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Description of *Longidorus azarbaijanensis* n. sp. (Dorylaimida: Longidoridae) from Iran

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Abstract

Longidorus azarbaijanensis n. sp. is described and illustrated using morphological and molecular data. It was recovered in West Azarbaijan province, northwestern Iran, from the rhizospheric soil of foxtail weed. The new species is characterized by having 5.4 to 6.8 mm long females, offset, anteriorly flat lip region and separated from the rest of the body by a shallow constriction, funnel-shaped amphidial pouches, the guiding ring at 23 to 27 µm from the anterior end, 73 to 81 and 44 to 50 µm long odontostyle and odontophore, respectively, 95.0 to 113.5 µm long pharyngeal bulb, didelphic-amphidelphic reproductive system with long tubular uteri lacking sperm cells, vulva located at 52.0% to 56.4%, conical tail dorsally convex, ventrally flat, with bluntly rounded wide tip, for juvenile developmental stages and absence of males. The general body shape of the new species is similar to that of five known species of the genus namely *L. euonymus, L. perangustus, L. persicus, L. protae* and *L. sturhani*. The morphological differences of the new species with the aforementioned species are discussed. For all the aforementioned species (except *L. protae,* currently lacking molecular data) the differences of the new species was also confirmed with differences in molecular sequences of D2-D3 expansion domains of 28S rDNA and the corresponding phylogenetic analyses. The partial sequence of the internal transcribed spacer 1 (ITS1) of the new species was also used in phylogenetic analyses. In partial 28S tree, the clade including the new species and six other species (*L. attenuatus, Longidorus* sp. and four above-mentioned species having molecular data for this fragment) was well supported in Bayesian inference. In the ITS1 tree, the new species formed a clade with *L. euonymus*, *L. perangustus* and *L. persicus*, as in 28S tree. This is one of the cases from which the morphologically similar species are separated using molecular sequences.

Key words

28S rDNA, Bayesian, ITS1, molecular, maximum likelihood, new species, phylogeny, taxonomy, West Azarbaijan province.

The genus *Longidorus* was erected by Micoletzky (1922) with *L. elongatus* (Micoletzky, 1922) as its type species. Decraemer and Geraert (2013) pointed out that around 155 species are known for the genus. However, Peneva et al. (2013) listed 158 species for the genus while describing *L. cholevae* (Peneva et al. 2013). Since 2013 till date, 10 further species are also described (Peneva et al. 2013; Trisciuzzi et al. 2015; Archidona-Yuste et al. 2016; Roshan-Bakhsh et al. 2016; Esmaeili et al. 2017). The list of valid species proposed by Peneva et al. (2013) is currently a useful resource on valid species, as, for example, it includes some species overlooked in the original key by Chen et al. (1997), and in the first supplement by Loof and

Chen (1999) (e.g., species described by Singh and Khan, 1996). It also includes the species transferred from other genera to the *Longidorus* (e.g., some species in Decraemer and Coomans, 2007). However, we were not able to find detailed illustrations of *L. nanus* (Romanenko, 1993) in the present study. The main morphological and morphometric features useful for the species delimitation in *Longidorus* by the classic approach are given by Chen et al. (1997). Exploiting molecular data, and especially using the ribosomal RNA genes sequences in taxonomic studies, mostly since 2001, has deeply influenced and improved species identification for this greatly biodiversified genus.

Besides direct feeding on root cells and direct damages, seven species (Decraemer and Geraert, 2013) transmit plant pathogenic nepoviruses. Also, this could still be an underestimation, needing further biological studies of extra species to inspect their ability in transmitting plant pathogenic viruses. Furthermore, the species could survive for long times in appropriate conditions in the field; and as the result, are a resource for further viral infections, and in conclusion, the aforementioned biological features emphasize on the correct identification of *Longidorus* species for the adoption appropriate controlling measures.

According to Ghaderi et al. (2012), 16 species of the genus are reported from Iran till 2011. Since 2011 till date, *L. perangustus* (Roshan-Bakhsh et al. 2016) and *L. persicus* (Esmaeili et al. 2017) have originally been described from Iran. During our recent nematological surveys in forests and grasslands of northwestern Iran, a population of an unidentified species was recovered from West Azarbaijan province that is described in the present paper as *L. azarbaijanensis* n. sp.

Material and methods

Sampling and morphological studies

About 45 soil samples were collected from a depth of 5 to 30 cm, in active plant roots area in West Azarbaijan province, northwestern Iran during July 2016. Nematodes were extracted from soil using a series of 20 and 60 mesh sieves (US standard mesh numbers equal to 850 and 250 µm sized openings, respectively). The specimens of interest were hand-picked under a Nikon SMZ1000 stereomicroscope, heat-killed by adding boiling 4% formaldehyde solution and transferred to anhydrous glycerin according to De Grisse (1969). In total, four populations of the genus *Longidorus* were recovered. The number of females of the three populations was less than 10, and their identification at the species level needed resampling and complementary studies. Measurements of the new species were made using a drawing tube attached to a Nikon Eclipse E600 light microscope. The juvenile developmental stages were identified according to Robbins et al. (1995). The digital images were prepared using an Olympus DP72 digital camera attached to an Olympus BX51 microscope powered with differential interference contrast. Drawings were made by hand using the drawing tube and were redrawn using CorelDRAW® software version 16.

