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Nothotylenchus andrassy n. sp. (Nematoda: Anguinidae) from Northern Iran

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Abstract

Nothotylenchus andrassy n. sp. is described and illustrated from moss (*Sphagnum* sp.) based on morphology and molecular analyses. Morphologically, this new species is characterized by a medium body size, six incisures in the lateral fields, and a delicate stylet (8–9 µm long) with clearly defined knobs. Pharynx with fusiform, valveless, non-muscular and sometimes indistinct median bulb. Basal pharyngeal bulb elongated and offset from the intestine; a long post-vulval uterine sac (55% of vulva to anus distance); and elongate, conical tail with pointed tip. *Nothotylenchus andrassy* n. sp. is morphologically similar to five known species of the genus, namely *Nothotylenchus geraerti*, *Nothotylenchus medians*, *Nothotylenchus affinis*, *Nothotylenchus buckleyi*, and *Nothotylenchus persicus*. The results of molecular analysis of rRNA gene sequences, including the D2–D3 expansion region of 28S rRNA, internal transcribed spacer (ITS) rRNA and partial 18S rRNA gene are provide for the new species.

Key words

18S rRNA, D2–D3 region, Molecular, Morphology, Moss, *Sphagnum* sp., ITS rRNA.

The genus *Nothotylenchus* Thorne, 1941 belongs to subfamily Anguinidae Nicoll, 1935 within the family Anguinidae Nicoll, 1935. Dong-Geun et al. (2005) showed that the type species of the genus, *Nothotylenchus acris*, is a parasite of strawberry (*Fragaria* × *ananassa* Duchesne). Nishizawa and Iyatomi (1955) also reported this nematode in association with strawberry diseases in Japan. *Nothotylenchus* species are morphologically closely related to members of *Ditylenchus* Filipjev, 1936, but they differ in morphology of the median pharyngeal bulb which is indistinct or non-muscular, non-valvate in *Nothotylenchus*. Furtuner and Maggenti (1987) and Sturhan and Brzeski (1991) did not give due taxonomic importance to the presence/absence of a muscular, valvate median pharyngeal bulb, even at the generic level, and considered *Nothotylenchus*, *Diptenchus* Khan et al., 1969, *Safianema* Siddiqi, 1981 and *Orrina* Brzeski, 1981 as synonyms of *Ditylenchus*. But some authors (Siddiqi, 2000; Andrássy, 2007) kept these genera valid. Relationships within Anguinidae were not well resolved in the phylogenetic analysis using the D2–D3 expansion segments of 28S, ITS, and partial 18S rRNA gene sequences (Subbotin et al., 2004). Here, we followed the classification scheme of Siddiqi (2000) and Andrássy (2007).

To date more than 48 species have been recognized for *Nothotylenchus*, although some species are considered as *species inquirendae* (Andrássy, 2007). Recently two new species of the genus, *Nothotylenchus persicus* Esmaeli et al., 2016 and *Nothotylenchus phoenixae* Esmaeili et al., 2017 were described in Iran from rhizosphere of grapevine (*Vitis* spp.) and date palm (*Phoenix dactylifera* L.), respectively. During a nematode survey on eastern forests of Guilan province, northern Iran, an unknown anguinid nematode population belonging to the genus *Nothotylenchus* was recovered from moss samples (*Sphagnum* sp.). Detailed observations using light microscopy and molecular assays indicated that this population differed from all previously described members of the genus and should be assigned to a new species. This publication includes a description

of *Nothotylenchus andrassy* n. sp. through morphological observation and molecular characterization by the partial 18S rRNA, D2–D3 expansion region of 28S rRNA and ITS rRNA gene sequences.

Materials and methods

Sampling, extraction, mounting, and drawing

Soil, root, and moss samples, were randomly collected from different regions of eastern forests of Guilan province, northern Iran during 2015. Nematodes were extracted from sample materials by the tray method (Whitehead and Hemming, 1965) and were soaked in a small amount of water for 48 hr. The extracted nematodes were observed and hand-picked using a stereomicroscope. Adult specimens for microscopic observation were killed by gentle heat and fixed in a solution of FGA 4:1:1 (formaldehyde, glycerin, and acetic acid) and processed to anhydrous glycerin (De Grisse, 1969). Permanent slides were made and examined using an Olympus BH2 light microscope. Morphometric data were obtained using a drawing tube and photomicrographs were taken using a digital camera. Line drawings were redrawn using CorelDraw® software version 17.

