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New data on a rare ektaphelenchid species, *Ektaphelenchus joyceae* Kaisa, Harman & Harman, 1995 (Rhabditida: Ektaphelenchinae) with its first molecular phylogenetic study

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Ektaphelenchus joyceae was repeatedly isolated from pine packaging woods imported from Korea and Japan to China. They are morphologically and morphometrically very similar and are characterized by having a total body length range of 539-718 μm, cuticle with fine transverse annuli and three lines in lateral fields, slightly offset lip region with six equally sized lips, hemizonid and excretory pore well posterior to metacorpus, 14.2-18.0 μm long stylet with visible lumen and lacking knobs, simple intestine ending in a blind sac, no visible rectum and anus, monodelphic-prodelphic reproductive system with small spheroid sperm cells inside the spermatheca, short post-vulval uterine sac (PUS) and lacking males. The recovered populations agreed well with the type population based on morphometric data and morphology of females. The molecular phylogenetic analyses were performed using small, large and internal transcribed spacer regions of ribosomal RNA genes for the first time and the feeding habit of the species was also observed and documented.

Key words: *Aphelenchoidea, morphology, morphometrics, new observations, phylogeny, taxonomy.*

INTRODUCTION

Till 2009, 24 species were known under the genus *Ektaphelenchus* Fuchs, 1937 (Hunt, 2009). Most of the species however, are established based on the classic criteria, and for several species, the type materials, light microphotographs and molecular data are not available. Since 2009 till now, five species: *E. ibericus* Gu, Wang, Chen & Wang 2013, *E. taiwanensis* Gu, Wang & Chen 2013 , *E. berbericus* Alvani, Mahdikhani-Moghadam, Giblin-Davis & Pedram 2016, *E. oleae* Miraeiz, Heydari, Adeldoost & Ye 2017 and *E. cupressi* Golhasan, Abdollahpour, Fang, Abolafia & Heydari, 2019 have also been added to the genus. The molecular data, are of much importance in depicting phylogenetic affinities of the species with other taxa, and correct identification of other populations. Thus, re-isolating of old species could provide opportunities to perform molecular phylogenetic studies and detailed morphological examinations.

To prevent the spread of the pine wood nematode, *Bursaphelenchus xylophilus* (Steiner & Buhrer, 1934) Nickle, 1970, and other pests through packaging wood materials, almost all packaging

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woods imported *via* Ningbo harbour have been sampled and inspected since 1997 (Gu *et al.,* 2006). In 2009 and 2010, an aphelenchoidid nematode species was repeatedly isolated from pine packaging woods imported from Korea and Japan. The close study revealed it is typologically an Ektaphelenchinae Paramonov, 1964 member and belongs to the rare species *Ektaphelenchus joyceae* Kaisa, Harman & Harman, 1995. This finding prompted us to perform a detailed morphological study of the recovered populations, and compare them with the type material. The molecular phylogenetic studies were also performed using the small subunit ribosomal DNA (SSU rDNA), partial large subunit ribosomal DNA D2-D3 (LSU rDNA D2-D3) and the internal transcribed spacer ribosomal DNA (ITS rDNA).

MATERIAL AND METHODS

Extraction, culturing attempts and morphological observation

Packaging woods were cut into small pieces no more than one cm wide. Nematodes were extracted using a modified Baermann funnel technique for 24 h. Measurements were made on specimens fixed in TAF and processed to glycerine following the method of Seinhorst (1959). Light micrographs were made using a Zeiss Imager Z1 microscope equipped with a Zeiss AxioCam MRm CCD camera. The feeding habit of the species was observed and recorded in water. To test the feeding and multiplication of the nematode on *Botrytis cinerea* Persoon, 1794, some individuals were put on the fungus grown on malt agar medium in 6-cm-diam. Petri dishes.

Molecular analyses

DNA samples were prepared according to Wang *et al.* (2011). Three sets of primers (Gu *et al.*, 2012) (synthesised by Invitrogen, Shanghai, China) were used in the PCR analyses to amplify the partial SSU, LSU D2-D3 and ITS (ITS1+5.8S+ITS2) rDNA genomic fragments. PCRs were performed as described by Li *et al.* (2008) and Ye *et al.* (2007). PCR products were separated on 1% agarose gels and visualized by ethidium bromide staining. The PCR products of sufficiently high quality were purified for cloning and sequencing by Invitrogen, Shanghai, China. The newly generated accession numbers were deposited into the GenBank database under the accession numbers: KC154092 (SSU, Japanese population, 918 nt long), KF452046 (LSU D2-D3, Korean population, 809 nt long), KC154094 (LSU D2-D3, Japanese population, 809 nt long), KC154093 (ITS, Japanese population, 1265 nt long) and KC160449 (ITS, Korean population, 1264 nt long).

For molecular phylogenetic analyses, the newly generated sequences were compared with sequences of other species available in GenBank using the BLAST homology search program. The three newly generated genomic sequences were matched with none of the currently available sequences deposited into the database. The selected sequences for reconstructing of each phylogenetic tree were aligned using the Q-INS-i algorithm of online version of MAFFT version 7 (http://mafft.cbrc.jp/alignment/server/) (Katoh & Standley, 2013). The Gblocks program (version 0.91b) with all the three less stringent parameters, a server tool at the Castresana Lab (http://molevol.cmima.csic.es/castresana/ Gblocks_server.html), was used for post-editing of the alignments, *i.e.* to eliminate poorly aligned regions or divergent positions. The model of base substitution was selected 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 analyses of SSU and LSU datasets, and a general time reversible model, including among-site rate heterogeneity (GTR + G) was used in analyses of ITS dataset. Bayesian analysis was performed using MrBayes v3.1.2 (Ronquist & Huelsenbeck, 2003) running the chains for 5×10^6 generations for two SSU and LSU D2-D3, and 2×10^6 generations for ITS dataset. After discarding burn-in samples, the remaining samples were retained for further analyses. The Markov chain Monte Carlo (MCMC) method within a Bayesian framework was used to estimate

the posterior probabilities of the phylogenetic trees (Larget & Simon, 1999) using the 50% majority rule. Convergence of model parameters and topology were assessed based on average standard deviation of split frequencies and potential scale reduction factor values. Adequacy of the posterior sample size was evaluated using autocorrelation statistics as implemented in Tracer v.1.6 (Rambaut & Drummond, 2009). A maximum likelihood (ML) tree was reconstructed by using RaxmlGUI 1.1 (Silvestro & Michalak, 2012) software using the same nucleotide substitution model as in the BI in 1000 bootstrap (BS) replicates for all three datasets. The output files of the phylogenetic programs used herein were visualized using Dendroscope V.3.2.8 (Huson & Scornavacca, 2012) and re-drawn in CorelDRAW software version 16. The Bayesian posterior probability (BPP) and ML BS values exceeding 0.50 and 50%, respectively, were given on appropriate clades in the format of BPP/ML BS.