Molecular studies

For the molecular phylogenetic studies, three female nematodes (labelled as fem.az.1-3) were picked out, studied individually on temporary slides, transferred to a small drop of TE buffer (10 mM Tris-Cl, 0.5 mM EDTA; pH 9.0, 100 QIAGEN Inc., Valencia CA) on separate clean slides and each was squashed using a clean slide cover glass. The suspension on each slide was collected by adding 45 µl TE buffer, each regarded as an independent DNA sample and stored at –20°C until used as polymerase chain reaction (PCR) template. Primers for the PCR amplification of the D2-D3 expansion domains of the 28S rDNA were: forward D2A (5′-ACAAGTACCGTGAGGGAAAGT-3′) (Nunn, 1992) and reverse KK28S-4 (5′-GCGGTATTTGCTACTA CCAYYAMGATCTGC-3′) (Kiontke et al. 2004) (several attempts to get the expected amplified fragments using the commonly used reverse primer D3B (5′-TGCGAA GGAACCAGCTACTA-3′) were not successful). The ITS1 fragment was amplified using the forward TW81 (5′-GTTTCCGTAGGTGAACCTGC-3′) and reverse AB28 (5′-ATATGCTTAAGTTCAGCGGGT-3′) (Joyce et al. 1994) primer pairs. PCR was carried out (for both the fragments) in a total volume of 30 µl (10.6 µl distilled water, 15 µl Master, 1.2 µl of each primer (10 pMol/µl), and 2 µl of DNA template). The thermal cycling program for both was as follows: denaturation at 94°C for 5 min, followed by 32 cycles of denaturation at 94°C for 30 sec, annealing at 52°C for 40 sec and extension at 72°C for 80 sec. A final extension was performed at 72°C for 10 min (Alvani et al., 2016; Pedram, 2017). PCR products were purified and sequenced directly for both strands using the same primers with an ABI 3730XL sequencer (Bioneer Corporation, South Korea). The recently obtained sequences of three females with isolate codes fem. az.1-3 were submitted to GenBank database under accession numbers: MF677863 and MG765549 for

partial 28S rDNA D2-D3 and ITS1 of female with isolate code fem.az.1, MG765547 and MF677864 for partial 28S rDNA D2-D3 and ITS1 of female with isolate code fem.az.2 and MG765548 for partial 28S rDNA D2-D3 of female with isolate code fem.az.3, respectively. The Basic Local Alignment Search Tool (BLAST) (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to inspect the relevance of the newly generated sequences with those already submitted into the database. For partial 28S tree, almost all available sequences of the genus were downloaded. The tree reconstructed from this big dataset was used to select sequences for the final 28S tree (the closely related species/sequences were selected to avoid a crowded tree). The available sequences for ITS1 rDNA of *Longidorus* spp. were also downloaded for reconstructing the corresponding phylogenetic tree. The sequences were aligned using the Q-INS-i algorithm of online version of MAFFT version 7 (http://mafft.cbrc.jp/alignment/server/) (Katoh and 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 the 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 the phylogenetic analyses of both 28S and ITS1 datasets. Bayesian analysis was performed with MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003) running the chains for two million generations (nruns=4). After discarding burn-in samples and evaluating convergence, the remaining samples were retained for further analyses. The Markov chain Monte Carlo method within a Bayesian framework was used to estimate the posterior probabilities of the phylogenetic trees (Larget and Simon, 1999) using the 50% majority rule. For ML analysis, the same dataset as for the Bayesian tree was used and it was analyzed using raxmlGUI version 1.1 (Silvestro and Michalak, 2011) using the same model of nucleotide substitution (GTR+G+I) as in the previous analysis. For phylogenetic analyses of 28S dataset (both Bayesian inference (BI) and maximum likelihood (ML) methods), the species *Nevadanema nevadense* (Alvarez-Ortega and Peña-Santiago, 2012) (JN242245) and *Prodorylaimus* sp. (EF207241) were used as outgroup taxa. The species *Xiphinema index* (Thorne and Allen, 1950) (HG969306) and *X. vuittenezi* (Luc et al. 1964) (HG969309) were the out-group taxa in ITS1 tree. The output files of the used phylogenetic programs were visualized using Dendroscope V.3.2.8 (Huson and Scornavacca 2012) and redrawn in CorelDRAW software version16. The Bayesian posterior probability (BPP) and maximum likelihood bootstrap (ML BS) values exceeding 50% are given on appropriate clades in the shape BPP/ML BS.

Results and discussion

Longidorus azarbaijanensis n. sp. (Figs. 1 and 2, Table 1).

The name of the new species refers to the original geographical distribution point.

