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Molecular and morphological characterization of *Paurodontoides siddiqii* n. sp. (Nematoda: Hexatylina, Sphaerulariidae) associated with bark samples of *Pinus eldarica* from western Iran

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Research Paper

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Abstract

A new species of *Paurodontoides*, *P. siddiqii* n. sp., is described and illustrated based on its morphological, morphometric, and molecular characters. The new species is characterized by a female 550–729 µm long, lip region continuous with body contour, stylet length 7.0–8.0 µm long or *c.* 1.0–1.2 times the lip region diameter, lateral fields with four smooth incisures, excretory pore at 85–125 µm from anterior end located at the base of the pharyngeal bulb or posterior to it, basal pharyngeal bulb with a short posterior extension projecting into the intestine, monodelphic–prodelphic reproductive system with prominent 19–22 µm long post-uterine sac, and elongate conoid tail with a filiform terminus. The new species is compared with two known species of the genus. It differs from the type species of the genus, *P. linfordi,* by having slightly shorter stylet, lateral field with smooth incisures, different position of the excretory pore, and absence of male. Compared to *P. latus*, the new species has a shorter body, shorter stylet, different position of the excretory pore, female tail shape and absence of male. The new species was also compared with close species of the genus *Paurodontus* because of lateral field marked with four lines, asymmetrical stylet knobs and absence of male. Molecular phylogenetic studies of the new species using partial sequences of 18S rDNA revealed that it forms a clade with a species of the genus *Ficotylus*. In phylogenetic analyses using partial sequences of the 28S rDNA D2-D3 domain, the new species formed a monophyletic group with a species of the genus *Veleshkinema* and *Sphaerularia* spp. (Sphaerulariinae).

Introduction

The genus *Paurodontoides* was proposed by Jairajpuri and Siddiqi (1969). They transferred *Neotylenchus linfordi* Hechler, 1962 to the genus *Paurodontoides* based on the following features: framework eight-sectored, lateral sectors reduced, stylet small (9–10 µm long in type species), knobs distinct in type species, asymmetrical, subventrals notched, lateral field with four incisures, basal pharyngeal bulb elongate pyriform, with a short stem-like basal extension opening into the intestine slightly ventrally, post-uterine sac (PUS) present, female tail elongate filiform, male tail conoid and completely enveloped by bursa. Siddiqi (1986) transferred *Neotylenchus latus* Thorne, 1935 to *Paurodontoides*. Andrássy (1976) considered *Paurodontoides* as a junior synonym of *Stictylus* Thorne, 1941. Sumenkova (1975), Geraert *et al.* (1985), and Fortuner and Raski (1987) accepted Andrássy's opinion and synonymized *Paurodontoides* and *Bealius* Massey & Hinds, 1970 with *Stictylus.* Siddiqi (1986, 2000) considered *Paurodontoides* as a valid genus and proposed the family Paurodontidae Thorne, 1941 as a junior synonym of Sphaerulariidae Lubbock, 1861. Some nematologists accepted Siddiqi's opinion (Chizhov, 2004; Andrássy, 2007; Handoo *et al.*, 2010; Esmaeili *et al.*, 2016 a, b; Golhasan *et al.*, 2016). According to the classification by Siddiqi (2000), *Paurodontoides* is a member of the subfamily Paurodontinae Thorne, 1941 belonging to the family Paurodontidae, in superfamily Sphaerularioidea Lubbock, 1861. The genus *Paurodontoides* includes two nominal species: *P. linfordi* (Hechler, 1962) Jairajpuri & Siddiqi, 1969 and *P. latus* (Thorne, 1935) Siddiqi, 1986. It comes very close to the genus *Paurodontus* Thorne, 1941 and the genera are separated from each other based on the position of the vulva from the anus and the nature of the bursa at the male tail.

Recently, two monotypic genera (*Abursanema* Yaghoubi, Pourjam, Pedram, Siddiqi & Atighi, 2014 and *Veleshkinema* Miraeiz, Heydari, Álvarez-Ortega, Pedram & Atighi, 2015) and five species (*Paurodontella iranica* Golhasan, Heydari & Miraeiz, 2016; *P. parapitica*