For ITS-RFLP profiles, suitable aliquots of the amplified ITS rDNA were digested for at least 3 h at 37°C using 10 U of each of the five restriction endonucleases (*Rsa* I, *Hae* III, *Msp* I, *Hinf* I and *Alu* I) (Takara, Japan) following the manufacturer's instructions. Fragments were resolved by electrophoresis in a 2.5% agarose gel and stained with ethidium bromide.

RESULTS

Ektaphelenchus joyceae

(Based on populations recovered from imported packaging woods from Japan and Korea)
(Figs 1 & 2)

Measurements, see Table 1.

Female. Body slightly ventrally arcuate, slightly more in the posterior region, tapering to conoid posterior body end (tail). Cuticle with fine, transverse striae. Lateral field *ca* 2.0-2.5 µm wide, with three incisures at mid-body. Lip region slightly offset by a constriction, with three equally sized lips in lateral view (six equally sized lips). Stylet moderately sclerotized, with distinct lumen all over its length, the conus *ca* 0.9 times the shaft, without basal knobs. Procorpus slender, flexible, its lumen well visible, median bulb oblong, longer than wide, its granular region about 50% of the length, the valve plates well developed, post-median, pharyngo-intestinal junction about one metacorpus valve length behind it. Nerve ring one-half to one body width posterior to median bulb. Excretory pore *ca* two metacorpus length posterior to its base. Hemizonid just anterior to excretory pore or sometimes at the same position with it. Pharyngeal gland lobe long, about six-seven times body width long, overlapping intestine dorsally, its nuclei not clearly seen. Intestine ending in a blind sac, rectum and anus absent. Reproductive system monodelphic-prodelphic, ovary outstretched, 179-262 µm long, oocytes in one to two rows, oviduct obscure, spermatheca longer than wide, sometimes two or three-lobed, containing small spheroid sperm cells, crustaformeria visible, uterus thick-walled, vagina short, moderately sclerotized, about one-third of vulval body width long, slightly inclined anteriorly, post-vulval uterine sac (PUS) short, about 4-10 µm long and vulva not protuberant with no flap. Posterior body end (tail) conoid with a sharply pointed terminus, its cuticle coarse and slightly swollen near the terminus, sometimes forms a mucron-like appearance.

Male. Not found.

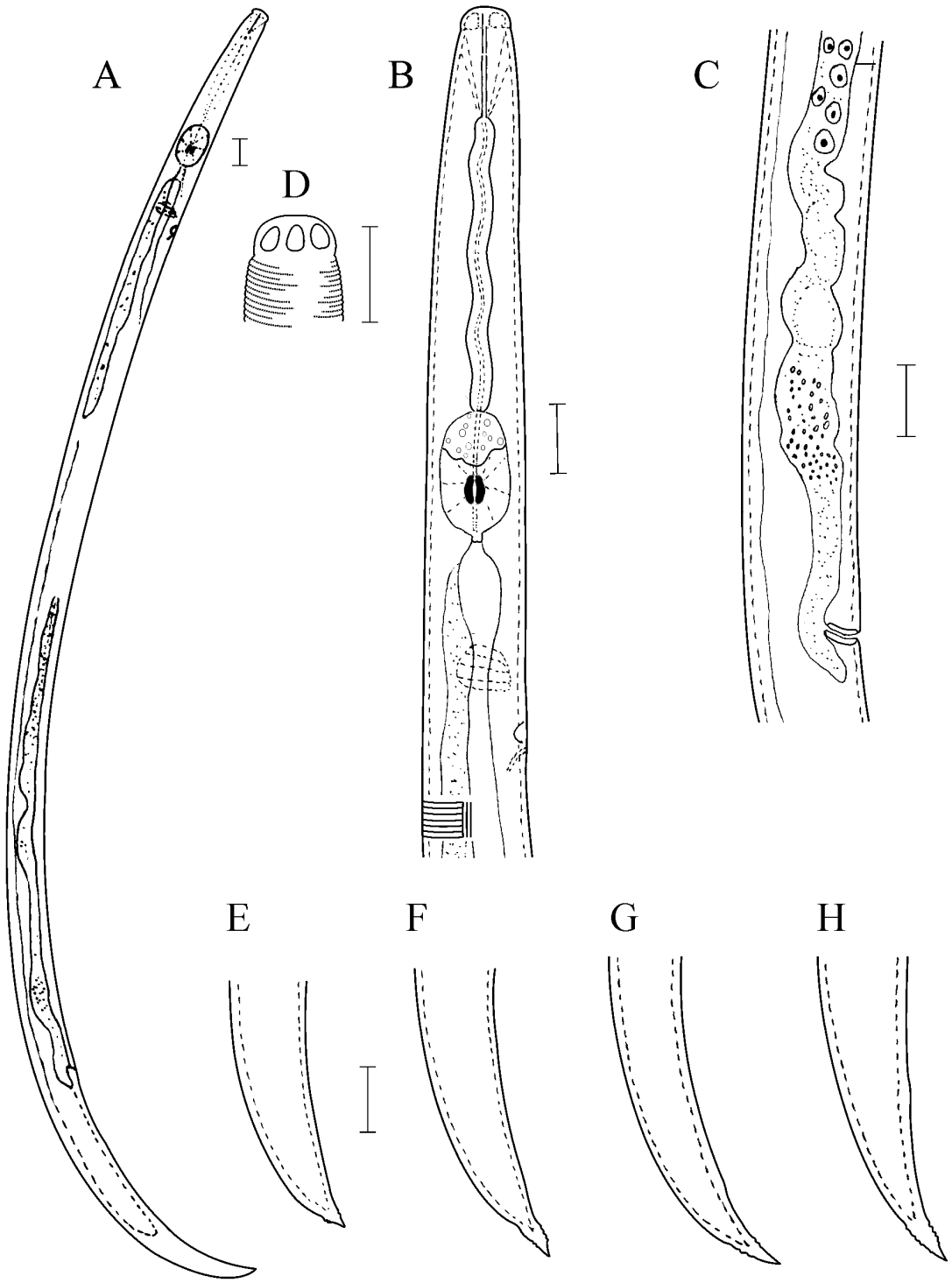

FIGURE 1. Line drawings of Japanese population of *Ektaphelenchus joyceae* Kaisa, Harman & Harman, 1995. (Female). Entire body (A), Pharynx (B), Vulval region (C), Lips in lateral view (D) and Posterior body end (tail) shape variation (E-H). (Scale bars = 10 µm.)