Description

Female

Body long and narrow, very gradually tapering towards both ends, more so towards the anterior end,

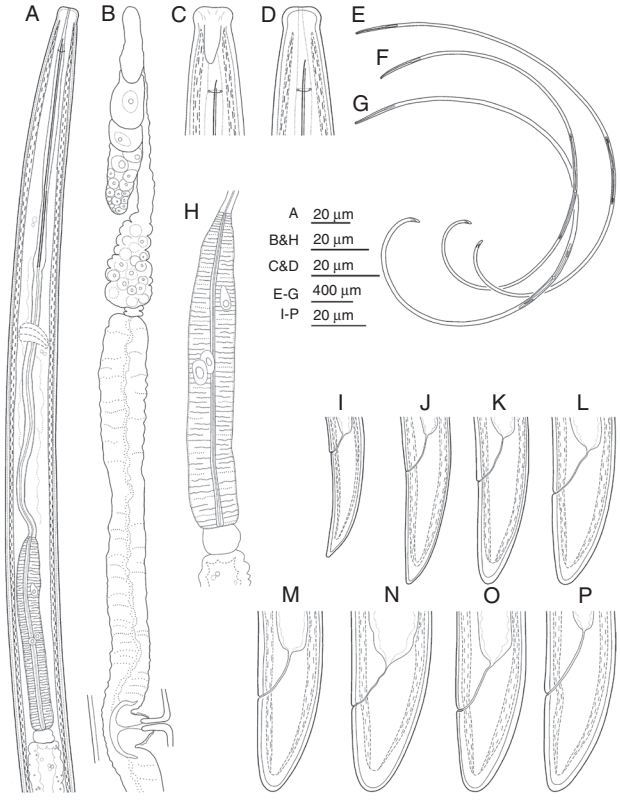

Figure 1: *Longidorus azarbaijanensis* n. sp. A: pharynx; B: anterior genital branch; C, D: female anterior end; E-G: entire female; H: pharyngeal bulb and the corresponding details; I-L: tail of juveniles from J1 to J4, respectively; M-P: female tail.

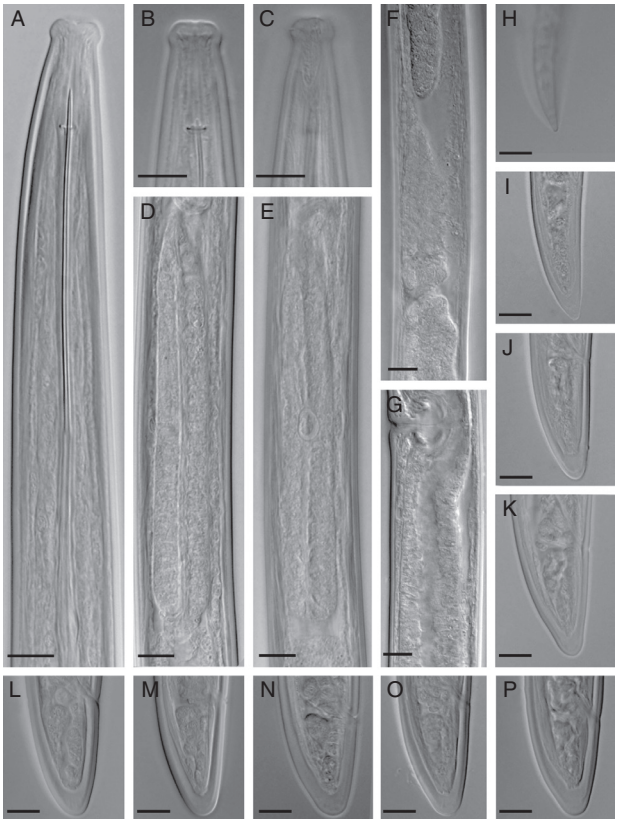

Figure 2: *Longidorus azarbaijanensis* n. sp. Female. A: anterior region; B: lip region; C: funnel-shaped amphidial pouch, observed only in one female; D: pharyngeal bulb and the dorsal pharyngeal gland's nucleus; E: one of ventrosublateral pharyngeal glands' nucleus; F: part of anterior genital branch; G: vulvar region and part of tubular uterus; H–K: tail of juveniles from J1 to J4, respectively; L–P: female tail. (Scale bars = 10 µm).

open C-shaped after heat relaxation, with posterior body end usually ventrally bent. Cuticle distinctly two layered with very fine transverse striation mostly visible at the dorsal side of the tail, 2.2 ± 0.3 (2.0-2.5) µm thick at the distance between anterior end and guiding ring, 1.8 ± 0.2 (1.5-2.0) µm at mid-body and 3.0 ± 0.5 (2.5-4.0) µm at the anus. Lateral chords 13.7 ± 4.6 (10-18) µm wide, occupying 26.3% to 46.9% of corresponding to the body diam. Amphidial foveae funnel-shaped (n = 3) (Fig. 2C), their outlet invisible. The lip region offset, anteriorly flat, separated from the rest of the body by constriction, 11.7 ± 0.7 (10.0-12.5) µm wide and 6.8 ± 1.1 (5-8) µm high. Guiding ring single, at a distance of 25.0 ± 1.2 (23-27) µm from the anterior end. Odontostyle relatively short, narrow, typical of the genus, 1.6 ± 0.1 (1.5-1.8) times as long as the odontophore, the latter with slightly swollen surrounding muscle at the base. Nerve ring at about the middle of the first half of the narrow part of the pharynx. Pharynx dorylaimoid, anterior slender part flexible, posteriorly expanding to a muscular terminal bulb occupying about 25.5 ± 1.3 (23.4-27.5)% of the total pharynx (neck region), with three glands nuclei. The dorsal gland nucleus (DN) smaller, at 24.5 ± 2.5 (22.5-29.5)%, and two ventrosublateral nuclei (S1N) at 50.5 ± 2.5 (48.0-54.5)% of the pharyngeal bulb length (location of glands nuclei according to Loof and Coomans, 1972). The cardia well developed, offset in all examined individuals (adjoining pharynx to the intestine), thick-plate-like, 11.0 ± 4.7 (9-20) µm high and 10.1 ± 3.4 (9-13) µm wide. Intestine simple, prerectum about 13.4 ± 3.7 (9.4-19.9) times and rectum about 1.1 ± 0.2 (0.9-1.4) times as long as the anal body width. Anus a small slit. The reproductive system didelphic–amphidelphic with both branches almost equally developed, each branch composed of a reflexed ovary of about 89.5 ± 19.5 (73-119) µm long, two partite oviductus 203 ± 28 (184-250) µm long with well-developed *pars dilatata oviductus*, a sphincter, tubular 161 ± 18 (135-182) µm long uterus, a 24.5 ± 2.5 (21-28) µm long vagina perpendicular to the body axis, surrounded by well-developed muscles and vulva in the shape of a transverse slit. Tail short, conical, dorsally convex, ventrally flat with a widely rounded terminus.