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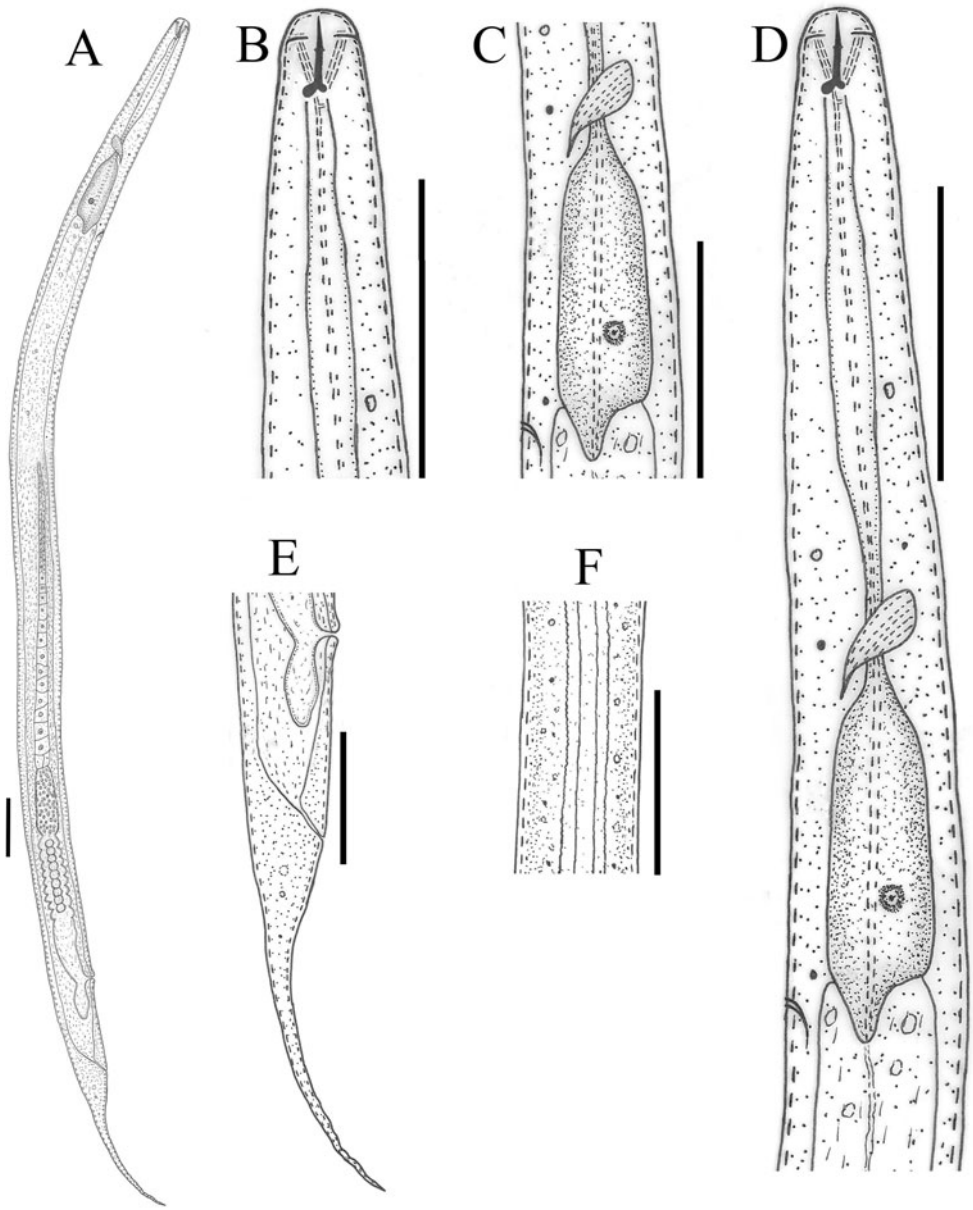

Fig. 1. Line drawing of female of *Paurodontoides siddiqii* n. sp. (A) Entire body; (B) anterior end; (C) basal bulb region; (D) pharynx; (E) vulva to tail end; (F) lateral fields. Scale bars: 30 μm.

Esmaeili, Heydari & Ye, 2016; *P. persica* Esmaeili, Heydari & Ye, 2017; *P. gilanica* Yaghoubi, Pourjam & Pedram, 2018; *Anguillonema amolensis* Mobasseri, Pedram & Pourjam, 2017; *A. iranicum* Yaghoubi, Pourjam & Pedram, 2018 and *Deladenus persicus* Miraeiz, Heydari & Golhasan, 2017) from the suborder Hexatylina Siddiqi, 1980 have been described from Iran.

During a nematology survey conducted in western Iran in 2017, a population of *Paurodontoides* was recovered from bark samples collected from dead or weakened pine trees. The population size ranged from one to 10 nematodes per 100 g of the bark. It belongs to the genus *Paurodontoides* mainly by having a short stylet with asymmetrical minute rounded knobs, with the dorsal knob smaller and anteriorly located compared to the subventral knobs, four incisures in the lateral fields, a short stem-like extension projecting into the lumen of intestine, and a prominent PUS. These traits prompted us to perform a detailed morphological and molecular study to compare this population with previously described species of the genus and also with some species in the genus *Paurodontus* having close morphology. The detailed observations revealed the recovered species is distinct from all species of both aforementioned genera, and it is described herein as *Paurodontoides siddiqii* n. sp.

The objectives of this study were to describe the newly recovered species and perform molecular phylogenetic analyses of it based on two partial rDNA 18S and 28S D2-D3 sequences.