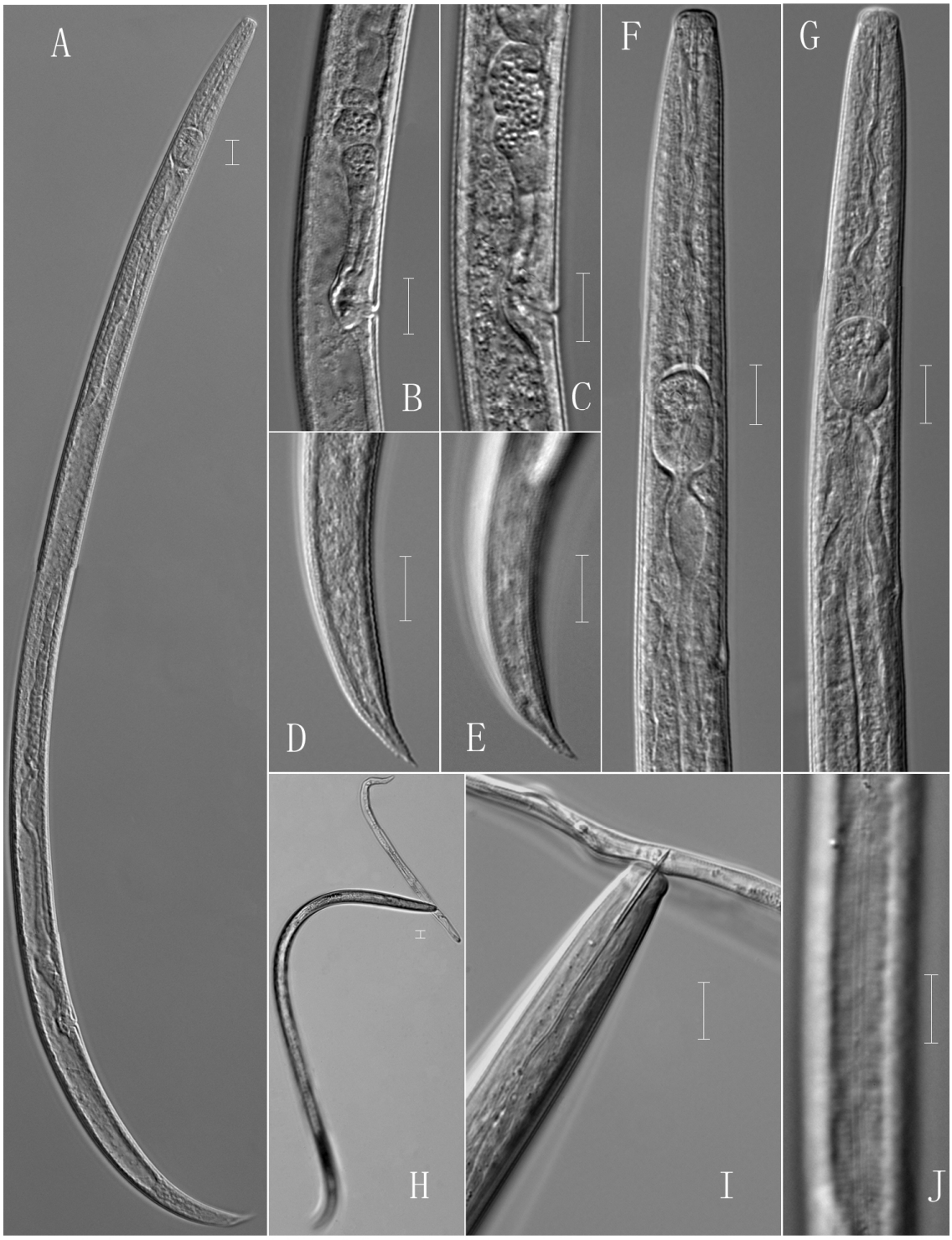

FIGURE 2. Light micrographs of Japanese population of *Ektaphelenchus joyceae* Kaisa, Harman & Harman, 1995 （Female）. Entire body (A), Vulval region (B & C), Posterior body end (tail) shape variation (D&E), Anterior body (F & G), Feeding on *Aphelenchoides* sp. (H&I) and Lateral lines (J). (Scale bars = 10 μm.)

TABLE 1. Morphometrics of the type and Japanese and Korean populations of *Ektaphelenchus joyceae* Kaisa, Harman & Harman, 1995. All measurements are in μm and in the format: mean ± s.d. (range).

Characters	Females		
	Type population	Japanese population	Korean population
n	32	13	11
L	640±60 (550-740)	600±44.2 (539-674)	650±36.3 (583-718)
a	34±3 (29-44)	35.0±1.8 (32.9-38.4)	35.4±1.6 (33.8-38.5)
b	9±1 (7-13)	8.2±0.3 (7.5-8.8)	9.0±0.5 (8.3-9.9)
b'	-	3.2±0.3 (2.7-3.5)	3.5±0.2 (3.3-3.8)
V	82±2 (77-86)	78.5±0.4 (77.9-79.1)	78.3±0.5 (77.6-79.2)
Max body diam.	-	17.2±0.9 (15.7-18.8)	18.4±1.3 (16.2-20.6)
Lip diam.	-	6.9±0.3 (6.2-7.2)	7.1±0.5 (6.3-8.0)
Lip height	-	3.0±0.2 (2.8-3.3)	3.0±0.3 (2.6-3.6)
Stylet length	16±1 (14-18)	16.2±0.8 (14.8-18.0)	15.6±0.7 (14.2-16.6)
Median bulb length	-	17.2±0.4 (16.7-17.6)	17.3±0.7 (16.1-18.5)
Median bulb diam.	-	10.0±0.6 (9.1-10.5)	10.1±0.8 (8.8-11.2)
Median bulb length/diam.	-	1.8±0.1 (1.6-1.9)	1.7±0.1 (1.6-1.9)
Excretory pore position	-	96.5±6.4 (85-103)	101.7±4.2 (94.2-108.0)
Ovary length	-	213.3±21.9 (179-239)	221.8±20.4 (201-262)
Post-vulval uterine sac length	-	5.6±1.2 (3.9-7.8)	6.9±1.8 (4.6-9.8)
Vulva to the end of blind sac	-	75.8±8.9 (61.6-89.0)	91.6±6.7 (81-102)

Habitat and locality
The Korean population (code: 42707) was isolated from packaging wood of *Pinus* sp. in 2009. The Japanese population (code: 39571) was isolated from packaging wood of *Pinus* sp. from Japan and inspected in the Ningbo Entry-Exit Inspection and Quarantine Bureau, P.R. China, in 2010.