Male

Not found.

Juveniles

All four juvenile developmental stages were recovered. Their general morphology looks similar to that of females, except for a smaller body size, presence of replacement odontostyle and not developed reproductive system. The stages were separated from each other according to Robbins et al. (1995). The scatter diagram representing the relationships between body length, functional and replacement odontostyle of females and juveniles is given in Figure 3. The first juvenile developmental stage (J1) is characterized by having a replacement odontostyle laying on the odontophore, its tip just close to the base of functional odontostyle. In the rest juvenile developmental stages (J2-J4), the tip of the replacement odontostyle is distantly located to the base of the

Table 1. Morphometrics of *Longidorus azarbaijanensis* n. sp. All measurements are in µm (except L in mm) and in the form: mean ± SD (range).

	Juvenile (Paratype)				Female	
Character	J1	J2	J3	J4	Holotype	Paratypes
n	1	2	8	3	–	13
L (mm)	–	–	3.2 ± 0.2	4.5 ± 0.1	5.4	6.0 ± 0.5
	1.6	2.3, 2.4	(3.0–3.6)	(4.4–4.6)		(5.4–6.8)
a	–	–	127.2 ± 7.1	148.5 ± 14.7	181.5	166.6 ± 13.4
	95.1	114.5, 99.7	(116.5–138.3)	(135.7–164.6)		(133.7–181.5)
b	–	–	9.0 ± 1.3	10.5 ± 0.6	14.6	15.1 ± 1.7
	6.9	6.5, 6.5	(7.7–11.4)	(9.9–11.1)		(12.1–17.3)
c	–	–	83.9 ± 10.1	128.2 ± 5.2	147.2	178.9 ± 21.4
	43.7	58.7, 56.8	(74.1–101.7)	(122.5–132.8)		(147.2–216.3)
c'	–	–	2.1 ± 0.3	1.6 ± 0.1	1.7	1.4 ± 0.2
	3.1	2.6, 2.6	(1.5–2.4)	(1.5–1.8)		(1.2–1.7)
V%	–	–	–	–	56	54.7 ± 1.5
	–	–	–	–		(52.0–56.4)
Lip region width	–	–	10.4 ± 0.6	11.3 ± 0.6	11.5	11.7 ± 0.7
	8	10, 10	(9.5–11.0)	(11–12)		(10.0–12.5)
Lip region height	–	–	–	–	8	6.8 ± 1.1
	–	–	–	–		(5–8)
Odontostyle length	–	–	61.3 ± 2.3	68.8 ± 1.9	77	75.6 ± 2.2
	52	57, 56	(58.0–64.5)	(67.5–71.0)		(73–81)
Odontophore length	–	–	45.0 ± 3.4	47.5 ± 5.9	44	47.5 ± 1.7
	31	41, 41	(42–52)	(42.5–54.0)		(44–50)
Replacement odontostyle	–	–	69.2 ± 2.0	78.7 ± 2.5	–	–
	58.5	60, 64	(66.5–73.0)	(76–81)		–
Stylet total length	–	–	106.1 ± 5.0	116.3 ± 6.0	121	123.0 ± 2.5
	83	97.5, 97.5	(99.0–116.5)	(110–122)		(120–129)
Anterior end to guiding ring	–	–	–	–	23	25.0 ± 1.2
	–	–	–	–		(23–27)
Pharynx length	–	–	357.5 ± 33.0	430.0 ± 15.6	372.5	401.2 ± 28.2
	235	350, 377	(311–405)	(417.5–447.5)		(372.5–468.8)
Pharyngeal bulb length	–	–	–	–	98	103.1 ± 5.8
	–	–	–	–		(95.0–113.5)
Pharyngeal bulb width	–	–	–	–	11	14.8 ± 2.5
	–	–	–	–		(11–21)