DNA extraction, polymerase chain reaction, and sequencing

Single nematode specimens were handpicked and examined individually by light microscopy and transferred to 10 µl of distilled water on a glass microscope slide, crushed with a pipette tip and collected in 50 µl AE buffer (10 mM *Tris*-Cl, 0.5 mM EDTA; pH 9.0, Qiagen, Valencia, CA) by pipette. DNA extracts were stored at −20°C until used as template for polymerase chain reaction (PCR) amplification.

A volume of 1 µl of extracted DNA was transferred to an Eppendorf tube containing: 2.5 µl 10× NH_4 reaction buffer, 0.75 µl $MgCl_2$ (50 mM), 0.25 µl dNTPs mixture (10 mM each), 0.75 µl of each primer (10 mM), 0.2 µl BIOTAQ DNA Polymerase (Bioline, UK) and ddH_2O to a final volume of 25 µl. The D2–D3 expansion segment of 28S rRNA gene was amplified using the forward D2A (5′-ACAAGTACCGTGAGGGAAAGTTG-3′) and reverse D3B (5′-TCGGAAGGAACCAGCTACTA-3′) primers (Nunn, 1992). The ITS region was amplified using forward primer TW81 (5′-GTTTCCGTAGGTGAACCTGC-3′) and reverse primer 5.8SM5 (5′-GGCGCAATGTGCATTCGA-3′) (Maafi et al., 2003; Vovlas et al., 2008), and the partial 18S was amplified using primers 1096F (5′-GGTAATTCTGGAGCTAATAC-3′), 1912R (5′-TTTACG GTCAG-AACTAGGG-3′) (Holterman et al., 2006). PCR cycle conditions were as follows: one cycle of 94°C for 2 min, followed by 35 cycles of 94°C for 30 sec, annealing temperature of 55°C for 45 sec, 72°C for 3 min, and finally one cycle of 72°C for 10 min. PCR products were purified after amplification using ExoSAP-IT (Affmetrix, USB Products), quantified using a Nanodrop spectrophotometer (Nanodrop Technologies) and used for direct sequencing in both directions using the primers referred to above.

Phylogenetic analyses

Newly obtained sequence of the D2–D3 expansion region of 28S, ITS, and partial 18S rRNA and available sequences of anguinid nematodes obtained from GenBank were used for phylogenetic reconstructions. The newly obtained and published sequences were aligned using Muscle (Edgar, 2004) with default parameters implemented in MEGA 5.0 (Tamura et al., 2011). Sequence alignment was edited using MEGA 5.0. The most appropriate model was determined using the Bayesian Information Criterion (BIC) implemented in the jModelTest program (Posada, 2008). Phylogenetic analyses of the sequence data set was performed based on Bayesian inference (BI) using MRBAYES3.1.2 (Ronquist and Huelsenbeck, 2003). The topologies were used to generate a 50% majority rule consensus tree. Posterior probabilities (PP) were given on appropriate clades. Trees were visualized using TreeView (Page, 1996).

Results

Nothotylenchus andrassy n. sp. (Table 1; Figs. 1, 2)

Description

Females

Body subcylindrical, tapering at both ends and almost straight upon fixation. Cuticle with transverse striae measuring *ca* 1.1 µm wide in mid-body region. Lateral fields with six incisures occupying a third of body diam. Lip region continuous with body 5 to 6 µm wide and 2 to 3 µm high. Stylet 8 to 9 µm long with well developed basal knobs, conical part occupying 44% to 50% of total stylet length. Orifice of dorsal pharyngeal gland at 1 to 1.5 µm posterior to stylet knobs. Pharynx with cylindrical corpus and valveless, non-muscular, fusiform median bulb which is indistinct in some specimens and connecting to an elongate pharyngeal basal bulb via a slender isthmus. Basal pharyngeal bulb is offset from intestine.

Table 1. Morphometrics of *Nothotylenchus andrassy* n. sp. All measurements in micrmeter and in the form: mean ± s.d. (range).