Materials and methods

Sampling, extraction, mounting and drawing

Specimens of *Paurodontoides siddiqii* n. sp. were obtained from bark samples of a dead Mondell pine tree (*Pinus eldarica* L.)

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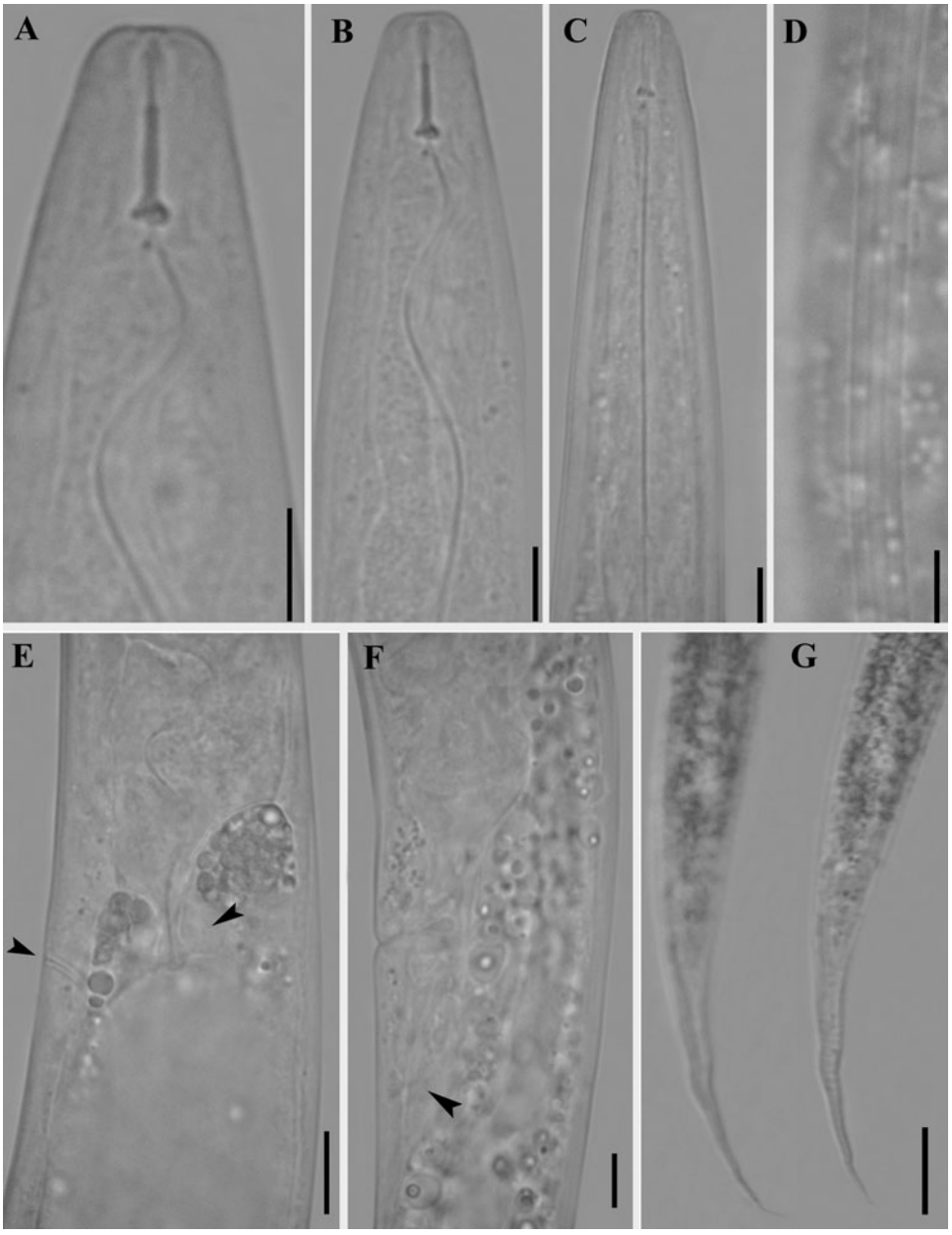

Fig. 2. Photomicrographs of female of *Paurodontoides siddiqii* n. sp. (A, B, C) Anterior end; (D) lateral fields; (E) basal bulb region, with arrowheads showing the excretory pore and basal pharyngeal bulb stem; (F) vulva region, with arrowhead showing the post-uterine sac; (G) posterior ends (tails). Scale bars: 5 µm.

collected in Kermanshah Province, western Iran in August 2017. To obtain a cleaner suspension of nematodes, the tray method (Whitehead and Hemming, 1965) of extraction was employed. Nematodes of interest were hand-picked, killed, fixed and transferred to anhydrous glycerin (De Grisse, 1969). Permanent slides were prepared, and studied under a light microscope (Nikon E200). Drawings were made using a drawing tube attached to the same microscope. Photographs of live nematodes were taken using a digital camera.