Diam. at pharynx base	–	–	–	–	26.5	29.4 ± 3.8
	–	–	–	–		(26–39)
- at mid–body	–	–	25.2 ± 1.4	30.7 ± 2.8	30	36.6 ± 5.4
	17	20.0, 24.5	(23–27)	(27.5–32.5)		(30.0–49.5)
- at anus	–	–	18.6 ± 1.5	22.0 ± 1.7	22	25.1 ± 2.6
	12	15.0, 16.5	(17–22)	(20–23)		(22–32)
- at guiding ring level	–	–	–	–	15	16.1 ± 0.8
	–	–	–	–		(15.0–17.5)
Prerectum length	–	–	–	–	437.5	331.5 ± 80.4
	–	–	–	–		(240.0–457.5)
Rectum length	–	–	20.1 ± 2.4	26.0 ± 1.7	26	27.4 ± 3.6
	16	13.5, 17.0	(15–22)	(24–27)		(21–33)
Lip region–vulva	–	–	–	–	3051.3	3303.6 ± 249.5
	–	–	–	–		(2881.3–3618.8)
Hyaline part of tail	–	–	–	–	7	7.8 ± 0.8
	–	–	–	–		(7–9)
Tail	–	–	38.4 ± 2.9	35.3 ± 0.6	37	34.0 ± 2.8
	37	39, 43	(34–43)	(35–36)		(30–38)

functional odontostyle. The tail of all stages is conical. J1 has a narrower tail compared with the tail of other three stages, with dorsally convex and ventrally slightly concave outline and rounded tip. Tail in J2 is dorsally convex and ventrally flat. Both J3 and J4 have a conical dorsally convex and ventrally flat tail with a broadly rounded tip; however, J3 has a slightly longer tail.

Type habitat and locality

Recovered from the soil samples collected about the rhizosphere of foxtail weed in natural grasslands and forests at 24 km distance to the city of Chaipareh, West Azarbaijan province, northwestern Iran, in July 2016. GPS coordinates: 38°59.373′ N, 44°48.721′ E.

Type material

Holotype female, seven paratype females and juveniles were deposited at Nematode Collection of the Faculty of Agriculture, Tarbiat Modares University, Tehran, Iran. Three paratype females were deposited at each of the following collections: UGent Nematode Collection of the Nematology Research Unit, Department of Biology, Ghent University, Ghent, Belgium and WANECO collection, Wageningen, The Netherlands (www.waneco.eu/).

Diagnosis and relationships

Longidorus azarbaijanensis n. sp. is a medium-length (5.4-6.8 mm) species lacking male in population. It is further characterized by having an offset lip region, anteriorly flat and separated from the rest body by constriction, funnel-shaped amphidial pouches, simple guiding ring located at 23 to 27 µm distance from the anterior end, 73 to 81 and 44 and 50 µm long odontostyle and odontophore, respectively,

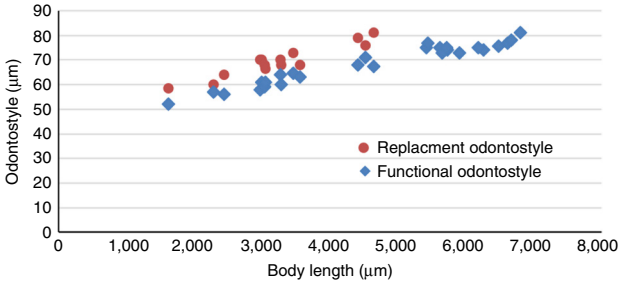

Figure 3: *Longidorus azarbaijanensis* n. sp. Correlation of functional and replacement odontostyle to body length in juveniles and females.

95.0 to 113.5 µm long pharyngeal bulb, well-developed offset cardia, short conical tail with widely rounded terminus and four juvenile developmental stages. The matrix code of the new species according to Chen et al. (1997) is: A23-B12-C2- D34-E4-F3-G34-H23-I1. With regard to similar polytomous codes yielded from the close morphology, the new species is similar to five known species of the genus namely: *L. euonymus* (Mali and Hooper, 1973) *L. perangustus*, *L. persicus*, *L. protae* (Lamberti and Bleve-Zacheo, 1977) and *L. sturhani* (Rubtsova et al. 2001).

The detailed comparisons with the aforementioned species are as follows:

Compared with *L. euonymus,* besides distant position in both 28S and ITS1 phylogenetic trees, the new species has a lip region separated from the rest of the body by a remarkable constriction (vs somewhat concave), slightly shorter body (5.4-6.8 vs 6.0-7.6 mm), less wider lip region (10.0-12.5 vs 14 µm), shorter odontostyle (73-81 vs 81-90 µm), anteriorly located guiding ring (23-27 vs 27-33 µm distance from anterior end), and narrower female tail terminus (vs broad).

The new species differs from *L. perangustus* by its shorter body (5.4-6.8 vs 6.3-8.9 mm), smaller a value (133-181 vs 207-341), slightly smaller b value (12.1-17.3 vs 16-24), posteriorly located vulva (V = 52.0-56.4 vs 47.0-52.8), slightly smaller c´ value (1.2-1.7 vs 1.6-2.6), shorter tail (30-38 vs 38-51 µm) and by absence of male (vs presence). The two species also occupy distant positions in both phylogenetic trees inferred in this study.

Compared witho *L. persicus*, the new species has a slightly shorter body (5.4-6.8 vs 6.5-7.8 mm), shorter odontostyle and odontophore (73-81 vs 80-93 and 44-50 vs 48-72 µm, respectively), shorter tail (30-38 vs 38-50 µm), shorter pharyngeal bulb (95.0-113.5 vs 130-218 µm), absence of male (vs presence), and completely different tail characters in first three juvenile developmental stages. Both species are well distant in 28S tree, while are close relatives in ITS1 tree.

Compared with *L. protae*, the new species has posteriorly located vulva (V = 52.0-56.4 vs 46-51), shorter odontophore (44-50 vs 50-60 µm), shorter pharyngeal bulb (95.0-113.5 vs 125-140 µm), basic difference in tail end morphology (narrowly rounded vs broadly rounded) and shorter tail in all juvenile developmental stages.