		Female	Male
Character	Holotype	Paratypes	Paratypes
n	`	6	4
L	778	754.7 ± 98.6 (681–962)	640 ± 70.7 (574–738)
a	38.9	35.3 ± 3.6 (30.3–38.9)	34.1 ± 1.7 (31.9–35.6)
b	6.1	5.9 ± 0.4 (5.4–6.4)	5.4 ± 0.4 (5.2–6.0)
c	13.2	11.7 ± 1.3 (8.9–13.2)	10.6 ± 1.7 (8.4–12.5)
c¢	4.5	5.0 ± 0.6 (4.4–6.0)	4.7 ± 0.9 (3.8–5.9)
V or *T*	81.6	80.7 ± 1.7 (77.1–82.4)	47.6 ± 1.8 (45.3–49.7)
Lip region height	2.0	2.0	2.3 ± 0.5 (2.0–3.0)
Lip region width	5.0	5.4 ± 0.5 (5.0–6.0)	5.3 ± 0.5 (5.0–6.0)
Stylet length	9.0	8.3 ± 0.5 (8.0–9.0)	8.1 ± 0.3 (8.0–8.5)
m[a]	44.4	45.7 ± 2.9 (43.8–50.0)	49.2 ± 3.9 (43.8–52.9)
E. pore from anterior end	103	99.0 ± 7.4 (92–112)	92 ± 3.9 (87–96)
Pharynx length	128	127.4 ± 16.2 (114–162)	117.3 ± 6.0 (109–123)
Max. body diam.	20	21.6 ± 3.7 (18–28)	18.8 ± 1.5 (18–21)
Vulval body diam. (VBD)	19	19.7 ± 3.3 (17–26)	–
Vulva–anus distance (V–A)	84	80.3 ± 14.8 (70–112)	–
Post-uterine sac (PUS) length	45	43.4 ± 5.6 (39–55)	–
PUS/Vulval body diam.	2.4	2.2 ± 0.2 (2.0–2.4)	–
PUS/V–A%	53.6	54.6 ± 4.3 (49.1–62.0)	–
Ovary length or testis	410	358.7 ± 69.6 (275–457)	305 ± 42.1 (273–367)
Anal (cloacal) body diam.	13	13.0 ± 2.3 (11–18)	13.0 ± 1.4 (12.0–15.0)
Spicule length	–	–	18.0 ± 0.6 (18.0–19.0)
Gubernaculum length	–	–	5.3 ± 0.5 (5.0–6.0)
Tail length	59	66.1 ± 18.6 (55–108)	62.3 ± 18.0 (46–88)
Bursa (% of tail)	–	–	28.0 ± 3.4 (24.6–32.6)

[a]Length of conus as percentage of total stylet length.

Excretory pore 92 to 112 µm from anterior end of body. Hemizonid about two to three body annuli wide and situated just anterior to excretory pore. Reproductive system prodelphic, ovary outstretched and oocytes arranged in a single row. Uterine quadricolumellar consisting of four rows of four cells, followed by an elongated spermatheca. Spermatheca filled with large rounded sperm cells in some specimens. Vulva a transverse slit with slightly protuberant lips. Vagina straight and reaching almost halfway across body. Post vulval uterine sac well developed occupying 49–62% of the vulva anus distance. Tail elongate, conical, regularly tapering toward a pointed tip, 4.4 to 6 times the anal body diam.

Males

Similar to female in general morphology with usually shorter body size. Lip region is slightly higher than female, 2 to 3 µm high and 5 to 6 µm wide. Cuticle with transverse striae measuring *ca* 1.1 µm wide in mid-body region. Stylet delicate with rounded basal knobs. Testis single, sometimes reflexed at anterior end. Spicules curved ventrally, 18 to 19 µm long.