Feeding habit
When observing the nematodes in the water under stereomicroscope, several specimens of *E. joyceae* were found feeding on *Bursaphelenchus mucronatus* Mamiya & Enda, 1979 and *Aphelenchoides* sp. The bodies of *B. mucronatus* and *Aphelenchoides* sp. were shrivelled after penetration of the stylet (Figs 2H, 2I).

Voucher material
20 females (slide number 42707-1 to 42707-5, 39571-1 to 39571-6) were deposited in the nematode collection of Ningbo Entry-Exit Inspection and Quarantine Bureau, China. One slide including three females (slide number 11555) was deposited in the Canadian National Collection of Nematodes, Ottawa, Ontario, Canada.

Remark
No remarkable morphological or morphometric differences were observed between the studied populations and the type population by Kaisa *et al.* (1995). A close examination of the type material of *E. joyceae* (Fig. 3) kindly provided by Prof. Zafar Handoo from United States Department of Agriculture Nematode Collection (USDANC), Beltsville, Maryland, USA, revealed it has three lines in the lateral field, as observed for the Japanese and Korean populations. Some features like unique posterior body end (tail) shape further confirmed the recovered population and the type population of *E. joyceae* are conspecific. Compared to the type population, no males were recovered for the two studied populations, but sperm were observed inside the female reproductive system, indicating probable amphimictic reproductive mode of these populations. Lacking of males, again could arise doubts on generic identity of the studied populations, but, the equally sized lips observed for some well-prepared females of the Korean and the Japanese populations, prompted us to provisionally put the recovered populations under the genus *Ektaphelenchus*, after comparisons with all valid *Deviбursaphelenchus* spp. The same general morphology and morphometric data ranges and posterior body end (tail end) characters supported the identity of these populations as *E. joyceae*.

Phylogenetic status and ITS-RFLP profiles
For reconstructing of phylogenetic trees, three independent datasets were prepared. The SSU dataset was composed of 38 aphelench species/isolates (including an isolate of *E. joyceae*), and three classic rhabditid species as outgroup taxa. The LSU D2-D3 dataset was composed of 51 species/isolates (including two isolates of *E. joyceae*), and three classic rhabditid taxa as outgroup species. The ITS dataset was also composed of 14 aphelench species/isolates, including two newly generated sequences for *E. joyceae* and an *Aphelenchoides* sp. as outgroup.
The SSU dataset had 1464 characters of which 738 characters were variable. Fig. 4. represents the phylogenetic tree inferred using this dataset. In this tree, *Ektaphelenchus joyceae* (KC154092) has formed a clade with *Ektaphelenchoides spondylis* Kanzaki, Giblin-Davis & Center, 2009 (AB849952) (BPP=1.00, ML BS= 97%), their clade being in sister relation with *Peraphelenchus orientalis* Kanzaki, Tanaka, Ikeda, Taki, Sugiura & Matsumoto, 2013 (AB786908). The nonmonophyly of two genera *Ektaphelenchus* and *Deviбursaphelenchus* Kakulia, 1967 is seen in this tree.

The LSU dataset had 489 characters of which 300 characters were variable. Fig. 5 represents that phylogenetic tree inferred using this dataset. In this tree, two Korean and Japanese populations

of *Ektaphelenchus joyceae* (KF452046, KC154094) have formed a clade with two species of *Devibursaphelenchus* (*D. hunanensis* (Yin, Fang & Tarjan, 1988) Braasch, 2009 and *D. wangi* Gu, Wang & Zheng, 2010) with maximal BPP and high (99%) ML BS. The nonmonophyletic nature of three genera *Ektaphelenchus*, *Ektaphelenchoides* Baujard, 1984 and *Devibursaphelenchus* is observed in this tree.

The ITS dataset had 634 characters of which 325 characters were variable. Fig. 6 represents the phylogenetic tree inferred using this dataset. In this tree, two Korean and Japanese populations of *Ektaphelenchus joyceae* (KC160449, KC154093) have formed a well-supported clade (0.99 BPP, 100 ML BS) with two isolates of *Devibursaphelenchus* (*D. hunanensis* and *D. wangi*). The nonmonophyletic nature of three genera *Ektaphelenchus*, *Ektaphelenchoides* and *Devibursaphelenchus* is again observed in this tree.

Amplification of the ITS region of *E. joyceae* resulted in a PCR product of 1265 bp. The patterns of restriction fragments produced by digestion of the PCR product with *Rsa* I, *Hae* III, *Msp* I, *Hinf* I and *Alu* I and their size are given in Fig. 7 and Table 2.