Compared with *L. sturhani*, besides remarkable differences in partial sequences of partial 28S and ITS1 rDNA and distant position in both phylogeny trees, the new species has a lip region separated from the rest of the body by a constriction (vs slight depression), less wider lip region (10.0-12.5 vs 14-17 µm), shorter odontophore (44-50 vs 57-78 µm), greater a value (133.7-181.5 vs 99-138), greater c value (147.2-216.3 vs 83-155) and shorter tail (30-38 vs 35-50 µm).

Molecular characterization and phylogenetic position of *Longidorus azarbaijanensis* n. sp.

Sequencings of 28S rDNA D2-D3 expansion domains of three females of the new species yielded three single fragments of 634 (MF677863) and 873 (MG765547 and MG765548) nt long. The longer size of the two latter sequences was due to the longer reads of the corresponding PCR products while sequencing. There were no differences (indels or gaps) in the overlapping region of the three aforementioned sequences. Sequencing of ITS1 rDNA fragment of two females yielded two single fragments of 831 nt long (accession numbers MF677864 and MG765549). Both ITS1 sequences were identical. For molecular phylogenetic analyses of the new species, one sequence of each newly generated aforementioned genomic sequences was used to avoid crowded trees (for accession numbers see Figs. 4 and 5). A BLAST search using one of the newly obtained partial sequences of 28S rDNA D2-D3 (MF677863) showed that it has no exact identity with available sequences in the database. The highest matched sequences were an unidentified species of *Longidorus* (KF242335) having 100% query coverage and 96% identity (27 indels/one gap) and *L. euonymus* (KX062667) having 100% query coverage and 96% identity (26 indels/one gap). The identity value with all other sequences was 95% and less. The BLAST search using one of the ITS1 sequences of the new species (MF677864) revealed that the highest identity and coverage belonged to two sequences of *L. euonymus* (KX062692 and KX062691) with 80% identity and 60% coverage (99 different nucleotides/20 gaps) and one sequence from two species *L. persicus* (KU747175) and *L. perangustus* (KT593863) with 76% and 80% identity and 89% and 58% coverage, respectively (184 and 102 different nucleotides/55 and 21 gaps, respectively).

For partial 28S phylogeny, further than 100 sequences of almost all sequenced species/populations of *Longidorus* were used (the resultant tree was not shown). The related clades and species to the new species were selected to reconstruct the smaller pruned tree. A total number of 33 species/populations of *Longidorus* mostly belonging to the clade I in D2-D3 tree given by Subbotin et al. (2014), including one sequence of the new species, two species of *Xiphinema* (Cobb, 1913) and two dorylaim species (the species name and accession numbers in Fig. 4) as the