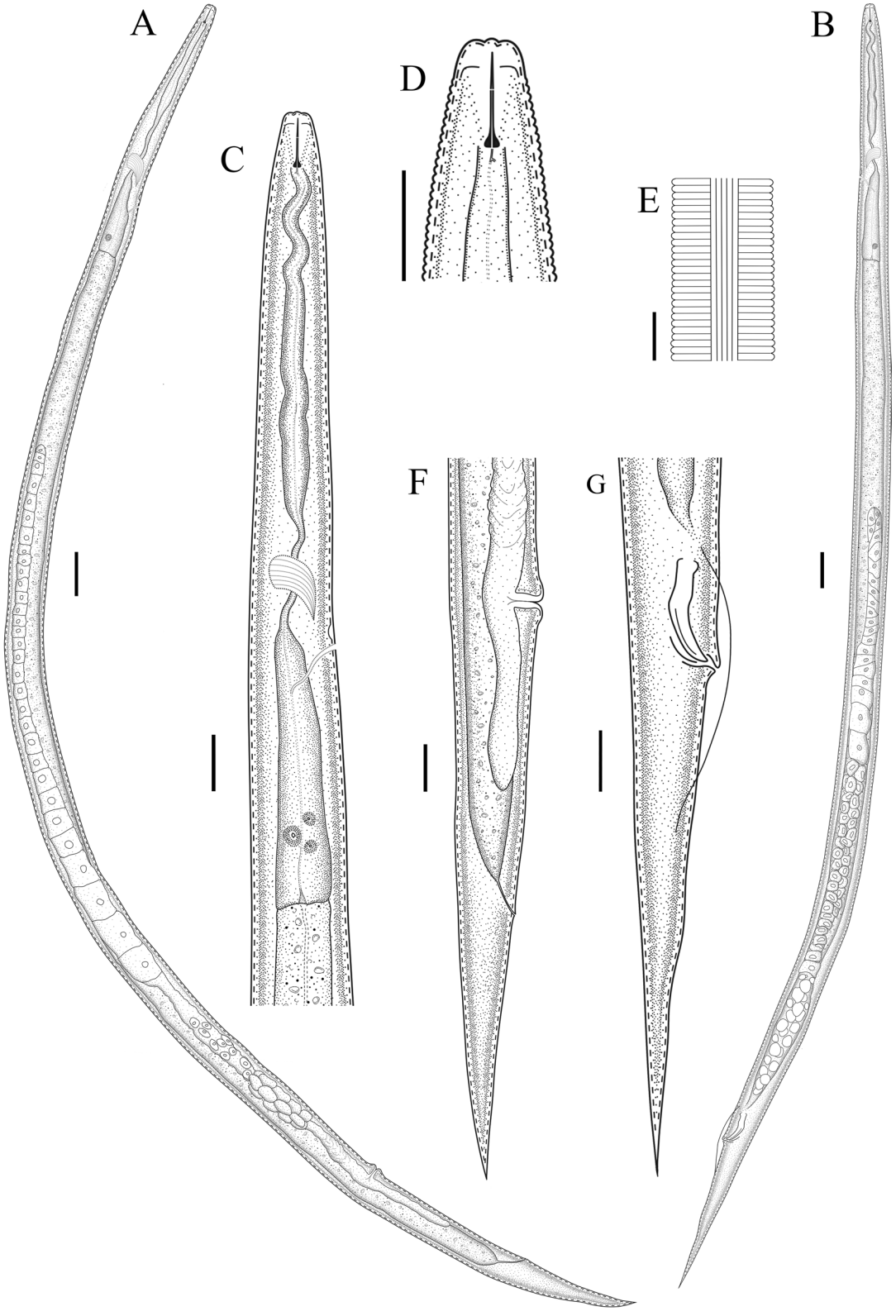

Figure 1: Line drawings of *Nothotylenchus andrassy* n. sp. A, Female body; B, Male body; C, Pharyngeal region of female; D, Female anterior end; E, Lateral field of female; F, Female posterior body; G, Male posterior body. (Scale bars: A and B = 20 µm, C–G = 10 µm).

