.1-6.9 *vs* 12-13), more posterior position of vulva (V = 67.4-73.3 *vs* 61-63), slightly set off lip region *vs* clearly set off, shorter post-uterine sac *ca* 0.3-0.6 body width *vs* 1.5, spicules with pointed rostrum *vs* rounded and male tail spicate *vs* filiform.

Compared to *E. musae*, the new species has a shorter stylet (9.0-12.5 *vs* 18.5-22.0 µm), shorter body length (355-487 *vs* 500-710 µm), lower b ratio (5.1-6.9 *vs* 7.4-9.8) and shorter post-uterine sac (3-7 *vs* 9-19 µm).

Compared to *E. sylvestris*, the new species has a shorter stylet (9.0-12.5 *vs* 18-23 µm), shorter body length (355-487 *vs* 644-843 µm), greater a ratio (31-38 *vs* 25-32), lower b ratio (5.1-6.9 *vs* 8-11), anterior position of vulva (V = 67.4-73.3 *vs* 75.0-76.5) and shorter vulva-posterior body end distance (97-151 *vs* 153-204 µm).

Compared to *E. kelardashtensis*, the new species has a shorter stylet (9.0-12.5 *vs* 13-16 µm), lower b ratio (5.1-6.9 *vs* 8.0-11.2), more posterior position of vulva (V = 67.4-73.3 *vs* 61.5-68.0), more anterior position of median bulb (MB = 52.5-76.1 *vs* 79.3-94.0), lower ratio of vulva-posterior body end to body width in vulva region (8.8-11.8 *vs* 12.6-17.4), lateral line with three incisures *vs* obscure, position of P2 (just posterior to cloacal aperture *vs* just anterior to cloacal aperture) shape of tail (conoid with spicate terminus *vs* conoid with sharply pointed terminus) and shape of spicules.

Compared to *E. pini*, the new species has a shorter stylet (9.0-12.5 *vs* 23 µm), shorter body (355-487 *vs* 720 µm), lower b ratio (5.1-6.9 *vs* 9) and midventral precloacal papilla (P1) and the second pair of subventral papillae (P2) *vs* their absence.

Type habitat and locality. Recovered from soil samples collected about the rhizosphere of an unidentified weed, in Khouzestan province, southwestern Iran during March 2013. GPS coordinates N32°27.450, E48°22.796.

Type material. Holotype female, five paratype females and paratype males deposited at Nematode Collection at 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 new species is named in honor of Walter Rühm, a pioneer in the taxonomy of bark-beetle associated nematodes.

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A. Yaghoubi, E. Pourjam, M.R. Atighi, M. Pedram. Молекулярная и морфологическая характеристика *Ektaphelenchoides ruehmi* sp. n. (Nematoda: Ektaphelenchinae) из юго-западного Ирана.

Резюме. Приводится описание строения, а также морфометрические и молекулярные данные для *Ektaphelenchoides ruehmi* sp. n. Новый вид отличается длиной тела у самок 355-487 µm, слегка обособленным головным концом, латеральными полями с тремя линиями, общей длиной стилета 9.0-12.5 µm, экскреторной порой на расстоянии 55-70 µm и гемизонидом на расстоянии 59-76 µm от головного конца, особой формой хвостового конца у самцов и самок и расположением папилл самца. По морфологи и морфометрии новый вид сходен с пятью известными видами рода: *E. attenuata*, *E. musae*, *E. sylvestris, E. kelardashtensis* и *E. pini* наличием длинного и нитевидного хвостового конца. По молекулярным признакама (нуклеотидным последовательностям D2D3 сегмента 28S рДНК), новый вид сходен с *E. kelardashtensis* (80.8% сходство по совпадающей части последовательности D2D3). В сравнении с *E. attenuata* новый вид имеет более короткое тело и стилет, меньшие значения индексов a и b, более короткий рудимент задней матки смещен кзади вульвой, особенностями формы хвостового конца и спикул. В сравнении с *E. musae* новый вид имеет более короткое тело, стилет и рудимент задней матки, меньшее значение индекса b. также у нового вида чаще c = встречаются самцы. В сравнении с *E. sylvestris* новый вид имеет более короткий стилет, меньшее значение индексов a и b, смещенную вперед вульву, большее расстояние между вульвой и задней частью тела, и часто встречающихся самцов. В сравнении с *E. kelardashtensis* новый вид имеет более короткий стилет, меньшее значение индексов b и MB, большее значение отношения расстояния от вульвы до задней оконечности тела к ширине тела на уровне вульвы, латеральное поле с тремя инцизурами, наличие среднебрюшной преклоакальной папиллы, иное расположение папиллы P2 и форму хвостового конца самцов и спикул. В сравнении с *E. pini*, новый вид имеет более короткое тело и стилет, меньшее значение b. У нового вида имеются преклоакальные папиллы P1 и вторая пара субвентральных папилл P2. Молекулярный анализ частичной последовательности D2D3-сегмента 28S рДНК длиной 757 п.н. показал обособленность *E. ruehmi* sp. n. Ближайший вид – *E. kelardashtensis*. Оба вида формировали ветвь при поддержке в Байесовой филограмме (обратной вероятности) 0.98 и 87% бутстрепа в анализе методом максимального правдоподобия.