Molecular characterization

For DNA extraction, an adult nematode was hand-picked and placed in a small drop of TE buffer (10 mM Tris–Cl, 0.5 mM EDTA; pH 9.0, Qiagen) on a clean slide and crushed using a sterilized razor blade. The suspension, as the DNA template, was collected by adding 20 µl TE buffer and stored at −20°C until used for polymerase chain reaction (PCR). A combination of forward primer 1096F (5′-GGT AAT TCT GGA GCT AAT AC-3′) and reverse primer 1912R (5′-TTT ACG GTC AGA ACT AGG G-3′) and forward primer 1813F (5′-CTG CGT GAG AGG TGA AAT-3′) and reverse primer 2646R (5′-GCT ACC TTG TTA CGA CTT TT-3′) were used for PCR amplification and DNA sequencing of SSU (Holterman *et al.*, 2006). The D2-D3 expansion segments of 28S rDNA were amplified using the D2A (5′-ACA AGT ACC GTG AGG GAA AGT TG-3′) and D3B (5′-TCG GAA GGA ACC AGC TAC TA-3′) primers (Nunn, 1992). PCR was performed in a final volume of 25 µl, which contained 12.5 µl 2X Go*Taq* DNA polymerase mix (Promega Corporation, Madison, WI, USA), 1.2 µl each of the

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forward and reverse primers (5 pM/µl), 8 µl distilled water and 2.1 µl of DNA template. The PCR cycle conditions for both markers were as follows: one cycle of 94°C for 2 minutes, followed by 35 cycles of 94°C for 30 s, annealing temperature of 55°C for 40 s, 72°C for 80 s, and finally one cycle of 72°C for 10 minutes. PCR products were purified after amplification using ExoSAP-IT (Affmetrix, USB products) and sequenced directly for both strands using the same primers with an ABI 3730XL sequencer (Macrogen Macrogen Corporation, South Korea). The newly obtained sequences were submitted to the GenBank database under accession numbers MG836263 for 18S rDNA and MG836264 for partial 28S rDNA D2-D3.

Phylogenetic analyses

The chromatograms of the newly obtained DNA sequences were edited with ChromasPro1.5 2003-2009 (Technelysium Pty Ltd, Helensvale, Australia). The available sequences of representatives of Hexatylina were retrieved from GenBank for 18S or 28S datasets. The downloaded sequences and the newly obtained sequences of the new species were aligned using ClustalW (http://workbench.sdsc.edu; Bioinformatics and Computational Biology group, Dept. Bioengineering, UC San Diego, CA). Outgroup taxa for each dataset were chosen according to previous study (Esmaeili *et al.*, 2016a). The model of base substitution was selected using MODELTEST version 3.06 (Posada and Crandall, 1998) based on the Akaike criterion (Arnold, 2010). Bayesian analysis was performed using MrBayes 3.1.0 (Huelsenbeck and Ronquist, 2001), running the chains for 1,000,000 generations and setting the 'burnin' at 25%. The Markov chain Monte Carlo (MCMC) method was used within a Bayesian framework to estimate the posterior probabilities (pp) of the phylogenetic trees (Larget and Simon, 1999) using the 50% majority-rule. The λ^2 test for homogeneity of base frequencies and phylogenetic trees were performed using PAUP* version 4.0 (Sinauer Associates, Inc. Publishers, Sunderland, MA).

Table 1. Morphometrics of female of *Paurodontoides siddiqii* n. sp. from Iran. All measurements are in µm and in the form mean ± SD (range).

	Female	
Character	Holotype	Paratypes
n	–	15
L	710	660 ± 54.3 (550–729)
a	32.3	31.3 ± 1.8 (27.5–34.3)
b	5.9	5.8 ± 0.5 (4.7–6.6)
c	7.8	7.2 ± 0.6 (6.5–8.3)
c'	6.5	6.5 ± 0.8 (5.3–8.4)
V	78	79.2 ± 1.4 (77.3–82.6)
Lip region height	2.0	2.1 ± 0.2 (2.0–2.5)
Lip region width	7.0	6.2 ± 0.7 (5.0–7.0)
Stylet length	7.0	7.9 ± 0.4 (7.0–8.0)
Nerve ring from anterior end	75	75.2 ± 6.8 (60–84)
Excretory pore from anterior end	116	102 ± 10.5 (85–125)
Pharynx length	120	114 ± 10.2 (89–128)
Post-uterine sac length	20	19 ± 2.1 (15–22)
Ovary length	382	294 ± 31.3 (276–382)
Body diameter at vulva (VBD)	18	18.8 ± 1.3 (18–22)
PUS/VBD	1.1	1.0 ± 0.1 (0.8–1.2)
Distance vulva–posterior end	143	137 ± 8.9 (115–150)
Anal body diameter	14	14.3 ± 1.8 (12–18)
Tail length	91	92.2 ± 6.0 (84–104)

n, number of specimens observed; *L*, body length; *a* = *L*/maximum width; *b* = *L*/pharyngeal length; *c* = *L*/tail length; *c'* = tail length/body diameter at anus; *V* = distance of vulva from anterior end × 100/*L*