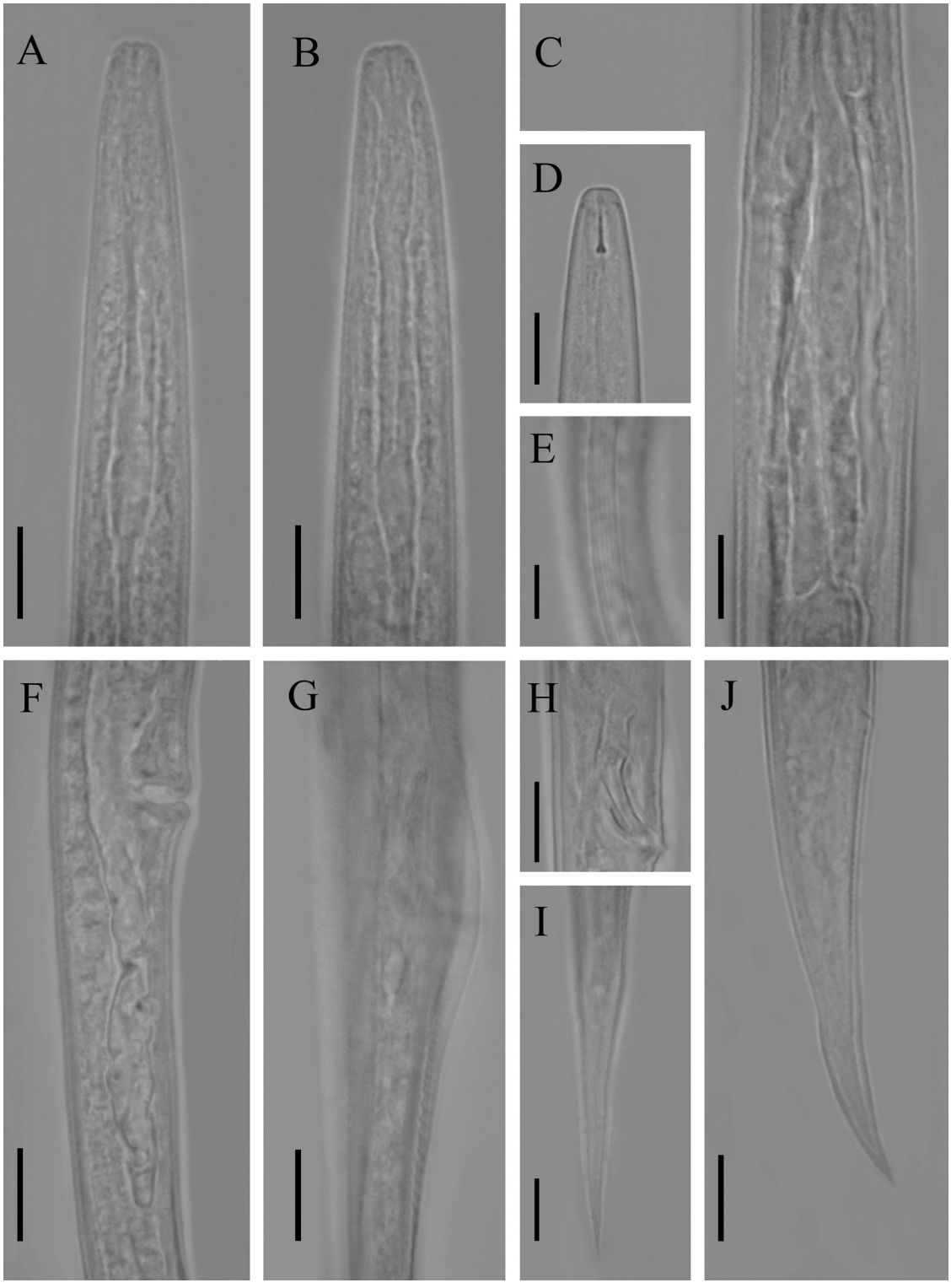

Figure 2: Photomicrographs of *Nothotylenchus andrassy* n. sp. A, Female pharyngeal region showing fusiform median bulb; B, Female pharyngeal region showing indistinct median bulb; C, Basal pharyngeal bulb region; D, Female anterior end; E, Lateral field; F, Vulva region showing post-uterine sac; G, Male posterior body showing leptoderan bursa; H, Spicules lateral view; I, Male posterior body (tail); J, Female posterior body (tail). (All scale bars = 10 µm).

