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

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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 a new species of Nothotylenchus from moss samples in northern Iran.
of *Nothotylenchus andrassyn* 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 collect-
ed 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 nema-
todes were observed and hand-picked using a stereo-
microscope. Adult specimens for microscopic observa-
tion 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 photomicro-
graphs 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 micro-
scope 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 poly-
merase chain reaction (PCR) amplification.

A volume of 1 μl of extracted DNA was transferred to
an Eppendorf tube containing: 2.5 μl 10× AE reaction
buffer, 0.75 μl MgCl₂ (50 mM), 0.25 μl dNTPs mix-
ture (10 mM each), 0.75 μl of each primer (10 mM), 0.2 μl
BIOTAQ DNA Polymerase (Bioline, UK) and ddH₂O
to a final volume of 25 μl. The D2–D3 expansion seg-
ment of 28S rRNA gene was amplified using the for-
ward D2A (5’-AGAACCTGC-3’)
and reverse D3B (5’-TCGGAAGGAACAGCTACTA-3’)
primers (Nunn, 1992). The ITS region was amplified
using forward primer TW81 (5’-ACAAGTACCGTGAGGGAAAGTTG-3’).
Reverse D2A (5’-ACAAGTACCGTGAGGGAAAGTTG-3’)
and reverse primer 5.8SM5 (5’-GG-CGCAATGTGCATTGCA-3’)
(Maafi et al., 2003; Vovlas et al., 2008), and the partial 18S was amplified using
primers 1096F (5’-GGTAATTCTGGAGCTAATAC-3’),
1912R (5’-TTTACGGTCGAACTAGGG-3’)
(Holter-
man et al., 2006). PCR cycle conditions were as fol-
lows: one cycle of 94°C for 2 min, followed by 35 cy-
cles 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 amplifica-
tion using ExoSAP-IT (Affymetrix, USB Products), quan-
tified 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 re-
gion of 28S, ITS, and partial 18S rRNA and available
sequences of anguiniid nematodes obtained from Gen-
Bank were used for phylogenetic reconstructions. The
newly obtained and published sequences were aligned
using Muscle (Edgar, 2004) with default parameters im-
plemented in MEGA 5.0 (Tamura et al., 2011). Sequence
alignment was edited using MEGA 5.0. The most ap-
propriate model was determined using the Bayesian
Information Criterion (BIC) implemented in the jModel-
Test 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. Posteri-
or probabilities (PP) were given on appropriate clades.
Trees were visualized using TreeView (Page, 1996).

Results

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

Description

Females

Body subcylindrical, tapering at both ends and al-
most straight upon fixation. Cuticle with transverse
striae measuring ca 1.1 μm wide in mid-body region.
Lateral fields with six incisions 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 oc-
cupying 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
drawing
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 quadricol umellar 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.

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

<table>
<thead>
<tr>
<th>Character</th>
<th>Female Holotype</th>
<th>Female Paratypes</th>
<th>Male Paratypes</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td></td>
<td>778</td>
<td>640 ± 70.7 (574–738)</td>
</tr>
<tr>
<td>L</td>
<td>38.9 ± 3.6 (30.3–38.9)</td>
<td>34.1 ± 1.7 (31.9–35.6)</td>
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</tr>
<tr>
<td>b</td>
<td>5.9 ± 0.4 (5.4–6.4)</td>
<td>5.4 ± 0.4 (5.2–6.0)</td>
<td></td>
</tr>
<tr>
<td>c</td>
<td>11.7 ± 1.3 (8.9–13.2)</td>
<td>10.6 ± 1.7 (8.4–12.5)</td>
<td></td>
</tr>
<tr>
<td>E. pore from anterior end</td>
<td>99.0 ± 7.4 (92–112)</td>
<td>92 ± 3.9 (87–96)</td>
<td></td>
</tr>
<tr>
<td>Lip region height</td>
<td>2.0 ± 0.5 (2.0–3.0)</td>
<td>2.3 ± 0.5 (2.0–3.0)</td>
<td></td>
</tr>
<tr>
<td>Lip region width</td>
<td>5.4 ± 0.5 (5.0–6.0)</td>
<td>5.3 ± 0.5 (5.0–6.0)</td>
<td></td>
</tr>
<tr>
<td>Stylet length</td>
<td>8.3 ± 0.5 (8.0–9.0)</td>
<td>8.1 ± 0.3 (8.0–8.5)</td>
<td></td>
</tr>
<tr>
<td>m²</td>
<td>45.7 ± 2.9 (43.8–50.0)</td>
<td>49.2 ± 3.9 (43.8–52.9)</td>
<td></td>
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<tr>
<td>Pharynx length</td>
<td>127.4 ± 16.2 (114–162)</td>
<td>117.3 ± 6.0 (109–123)</td>
<td></td>
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<tr>
<td>Max. body diam.</td>
<td>21.6 ± 3.7 (18–28)</td>
<td>18.8 ± 1.5 (18–21)</td>
<td></td>
</tr>
<tr>
<td>Vulval body diam. (VBD)</td>
<td>19 ± 3.3 (17–26)</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Vulva–anus distance (V–A)</td>
<td>80.3 ± 14.8 (70–112)</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Post-uterine sac (PUS) length</td>
<td>43.4 ± 5.6 (39–55)</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>PUS/Vulval body diam.</td>
<td>2.2 ± 0.2 (2.0–2.4)</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Ovary length or testis</td>
<td>358.7 ± 69.6 (275–457)</td>
<td>305 ± 42.1 (273–367)</td>
<td></td>
</tr>
<tr>
<td>Anal (cloacal) body diam.</td>
<td>13.0 ± 2.3 (11–18)</td>
<td>13.0 ± 1.4 (12.0–15.0)</td>
<td></td>
</tr>
<tr>
<td>Spicule length</td>
<td>–</td>
<td>18.0 ± 0.6 (18.0–19.0)</td>
<td></td>
</tr>
<tr>
<td>Guberniculum length</td>
<td>–</td>
<td>5.3 ± 0.5 (5.0–6.0)</td>
<td></td>
</tr>
<tr>
<td>Tail length</td>
<td>66.1 ± 18.6 (55–108)</td>
<td>62.3 ± 18.0 (46–88)</td>
<td></td>
</tr>
<tr>
<td>Bursa (% of tail)</td>
<td>–</td>
<td>28.0 ± 3.4 (24.6–32.6)</td>
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</tbody>
</table>

²Length of conus as percentage of total stylet length.
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).
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Gubernaculum simple, 5 to 6 μm 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 (8–9 μm long) with clearly defined knobs; Pharynx with cylindrical corpus, fusiform, valveless and

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.
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 versus 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 versus 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. 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 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.
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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.
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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**References**


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