Results

Paurodontoides siddiqii *n. sp. (figs 1 and 2; see table 1 for measurements)*

Description

Female. Body straight to slightly ventrally arcuate when heat-relaxed. Cuticle weakly annulated. Lateral field 3–4 µm wide at mid-body, with four incisures (i.e. three bands) at vulval region, not areolated. Lip region flattened, smooth and continuous with body contour. Stylet length almost equal to head width, delicate with asymmetrical knobs, the subventral knobs larger than the dorsal knob and posterior to it. The dorsal gland orifice (DGO) just posterior to the subventral knobs. Procorpus wide, muscular, metacorpus not well developed, without refractive valve, isthmus narrow, basal bulb large, with short stem-like extension projecting into the lumen of the intestine, 5–8 µm long. Neck region (anterior end to base of pharynx) 89–128 µm long, comprising 16–21% of total body length. Nerve ring surrounds isthmus at 60–84 µm distance from anterior end. Excretory pore near the base of the pharyngeal bulb or posterior to it, with moderately sclerotized duct. Hemizonids distinct, 2–3 µm long, anterior to excretory pore. Reproductive system monodelphic–prodelphic, occupying 77–80% of the body length, ovary outstretched, with double and single rows of oocytes in proximal and distal part respectively, oviduct cellular, spermatheca rounded to ellipsoid, axial, filled with spheroid sperm cells, crustaformeria composed of 8–10 rows of cells, vulva a transverse slit, vagina extending into body for slightly less than 50% body diameter, PUS prominent, occupying 33–52% of distance from vulva to anus and *c.* 0.9 times the corresponding body diameter long. Vulva–anus distance 40–52 µm long. Tail elongate-filiform.

Male. Not found.

Type host and locality. Isolated from wood and bark samples of a dead tree of *Pinus eldarica* in Torshekiban forest, city of Gilan-e-Gharb, Kermanshah Province, western Iran, in August 2017. GPS coordinates: 33°59′N, 46°12′E, 1248 m a.s.l.

Type material. Holotype female (slide 001PSS) together with seven paratype specimens (five females, slides: 002PSS–004PSS) were deposited in the Nematode Collection of the Department of Plant Protection, College of Agriculture and Natural Resources, University of Tehran, Karaj, Iran. Three paratype females deposited in the USDA Nematode Collection, Beltsville, MD, USA, and five paratype females deposited in the National Nematode Collection of the Department of Nematology, Iranian Research Institute of Plant Protection, Tehran, Iran.

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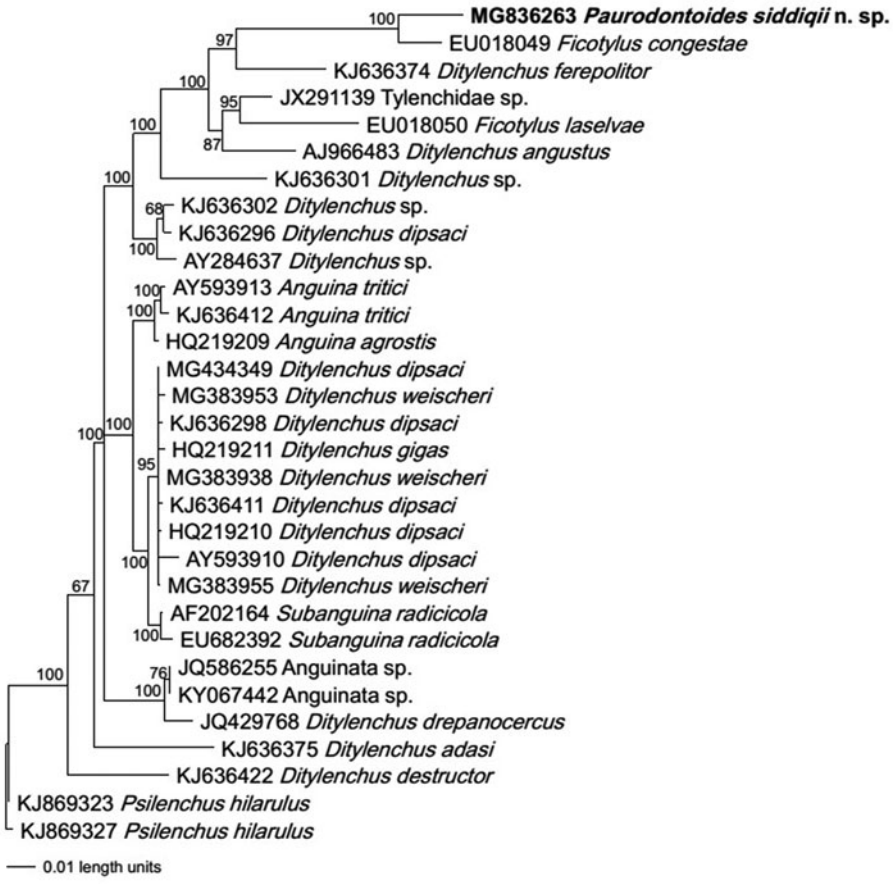