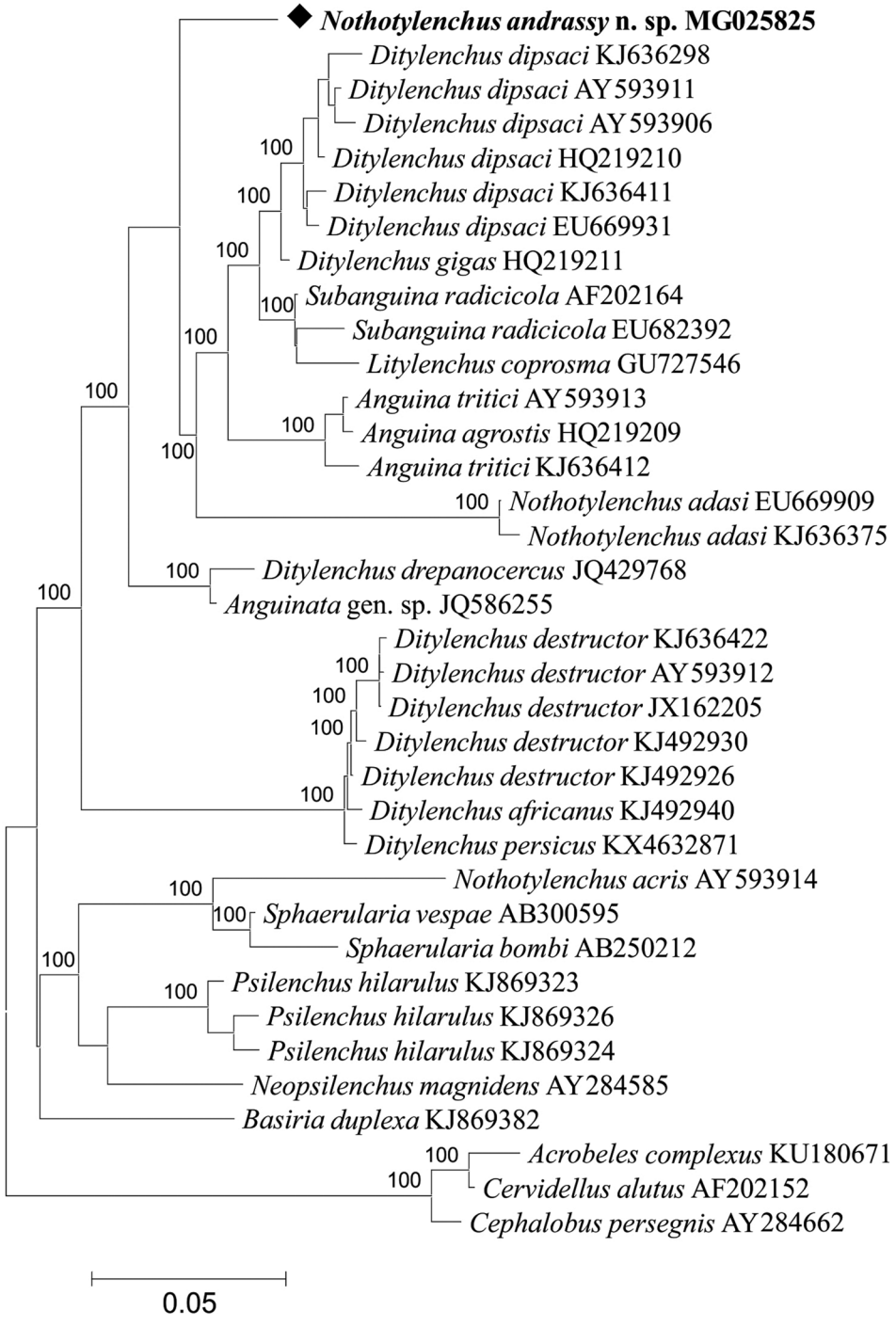

Figure 3: The molecular phylogenetic tree generated from the partial 18S rRNA inferred from Bayesian analyses under GTR+G+I model. Posterior probability values exceeding 50% are given on appropriate clades. The new species is in bold font.

Gubernaculum simple, 5 to 6µm long and slightly less than one third the length of the spicules. Bursa short, leptoderan, beginning almost opposite the proximal end of the spicules and covering 25 to 33% of tail length. Tail elongate, conoid, usually straight, with pointed tip, 4 to 6 times the anal body diam.

Diagnosis and relationships

Nothotylenchus andrassy n. sp. is characterized by a medium body size, six incisures at the lateral fields, a delicate stylet (8–9µm long) with clearly defined knobs; Pharynx with cylindrical corpus, fusiform, valveless and

Figure 4: The molecular phylogenetic tree generated from the D2–D3 of 28S rRNA inferred from Bayesian analyses under GTR + G + I model. Posterior probability values exceeding 50% are given on appropriate clades. The new species is in bold font.

sometimes indistinct median bulb and elongated basal pharyngeal bulb. Vulva at 77% to 82% of body length; a long post-vulval uterine sac (55% of the vulva–anus distance) and elongate, conical tail with pointed tip. *Nothotylenchus andrassy* n. sp. is morphologically and morphometrically similar to *Nothotylenchus geraerti* Kheiri, 1971, *Nothotylenchus medians* Thorne and Malek, 1968, *Nothotylenchus affinis* Thorne, 1941, *Nothotylenchus buckleyi* Das, 1960, and *N. persicus*. The new species differs from *N. geraerti* mainly by more elongate basal pharyngeal bulb, longer spicules (18–19 vs. 16 µm) and tail tip pointed vs. rounded. It differs from *N. medians* by slightly longer stylet (8–9 vs. 6.5–8 µm), slightly longer spicules (18–19 vs. 15–18 µm), shorter bursa as percentage of tail length (25–33 vs. 27–84%) and tail tip pointed vs. rounded. It can be distinguished from *N. affinis* by longer post-vulval uterine sac (2–2.4 vs. 1.1–1.3 times vulval body diam.), slightly longer spicules (18–19 vs. 15–17 µm); slightly shorter bursa length as percentage of tail length (25–33 vs. 50%) and tail tip pointed versus rounded. *Nothotylenchus andrassy* n. sp. can be distinguished from *N. buckleyi* by longer body length (0.68–0.96 vs. 0.43 mm in females and 0.57–0.74 vs. 0.45 mm in males); shorter stylet (8–9 vs. 11 µm), posterior position of the excretory pore (opposite posterior one third of basal pharyngeal bulb vs. opposite middle of isthmus), more posteriorly located vulva (V = 77–82% vs. 71%); and longer spicules (18–19 vs. 15 µm). Finally it can be distinguished from *N. persicus* by longer stylet (8–9 vs. 5–6 µm), anterior position of the excretory pore (opposite posterior one third of basal pharyngeal bulb vs. posterior to basal bulb), longer post-vulval uterine sac (2–2.4 vs. 0.4–0.6 times vulval body diam.) and slightly shorter spicules (18–19 vs. 21–22 µm).