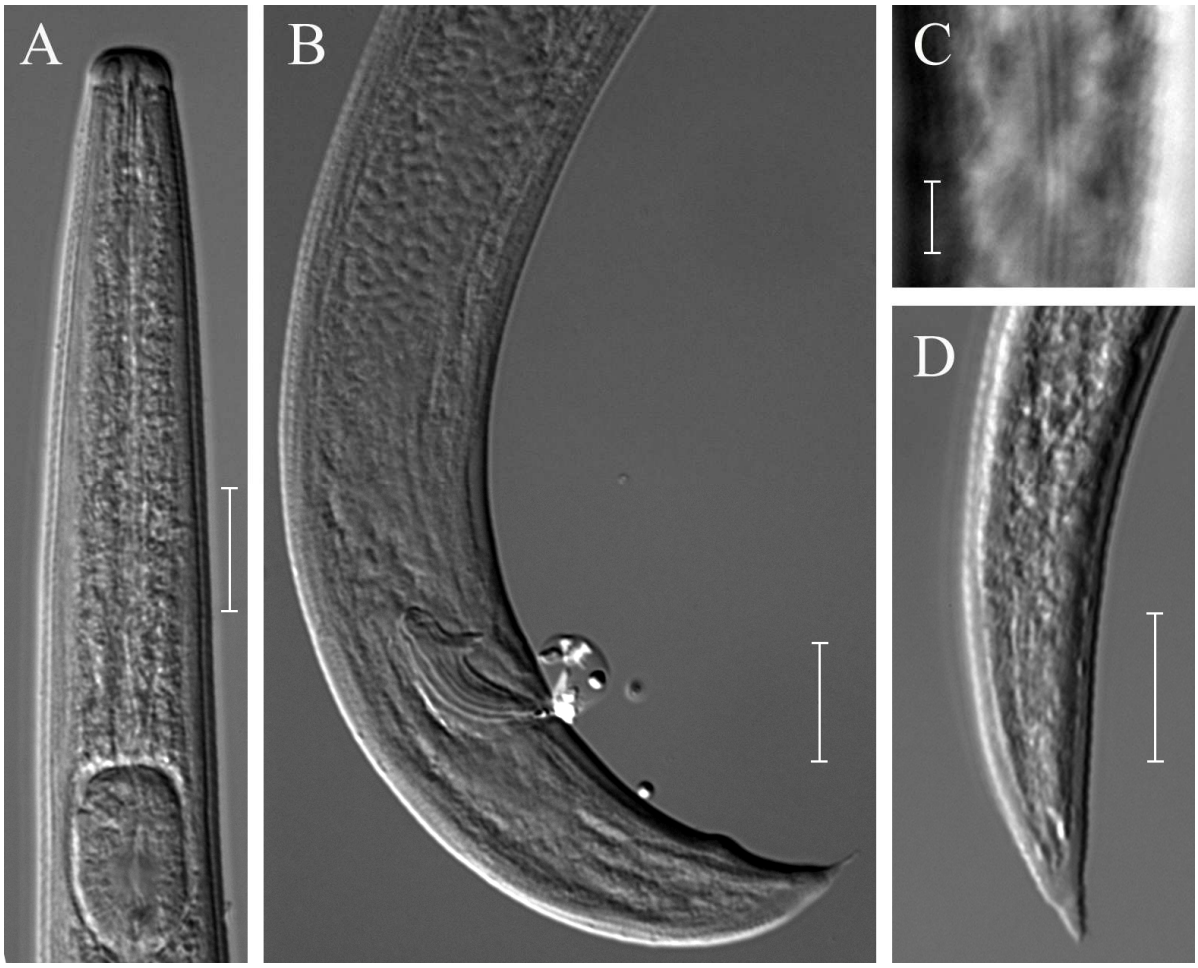

FIGURE 3. Light micrographs of the type population of *Ektaphelenchus joyceae* Kaisa, Harman & Harman, 1995. Anterior body region (A), Male posterior body (B), Lateral field having three lines (C) and Female posterior body region (D). (A, B, D: scale bars = 10 µm; C: scale bar=5 µm.)

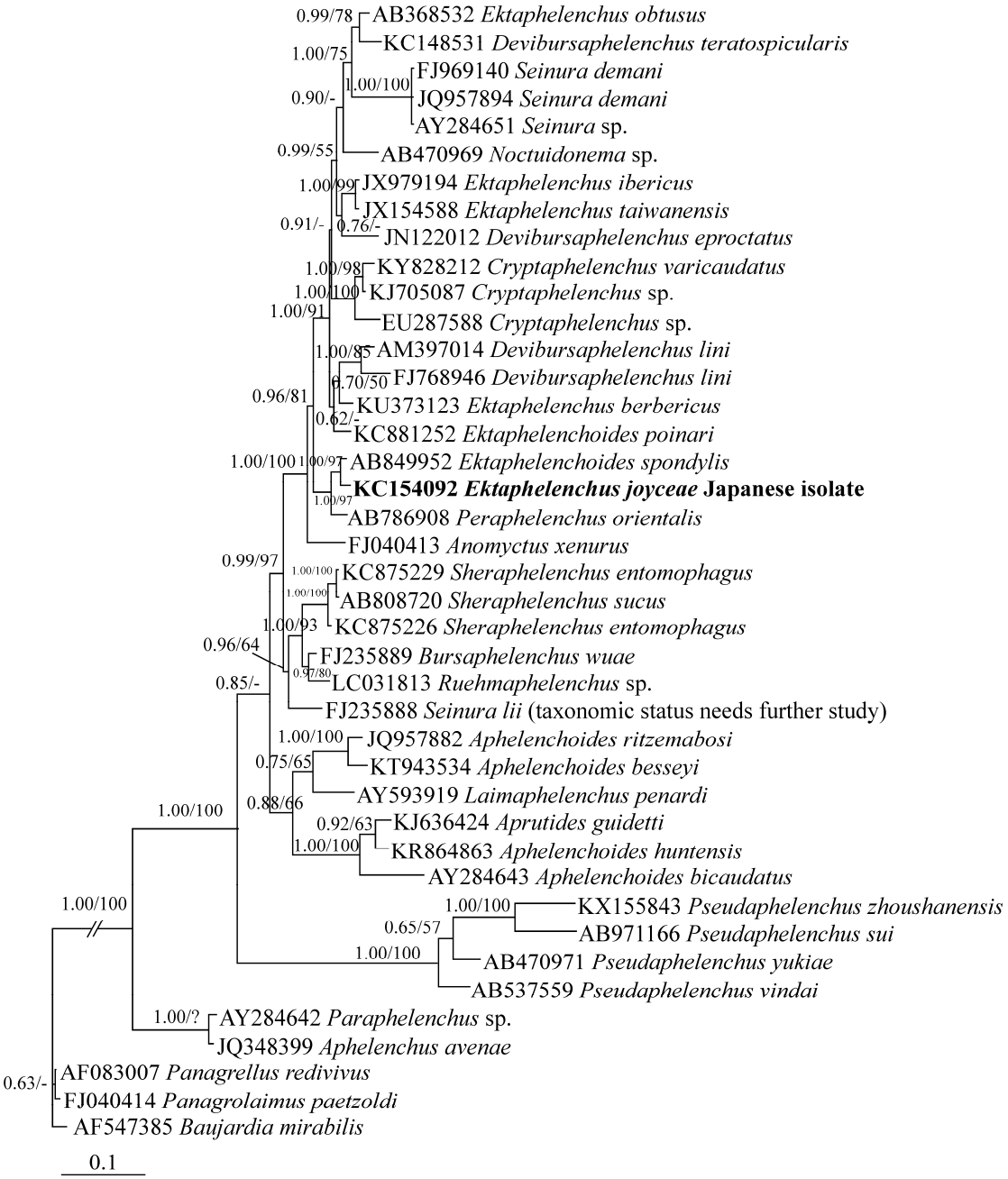

FIGURE 4. Bayesian 50% majority rule consensus tree inferred from SSU rDNA of Japanese population of *Ektaphelenchus joyceae* Kaisa, Harman & Harman, 1995 under the GTR + G + I model. Bayesian posterior probabilities (BPP) and maximum likelihood bootstrap (ML BS) values more than 50% are given for appropriate clades in the form: BPP/ML BS. New sequence is in bold font.