Fig. 3. Bayesian consensus tree inferred from 18S under GTR+I+G model (-lnL = 5899.2671; AIC = 11,818.5342; freqA = 0.2451; freqC = 0.2176; freqG = 0.2709; freqT = 0.2664; R(a) = 1.4026; R(b) = 4.5611; R(c) = 2.166; R(d) = 0.3455; R(e) = 9.0799; R(f) = 1; Pinva = 0.5473; Shape = 0.8251). Posterior probability values exceeding 50% are given on appropriate clades.

Diagnosis and relationships

Paurodontoides siddiqi n. sp. is characterized by 660 (550–729) µm long females having a lateral field with four lines, stylet 7.9 (7–8) µm long with asymmetrical knobs, the subventral knobs larger than the dorsal and posterior to it, excretory pore at 102 (85–125) µm from anterior end, basal bulb with short stem-like extension projecting into the intestine and elongate-filiform tail.

According to Siddiqi (2000), *Paurodontoides* contains two known species, namely the type species, *P. linfordi*, and *P. latus*.

The new species differs from the type species, *P. linfordi*, by having slightly shorter stylet (7–8 *vs* 9–10 µm), lateral field with smooth outer incisures *vs* crenate, the position of the excretory pore (at the level with, or posterior to basal bulb-intestine junction *vs* anterior to it), and absence of male in population *vs* presence.

Compared to *P. latus*, the new species has a shorter body (550–729 *vs* 700–1100 µm), shorter stylet (7–8 *vs* 10–12 µm), greater *a* (27.5–34.3 *vs* 17 in the female), lower *c* (6.5–8.3 *vs* 18 in the female), more anteriorly located vulva (*V* = 78 *vs* 85), position of excretory pore (at the level with, or posterior to basal bulb–intestine junction *vs* anterior to it), female tail shape (elongate-filiform *vs* conical, ventrally bent, with small rounded tip) and absence of male *vs* presence.

Because of close morphological similarities between two genera, *Paurodontoides* and *Paurodontus* Thorne, 1941 (both genera are differentiated based on the position of vulva from anus and the nature of bursa at male tail), the new species was also compared with similar species under *Paurodontus*.

By having a prominent PUS (*vs* a short PUS in *Paurodontus* spp.), stylet knob characters (asymmetrical in the case of the new species and type species of the genus), and vulva at less than two body widths from anus (*vs* more than two body widths), the new species was assigned to the genus *Paurodontoides*.

In comparison with four known species of the genus *Paurodontus* having four lines in lateral field, the new species has asymmetrical stylet knobs (*vs* symmetrical).

From *P. brassicae* Das & Shivaswamy, 1980, the new species differs by having lower *c* (5.3–8.4 *vs* 9.6–12.1) and by the position of the excretory pore (at the level or posterior to pharyngeal bulb–intestine junction *vs* anterior); from *P. chawdhuri* Husain & Khan, 1965 by a shorter body (550–729 *vs* 750–930 µm), shorter stylet (7–8 *vs* 11 µm), lower *b* (4.7–6.6 *vs* 7.8–8.8) and lower *c* (5.3–8.4 *vs* 11.5–23.2); from *P. gracilis* Thorne, 1941 by absence *vs* presence of chamber encircling pharyngeal bulb, prominent PUS *vs* rudimentary, ovary arranged in double *vs* single file; and from *P. similis* Siddiqi, 1961 by having shorter stylet (7–8 *vs* 9–10 µm), lower *c* (5.3–8.4 *vs* 9.5–10.5) and tail with pointed tip *vs* rounded.

Etymology

Named in honor of Prof. Mohammad Rafiq Siddiqi, the pioneer taxonomist of hexatylenchid nematodes.

Phylogenetic position of Paurodontoides siddiqii *n. sp. within Sphaerularioidea*

The partial 18S rDNA gene sequence of *P. siddiqii* n. sp. (GenBank accession number MG836263) had < 96% identity with available DNA sequences deposited in GenBank. The highest