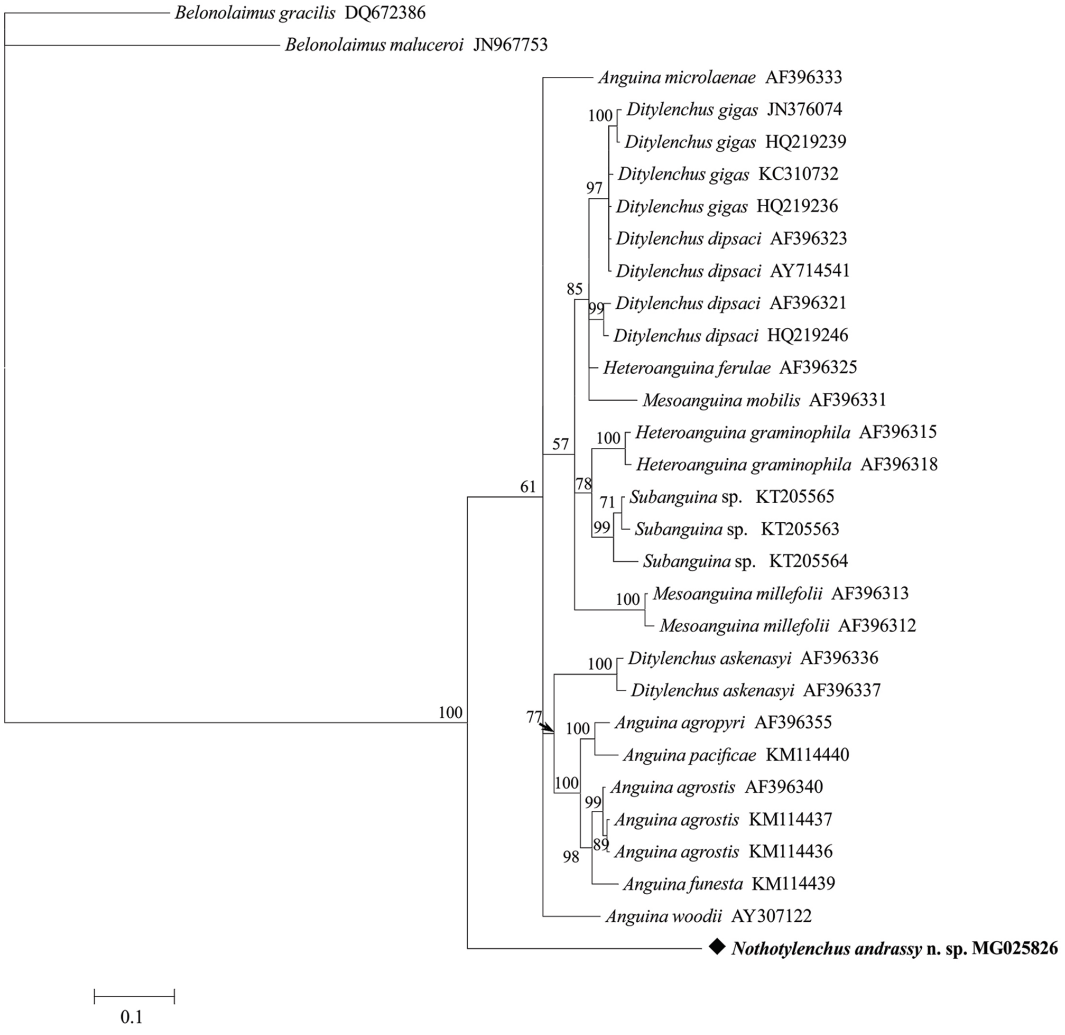

Figure 5: The molecular phylogenetic tree generated from the ITS rRNA inferred from Bayesian analyses under GTR + G + I model. Posterior probability values exceeding 50% are given on appropriate clades. The new species is in bold font.