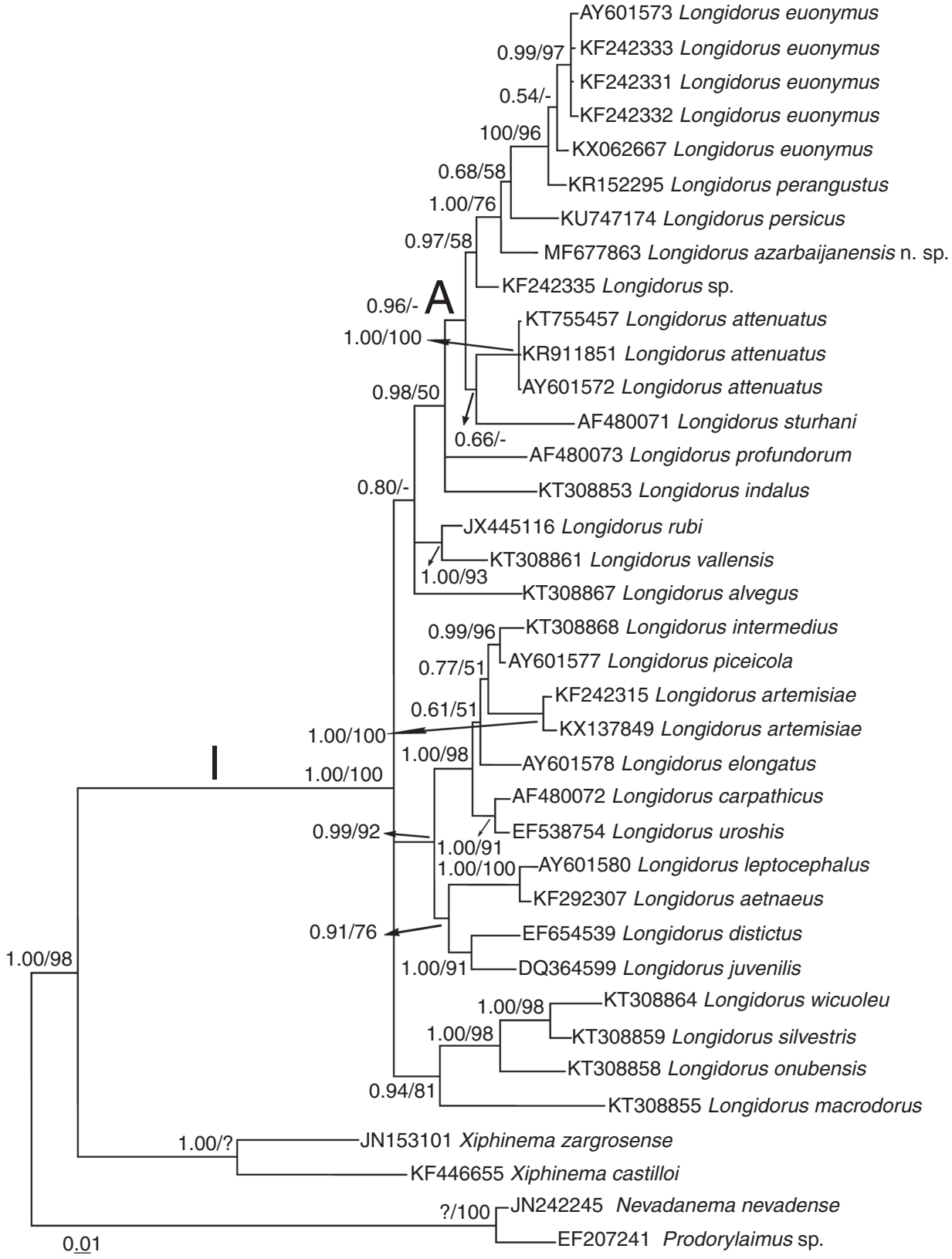

Figure 4: Bayesian tree inferred under the GTR+G+I model from 28S rDNA D2-D3 expansion domains of *Longidorus azarbaijanensis* n. sp. Posterior probability and bootstrap values exceeding 50% are given on appropriate clades in the form BPP/BS.

outgroup taxa, were used to reconstruct the final tree. The alignment of this dataset included 749 total characters with 335 variable and 261 parsimonious informative characters. The average nucleotide composition was as follows: T, 21.5%, C, 23.1%, A, 24.5%, and G, 31.0%. Figure 4 represents the Bayesian phylogenetic tree inferred using the above-mentioned dataset. The clade A (whit 0.96% BPP and no ML BS support), inside the major clade I *sensu* Subbotin et al. (2014) includes the new species, five sequences of *L. euonymus* (KX062667, KF242332, KF242331, KF242333, AY601573), one sequence of *L. perangustus* (KR152295), *L. persicus* (KU747174) and *Longidorus* sp. (KF242335), three sequences of *L. attenuatus* (KT755457, KR911851, AY601572) and one sequence of *L. sturhani* (AF480071). Surprisingly, four species (*L. euonymus, L. perangustus, L. persicus* and *L. sturhani*) out of the five morphologically close species are in close phylogenetic relation with the new species in this tree. The species *L. protae,* the fifth morphologically close species, does currently have no available 28S sequence(s).

A total number of 18 species/populations of *Longidorus* including one sequence of the new species and two *Xiphinema* species as the outgroup taxa (the species names and accession numbers in Fig. 5) were used in the molecular phylogenetic analyses using ITS1 sequences. The alignment of this dataset included 720 total characters with 484 variable and 348 parsimonious informative characters. The average nucleotide composition was as follows: T, 26.2%, C, 20.8%, A, 24.1%, and G, 28.9%. Figure 5 represents the Bayesian phylogenetic tree inferred using the above-mentioned dataset. The clade B in this tree includes the new species, two sequences of *L. euonymus* (KX062692 and KX062691), one sequence of *L. perangustus* (KT593863) and one sequence of *L. persicus* (KU747175). This clade receives maximal BPP and high ML BS values (1.00 and 99%, respectively). Inside the clade B, *L. azarbaijanensis* n. sp. is phylogenetically more close to *L. persicus* both of which forming a monophyletic group with high BPP and ML BS value (0.99 and 90%, respectively). From the five morphologically similar species (as already discussed), four species *L. euonymus, L. perangustus, L. persicus* and *L. sturhani* are sequence for their ITS1 fragment (KX062691 and KX062692 for two sequences of *L. euonymus*, KT593863 for *L. perangustus*, KU747175 for *L. persicus* and FJ009680 for *L. sturhani*). Similar to 28S tree, the new species has a close phylogenetic relation

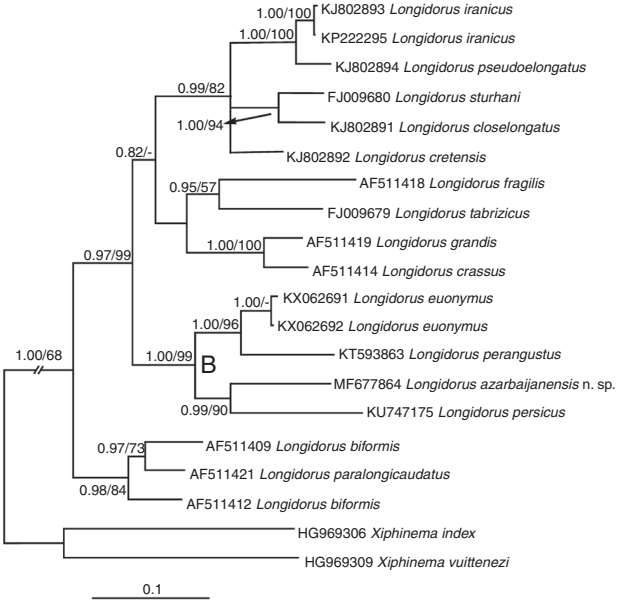

Figure 5: Bayesian tree inferred under the GTR+G+I model from ITS1 rDNA partial sequences of *Longidorus azarbaijanensis* n. sp. Posterior probability and bootstrap values exceeding 50% are given on appropriate clades in the form BPP/BS.

with three species *L. euonymus, L. perangustus* and *L. persicus* (members of clade B). In conclusion, this is one of the cases in which a group of morphologically similar species are close relatives in molecular phylogenetic analyses as well.