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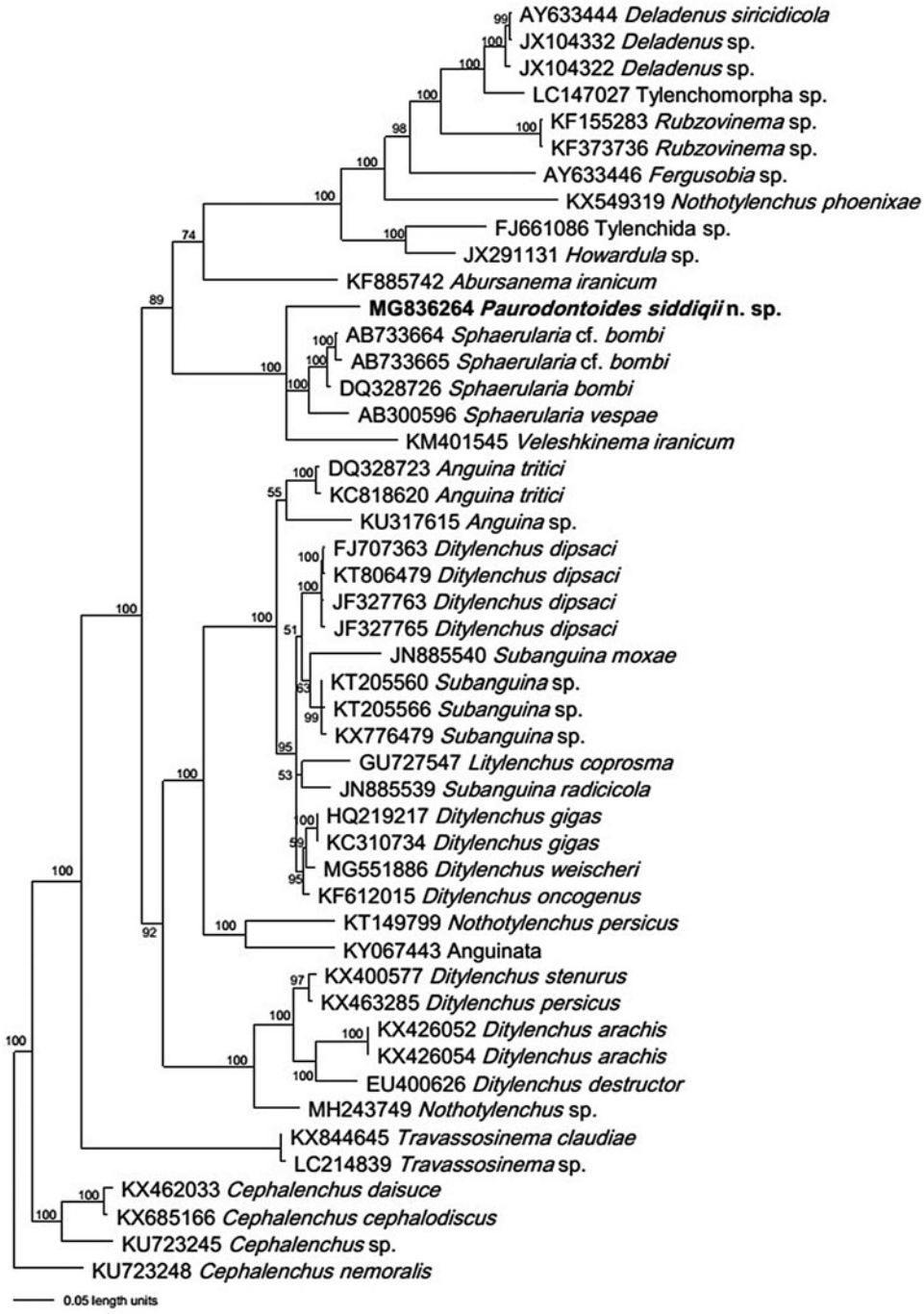

Fig. 4. Bayesian consensus tree inferred from 28S D2/D3 under GTR+I+G model (-lnL = 9676.082; AIC = 19372.1641; freqA = 0.2129; freqC = 0.2017; freqG = 0.2956; freqT = 0.2899; R(a) = 1.0709; R(b) = 7.4588; R(c) = 2.4965; R(d) = 0.9473; R(e) = 10.8704; R(f) = 1; Pinva = 0.2218; Shape = 1.1544). Posterior probability values exceeding 50% are given on appropriate clades.

matched sequence was that of *Ficotylus congestae* Davies, Ye, Giblin-Davis and Thomas, 2009 (EU018049), with 96% identity (34 indels, 23 gaps). The 28S D2-D3 sequence of *P. siddiqii* n. sp. (MG836264) was < 82% homologous from any available DNA sequences from GenBank. The BlastN search revealed the highest match was with *Sphaerularia* spp. (AB300596, DQ328726, AB733665 and AB733664), with 80–82% identity (44–72 indels, 36–49 gaps).

The phylogenetic tree inferred using the partial 18S rDNA by Bayesian inference (BI) using the GTR + I+G model is presented in fig. 3. This tree includes 29 ingroup and two outgroup taxa (the species names and accession numbers in the tree). *Psilenchus hilarulus* de Man, 1921 (KJ869323, KJ869327) in the family Tylenchidae was used as the outgroup taxon in phylogenetic analysis. A λ^2 test ($\lambda^2 = 12.704562$ (df = 90), $P = 1.00000000$) revealed the highest homogeneity in 18S sequences among the species selected. This tree revealed that the new species forms a clade with *Ficotylus congestae* (EU018049), with 100% support. The species *Ditylenchus ferepolitor* (KJ636374) is the sister taxon to this clade.