Type habitat and locality

The new species was recovered from moss samples (*Sphagnum* sp.) in Leila koh region, Langarud, Guilan province (GPS coordinates: 37° 10′ 29′ N, 50° 7′′ 19′′ E), northern Iran.

Type material

Holotype female (slide ANA001) together with four paratype specimens: Two females, two males (slides ANA001, ANA002) deposited in the Nematode Collection of the Department of Plant Protection, College of Agriculture and Natural Resources, University of Tehran, Karaj, Iran. Two female and two male paratypes deposited at Royal Belgian Institute of Natural Sciences, Brussels, Belgium. Paratype females deposited in the National Nematode Collection of the Department of Nematology, Iranian Research Institute of Plant protection, Tehran, Iran.

Etymology

The new species is named in honor of Dr István Andrássy, a pioneering scientist in the systematics of nematodes.

Molecular phylogeny

Amplification of D2–D3 expansion segments of 28S, ITS, and the partial 18S rRNA yielded a single fragment of 615 bp, 430 bp, and 775 bp, respectively.

The molecular phylogenetic trees were obtained from Bayesian analysis under the GTR + I + G model

(Tavaré, 1986) to infer the relative placement of the new species among other species of *Nothotylenchus* and other genera of Anguinidae. The trees inferred by partial 18S, D2–D3 segments of 28S and ITS rRNA are shown in Figs. 3–5, respectively. In these trees, *N. andrassy* n. sp. is shown in bold font.

The BlastN search of partial 18S rRNA gene sequence of *N. andrassy* n. sp. (GenBank MG025825) revealed the highest match with sequences of *Ditylenchus dipsaci* (Kühn, 1857) Filipjev, 1936 (KJ636298 and HQ219210) with 97% identity. The 28S rRNA D2–D3 sequence of *N. andrassy* n. sp. (GenBank MG025824) revealed the highest match with sequences of *Ditylenchus persicus* Esmaeili et al., 2017 (KX463285) with 98% identity. The ITS rRNA sequence of *N. andrassy* n. sp. (GenBank MG025826) revealed the highest match with sequences of *Ditylenchus gigas* Vovlas et al., 2011 (JN376074, HQ219239, HQ219236, KC310732) with 85% identity and less than 85% homology with other available DNA sequences from GenBank.

The molecular phylogenetic tree generated from the partial 18S rRNA included 32 in-group and three outgroup taxa. In this tree *N. andrassy* n. sp. clustered with *Nothotylenchus adasi* Sykes, 1980 (EU669909, KJ636375) and also a clade containing some anguind species, i.e., *D. dipsaci*, *D. gigas*, *Subanguina radicicola* (Greeff, 1872) Paramanov, 1967, *Litylenchus coprosma* Zhao et al., 2011, *Anguina tritici* (Steinbuch, 1799) Chitwood, 1935 and *Anguina agrostis* (Steinbuch, 1799) Filipjev, 1936. *Nothotylenchus andrassy* n. sp. differs from *N. adasi* by having morphological and morphometric differences such as six versus four lines in lateral fields, shorter stylet (8–9 vs. 11–13 µm), shorter spicules (18–19 vs. 22–24 µm), more posteriorly located vulva (77%–82% vs. 68%–76%) and sharply pointed tail tip versus rounded or dull tail tip. The other *Nothotylenchus* species with available 18S rRNA sequence, *N. acris* Thorne, 1941 (AY593914) is in a monophyletic clade having posterior probability support of 100% with *Sphaerularia vespae* Kanzaki et al., 2007 (AB300595), and *Sphaerularia bombi* Dufour, 1837 (AB250212).

The molecular phylogenetic tree generated from D2–D3 expansion segments of 28S rRNA included 36 in-group and three outgroup taxa. In this tree *N. andrassy* n. sp. clustered with *D. persicus* (KX463285) in a clade with 73% posterior probability. It differs morphologically from that species by having valveless fusiform, sometimes indistinct versus valvate, well developed median pharyngeal bulb and some other characters. The other *Nothotylenchus* species with available 28S rRNA sequence, *N. persicus* (KT149799), clustered with *Neotylenchus* sp. and *Ficotylus congestae.*

The molecular phylogenetic tree generated from ITS rRNA included 27 in-group and two outgroup taxa. In this tree all species of Anguinidae grouped in a 100% supported monophyletic clade and *N. andrassy* n. sp. was separated from other anguinid species as the only species in the genus in this tree.

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