Conclusion

Different aspects of biology, taxonomy or pathogenicity of *Longidorus* spp. on plants are attractive for biologists, zoologists and plant pathologists. The genus encompasses plant parasitic species causing the formation of small galls and hypertrophy on roots of the host plants; the feeding cells are also unicellular (Wyss, 2010). Darkening of tissues, cortical hyperplasia and lateral root proliferation however are reported (Cohn, 1975). A general preference of herbaceous plants, and feeding on root tip (Hunt, 1993) and sometimes, from other parts of roots (Cohn, 1970) is documented for *Longidorus.* The new species was however recovered in association with grasses in natural grasslands of West Azarbaijan province and surprisingly, several other species of the genus were also recovered from the region that are under supplementary study, due to their insufficient number of females. On the basis of

our knowledge, there were no evidences of growing of woody trees in the region for a long time and the foxtail weed could be the natural host for the new species. On the other hand, except some classic pathogenicity tests that were already cited and are accessible in the literature, the elaborate mechanisms involved in the pathogenicity of these nematodes, the involved effectors or activated pathways, that are well studied in case of cyst forming or root knot nematodes, are still to be studied and clarified for longidorids. The plant pathogenic virus transmission ability of some species is another issue, discussed by several authors (e.g., Decraemer and Robbins, 2007).

From the taxonomic points of view, the list of valid species is given in several taxonomic studies (see Introduction). Fortunately, most of the recent studies include genomic or non-genomic DNA sequences and the corresponding phylogenetic trees. The difficulties in species identification in *Longidorus* are well known for almost all nematologists, and the use of informative genomic or non-genomic sequences in some cases are the only way to draw species boundaries. In a recent study, Subbotin et al. (2014) proved the usefulness of even a short fragment of 28S rDNA in the identification of *L. artemisiae* (Rubtsova et al., 1999). The D2-D3 sequence of the new species had 27-33 indels/gaps compared with the same sequences of *L. euonymus* isolates in Figure 4, and 32, 30 and 62 indels/gaps compared with the same sequences of *L. perangustus*, *L. persicus* and *L. sturhani* in the aforementioned tree, respectively. Based on the present observations, such number of different nucleotides could be trusted to separate morphologically close species.

Close morphology of some other species such as *L. aetnaeus* (Roca et al., 1986) and *L. leptocephalus* (Hooper, 1961) is already known and their close phylogenetic relation and morphological differences are also discussed (Bakhshi Amrei et al., 2013). Further, molecular phylogenetic analyses using other genomic or non-genomic sequences in such cases are recommended to empower species boundaries of such close taxa. In some cases, juvenile characters like tail morphology could also be helpful.

In our partial 28S rDNA phylogeny reconstructed using almost all available sequences of *Longidorus* spp. available in GenBank database, the new species felt into the clade I *sensu* Subbotin et al. (2014) (the tree not shown). The pruned tree illustrated in Figure 4 represents the relation of the new species with other species, and surprisingly, the three species with close morphology, i.e., *L. euonymus*, *L. perangustus* and *L. persicus*, having overlapping morphometric data ranges, that is common between species of the genus (Ye and Robbins, 2004), are in close phylogenetic relation with the new species. The other morphologically similar species, *L. sturhani*, has occupied distant placement in this tree. In phylogenetic analyses using ITS1 sequences, however, the close phylogenetic relation of the new species and the three species *L. euonymus*, *L. perangustus* and *L. persicus* is seen. The differences between the topology of our tree and the ITS1 tree inferred by Gutiérrez-Gutiérrez et al. (2013) are due to the differences in taxon sampling and/or different methods used while aligning and post-editing.

In the present study, a new species was added to the clade I of 28S rDNA D2-D3 tree *sensu* Subbotin et al. (2014). The new species had overlapping morphometric data ranges with closely related species and separated from them in molecular phylogenetic analyses.

Remarks

A funnel-shaped amphidial pouch was well observed in three females of the new species. Its shape was not well observed for all examined females, and thus, this trait was not used in morphological comparisons. He et al. (2005) showed, however, that the species of *Longidorus* having the close morphology of amphidial pouches usually have close phylogenetic relations too. The species close to the new species, *L. euonymus* and *L. persicus* have bilobed amphidial pouches. A future study using the maximal number of species' sequences and mapping of amphidial pouches' shape on the resultant tree is needed to further validate this relation. The ventral placement of the dorsal pharyngeal gland nucleus as illustrated in Figure 1H and shown in Figure 2D is a common phenomenon in longidorids, due to rotation of the body or pharyngeal bulb. For the morphology of the lip region, we preferred to describe it as "separated from the rest of the body by a shallow constriction", as shown in Figure 2A. However, others could have other types of interpretations/descriptions. At the moment, no male was recovered for the new species; neither sperm was observed inside the body of the females of the new species. Future sampling could determine if males occur for *L. azarbaijanensis* n. sp. or not.

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