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The Bayesian phylogenetic tree inferred using the 28S D2-D3 dataset using the GTR+I+G model is presented in fig. 4. This tree contained 44 ingroup and four outgroup taxa (the species names and accession numbers in the tree). This tree is rooted with *Cephalenchus* sp. (KU723245), *C. nemoralis* Mizukubo and Minagawa, 1985 (KU723248), *C. cephalodiscus* Sultan and Jairajpuri, 1982 (KX685166) and *C. daisuce* Mizukubo and Minagawa, 1985 (KX462033). A λ^2 test ($\lambda^2 = 142.028327$ (df = 141), $P = 0.45986390$) revealed a medium level of homogeneity with high variation in 28S sequences among the species selected. In this tree, members of the superfamily Sphaerularioidea have divided into two main monophyletic groups, anguind and sphaerulariid species, with 100% support. In this tree, the new species form a clade with members of the Sphaerulariidae (*Veleshkinema iranicum* (KM401545) and *Sphaerularia* spp.).

Discussion

According to Siddiqi, the families Sphaerulariidae and Paurodontidae are synonymous, and the study of this new species (as a member of Paurodontidae) in our phylogenetic trees based on two different regions of the rDNA gene supports his opinion. Results from our phylogenetic analysis are in agreement with Yaghoubi *et al.* (2014), Miraeiz *et al.* (2015), Esmaeili *et al.* (2017) and Mobasseri *et al.* (2017), indicating the close relationships amongst the genera of Sphaerulariidae and Paurodontidae.

The genus *Paurodontoides* is one of the rare Tylenchomorpha De Ley & Blaxter, 2002 genera. It is characterized mainly by having stylet knobs, excretory pore almost at the level with nerve ring, basal bulb with a stem-like extension projecting into the intestine, presence of a prominent PUS, simple vulval lips and bursa completely enclosing tail tip (Siddiqi, 2000). However, the assigning of *Paurodontoides* to Paurodontinae by Andrássy (2007) could be logical, as the characters of pharynx (basal bulb with a stem-like extension projecting into the intestine in various positions) support such a taxonomic placement. There are also similarities in their biology (fungus-feeding generation is well known and nothing is known about entomoparasitic forms). Currently, only two species of *Paurodontoides* are described (Siddiqi, 2000), both of which are poorly described in the shape of original descriptions (no other reports or redescriptions are available), and light microphotographs or molecular data are not available.

The new species belongs to the genus *Paurodontoides* on the basis of commonly shared characters, i.e. an elongate fusiform and not well-developed median pharyngeal bulb, basal bulb with a stem-like extension projecting into the intestine, and presence of a prominent PUS. The new species shares some morphological characters (e.g. nonvalvate median pharyngeal bulb and a stem-like basal bulb with an extension projecting into the intestine) with some other members of the family Paurodontidae, including *Abursanema*, *Bealius*, *Misticius* Massey, 1967 and *Paurodontella* Husain & Khan, 1968. *Paurodontoides siddiqii* n. sp. can be distinguished from *Abursanema* by having a stylet with basal knobs (*vs* without knobs). It differs from *Misticius* by its excretory pore opening at the end of basal bulb region (*vs* near the stylet base). It can be distinguished from *Bealius* by having a prominent PUS (*vs* absent), and it can be distinguished from *Paurodontella* by presence of prominent PUS (*vs* absent or rudimentary).

Recently, molecular markers and phylogenetic inferences have been proven to be an effective method of delimiting species of the complex and morphologically conserved sphaerularioid group members (Yaghoubi *et al.*, 2014; Golhasan *et al.*, 2016; Miraeiz *et al.*, 2015; Esmaeili *et al.*, 2016a, b, 2017). According to our 18S and partial 28S trees (figs 3 and 4) and other previous studies (Koshel *et al.*, 2014; Yaghoubi *et al.*, 2014; Miraeiz *et al.*, 2015; Esmaeili *et al.*, 2016a, b, 2017; Golhasan *et al.*, 2016; Mobasseri *et al.*, 2017), most genera and families of Hexatylina are not monophyletic, using rDNA sequences; however, the molecular sequences are not available for most representatives of this suborder (*sensu* Siddiqi, 2000).

In this study a new species of *Paurodontoides* was described using morphological and molecular data. Based on morphological similarities, the new species was assigned to the genus. This is the first species in *Paurodontoides* with DNA sequence data. In the future, more DNA sequences are needed from two closely related genera, *Paurodontoides* and *Paurodontus*, to examine their phylogenetic relationships. This could shed light on the taxonomic status of species. Although several families and genera of this group of nematodes are not monophyletic based on ribosomal DNA sequence data, they are still useful to separate different species.

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Conflict of interest. None.

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