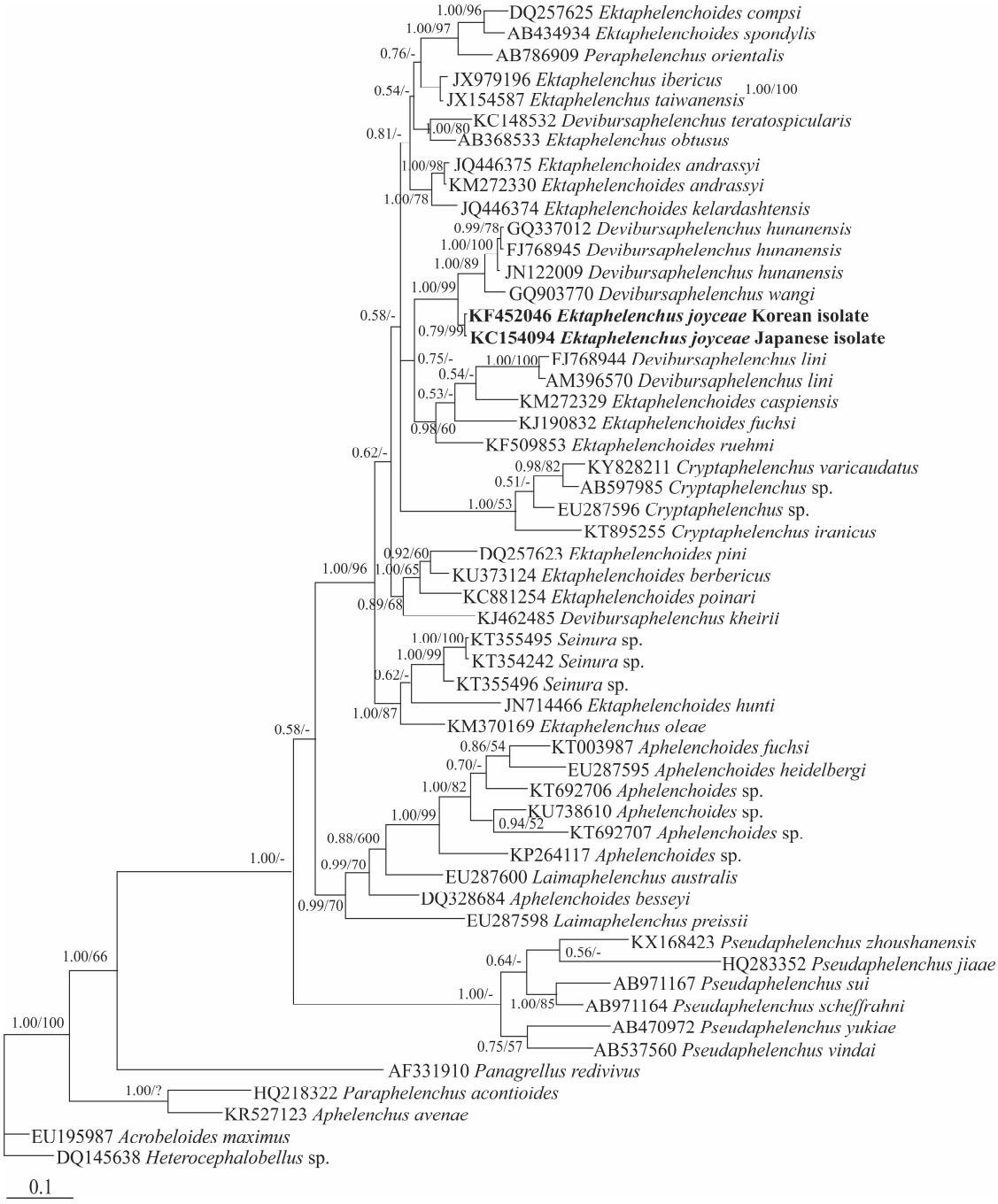

FIGURE 5. Bayesian 50% majority rule consensus tree inferred from LSU rDNA D2-D3 of Japanese and Koran populations of *Ektaphelenchus joyceae* Kaisa, Harman & Harman, 1995 under the GTR + G + I model. Bayesian posterior probabilities (BPP) and maximum likelihood bootstrap (ML BS) values more than 50% are given for appropriate clades in the form: BPP/ML BS. New sequences are in bold font.

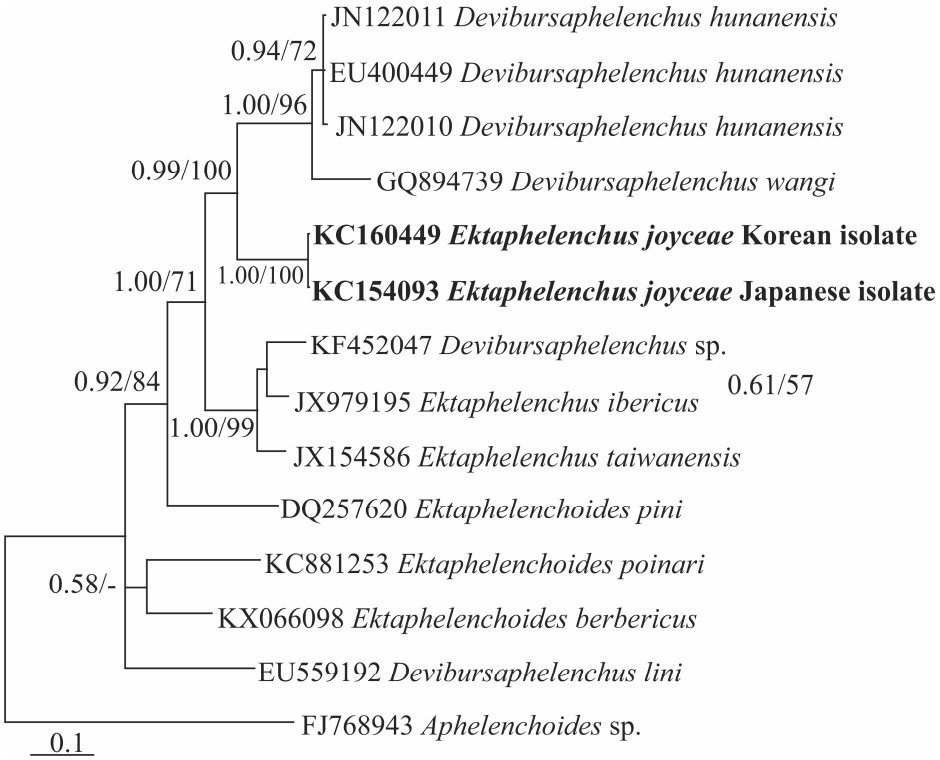

FIGURE 6. Bayesian 50% majority rule consensus tree inferred from ITS sequences of Japanese and Koran populations of *Ektaphelenchus joyceae* Kaisa, Harman & Harman, 1995 under the GTR + G model. Bayesian posterior probabilities (BPP) and maximum likelihood bootstrap (ML BS) values more than 50% are given for appropriate clades in the form: BPP/ML BS. New sequences are in bold font.

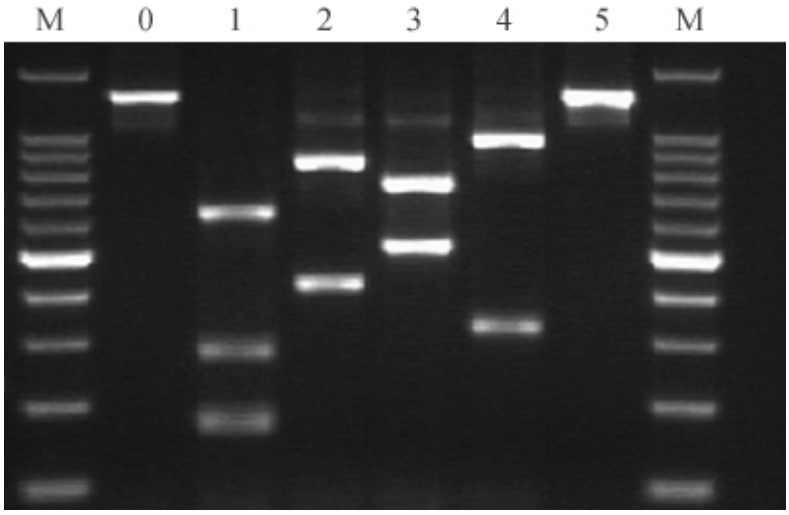

FIGURE 7. ITS-RFLP patterns of *Ektaphelenchus joyceae* Kaisa, Harman & Harman, 1995. Restriction fragments were obtained by digestion of the amplified ITS rDNA fragment (0) with *Rsa* I (1), *Hae* III (2), *Msp* I (3), *Hinf* I (4) and *Alu* I (5). M = DNA marker (100 bp ladder, Invitrogen Life Technologies).

TABLE 2. Sizes of DNA restriction fragments obtained for *Ektaphelenchus joyceae* Kaisa, Harman & Harman, 1995 in ITS-RFLP analysis.

Species		PCR product (bp)	Restriction fragments (bp)				
			Rsa I	*Hae* III	*Msp* I	*Hinf* I	*Alu* I
Ektaphelenchus	*joyceae*	1265	629	847	746	949	1252
			280	418	519	316	13
			183				
			173				

DISSCUSSION

Most old aphelench genera and species are only known with their type populations, described based on classic criteria, and light microphotographs, and more importantly, their molecular data are not currently available. Re-isolating of such taxa provides opportunities for their molecular phylogenetic studies and biological examinations. In present study, two populations of an ektaphelenchid species were repeatedly isolated from wood materials imported from Japan and Korea to China. Besides their identical morphology and morphometric data ranges, sequencing of their partial LSU D2-D3 and ITS fragments revealed they are conspecific. The predatory feeding habit of the species was also observed and documented. Their morphological comparison with valid *Devibursaphelenchus* and *Ektaphelenchus* species revealed they are very similar to a previously described species, *Ektaphelenchus joyceae,* and also close examination of the type material of the species revealed the lateral line number and tail end morphology are shared between the populations and in conclusion, the recovered Korean and Japanese populations were assigned to *E. joyceae.*

In accordance with Alvani *et al.* (2016), the nonmonophyletic nature of the three ektaphelenchid genera, *Ektaphelenchoides*, *Ektaphelenchus* and *Devibursaphelenchus* was observed in present study too.

The ITS-RFLP patterns of *E. joyceae* were different from those for *D. lini*, *D. wangi* and *D. hunanensis* (Burgermeister *et al.*, 2009; Gu *et al.*, 2010).

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LITERATURE CITED

Alvani, S., Mahdikhani-Moghadam, E., Giblin-Davis, R.M., Pedram, M., 2016. Description of *Ektaphelenchus berbericus* n. sp. (Rhabditida: Ektaphelenchinae) from eastern Iran. Nematology 18, 1063-1077.

Baujard, P., 1984. Remarques sur la sous-famille des Ektaphelenchinae Paramonov, 1964 et proposition *d'Ektaphelenchoides* n. gen. (Nematoda: Aphelenchoididae). Revue de Nématologie 7, 147-171.

Braasch, H., 2009. Re-establishment of *Deviбursaphelenchus* Kakuliya, 1967 (Nematoda, Aphelenchoididae) and proposal for a new combination of several *Bursaphelenchus* species. Journal of Nematode Morphology and Systematics 12, 1-5.

Burgermeister,W., Braasch, H., Metge, K., Gu, J.F., Schröder, T., Woldt, E., 2009. ITS-RFLP analysis, an efficient tool for differentiation of *Bursaphelenchus* species. Nematology 11, 649-668.

Fisher, M., 1894. Uber eine Clematis -krankheit. Bericht aus dem Physiolischen Laboratorium des Landwirthschaftlichen, Instituts der Universitat Halle 3, 1-11.

Golhasan, B., Abdollahpour, M., Fang, Y., Abolafia, J., Heydari, R. Description of *Ektaphelenchus cupressi* n. sp. (Nematoda: Ektaphelenchinae) from Iran. Nematology, In press.

Gu, J., Braasch, H., Burgermeister, W., Zhang, J., 2006. Records of *Bursaphelenchus* spp. intercepted in imported packaging wood at Ningbo, China. Forest Pathology 36, 323-333.

Gu, J., Wang, J., Zheng, J., 2010. *Deviбursaphelenchus wangi* sp. n. feeding on *Aphelenchoides* sp. Russian Journal of Nematology 18, 49-57.

Gu, J., Wang, J., Zheng, J., 2012. Description of *Bursaphelenchus arthuroides* sp. n. (Nematoda: Aphelenchoididae), a second parthenogenetic species of *Bursaphelenchus* Fuchs, 1937. Nematology 14, 51-63.

Gu, J., Wang, J., Chen, X., 2013. Description of *Ektaphelenchus taiwanensis* sp. n. (Nematoda: Ektaphelenchinae) found in packaging wood from Taiwan. Nematology 15, 329-338.

Gu, J., Wang, J., Chen, X., Wang, X., 2013. Description of *Ektaphelenchus ibericus* n. sp. (Nematoda: Ektaphelenchinae) found in packaging wood from Spain. Nematology 15, 871-878.

Hunt, D.J., 2009. A checklist of the Aphelenchoidea (Nematoda: Tylenchina). Journal of Nematode Morphology and Systematics 10, 99-135.

Huson, D.H., Scornavacca, C., 2012. Dendroscope 3: an interactive tool for rooted phylogenetic trees and networks. Systematic Biology 61, 1061-1067.

Kaisa, T.R., Harman, A.L., Harman, D.M., 1995. Two new species of Aphelenchoididae (Nemata) from declining red pine in Maryland. Journal of Nematology 27, 213-221.

Kakulia, G.A., 1967. New nematode genus *Deviбursaphelenchus* n. g. (Nematoda: Aphelenchoididae). Soobschcheniya Akademii Nauk Gruzinskoi SSR 46, 439-443. [In Russian]

Katoh, K., Standley, D.M., 2013. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. Molecular Biology and Evolution 30, 772-780.

Kanzaki, N., Giblin-Davis, R.M., Center, B.J., 2009. Description of *Ektaphelenchoides spondylis* n. sp.(Nematoda: Ektaphelenchinae) isolated from Spondylis buprestoides (Coleoptera: Cerambycidae) in Japan. Nematology 11, 181-188. http://dx.doi.org/10.1163/156854109X429529

Kanzaki, N., Giblin-Davis, R.M., 2012. Chapter 7: Aphelenchoidea. *In*: R. Manzanilla-Lopez & N. Marbán-Mendoza (Eds.), *Practical Plant Nematology*. Biblioteca Básica de Agricultura, Guadalajara, Mexico, pp. 161–208.

Kanzaki, N., Tanaka, R., Ikeda, H., Taki, H., Sugiura, S., Matsumoto, K., 2013. Phylogenetic status of insect parasitism in the subfamily Entaphelenchinae Nickle with description of *Peraphelenchus orientalis* n. sp. (Tylenchomorpha: Aphelenchoididae). The Journal of Parasitology 99, 639-649.

Larget, B., Simon, D.L., 1999. Markov chain Monte Carlo algorithms for the Bayesian analysis of phylogenetic trees. Molecular Biology and Evolution 16, 750-759.

Li, H., Trinh, P.Q., Waeyenberge, L., Moens, M., 2008. *Bursaphelenchus chengi* sp. n. (Nematoda: Parasitaphelenchidae) isolated at Nanjing, China, in packaging wood from Taiwan. Nematology 10, 335-346.

Mamiya, Y., Enda, N., 1979. *Bursaphelenchus mucronatus* n. sp. (Nematoda: Aphelenchoididae) from pine wood and its biology and pathogenicity to pine trees. Nematologica 25, 353-361.

Miraeiz, E., Heydari, R., Adeldoost, Y., Ye, W., 2017. Description of *Ektaphelenchus oleae* n. sp. (Rhabditida: Seinurinae) from Iran. Nematology 19, 1123-1134.

Nickle, W.R., 1970. A taxonomic review of the genera of the Aphelenchoidea (Fnchs, 1937) Thorne, 1949 (Nematoda: Tylenchida). Journal of Nematology 2, 375-392.

Nylander, J.A.A., 2004. *MrModeltest v2. Program distributed by the author*. Evolutionary Biology Centre, Uppsala University.

Rambaut, A., Drummond, A.J., 2009. Tracer v1.6. Available from: http://beast.bio.ed.ac.uk/Tracer.

Paramonov, A.A., 1964. *Fundamentals of phytohelminthology. Vol. II. Taxonomy of phytonematodes*. Nauka, Moscow, 466 pp. [In Russia]

Ronquist, F., Huelsenbeck, J.P., 2003. MRBAYES3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19, 1572-1574.

Seinhorst, J.W., 1959. A rapid method for the transfer of nematodes from fixative to anhydrous glycerin. Nematologica 4, 67-69.

Silvestro, D., Michalak, I., 2012. raxmlGUI: a graphical front-end for RAxML. Organisms Diversity and Evolution 12, 335-337.

Steiner, G., Buhrer, E.M., 1934. *Aphelenchoides xylophilus*, n. sp., a nematode associated with blue-stain and other fungi in timber. Journal of Agricultural Reseach 48, 949-951.

Wang, J., Zhang, J., Gu, J., 2011. Method for extract DNA from a single nematode. Plant Qurarantine 25, 32-35. [In Chinese]

Ye, W., Giblin-Davis, R.M., Braasch, H., Morris, K., Thomas, W.K., 2007. Phylogenetic relationships among *Bursaphelenchus* species (Nematoda: Parasitaphelenchidae) inferred from nuclear ribosomal and mitochondrial DNA sequence data. Molecular Phylogenetics and Evolution 43, 1185-1197.

Yin, K., Fang, Y., Tarjan, A.C., 1988. A key to species in the genus *Bursaphelenchus* with a description of *Bursaphelenchus hunanensis* sp. n. (Nematoda: Aphelenchoididae) found in pine wood in Hunan Province, China. Proceedings of Helmithological Society of Washington 55, 1-11.