First report of stem bulb nematode, *Ditylenchus weischeri* Chizhov, Borisov & Subbotin, 2010 (Tylenchida: Anguinidae), from Iran

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Accepted for publication 06 February 2023

Summary. During a survey on the identification of plant-parasitic nematodes of several potato fields in Iran, *Ditylenchus weischeri* was found from the rhizosphere of weeds in Markazi province. Nematodes were extracted from soil using the tray method and then fixed according to the standard protocols. The morphological characters of these nematodes agreed with the description of *D. weischeri*. In addition, molecular analysis of this sample using 18S rRNA and 28S rRNA gene sequences indicated 99.7-100% and 99.9-100% similarity, respectively, to those of several isolates of *D. weischeri*. The Iranian population formed a strongly supported clade with several isolates of *D. weischeri* in 18S and 28S rRNA gene trees. This is the first report of *D. weischeri* from Iran.

Key words: 18S rDNA, 28S rDNA, *Ditylenchus*, Markazi province, molecular, morphology, taxonomy.

The *Ditylenchus* Filipjev, 1936 species have diverse feeding behaviour being mostly mycophagous, feeding on mosses and lichens, and some of them are plant-parasitic attacking numerous flowering plants (Sturhan & Brzeski, 1991; Subbotin *et al.*, 2005). Species such as *D. africanus* Wendt, Swart, Vrain & Webster, 1995; *D. angustus* (Butler, 1913) Filipjev, 1936; *D. arachis* Zhang, Liu, Janssen, Zhang, Xiao, Li, Couvreur, & Bert, 2014; *D. destructor* Thorne, 1945; *D. dipsaci* (Kühn, 1857) Filipjev, 1936 and *D. gigas* Vovlas, Troccoli, Palomares-Rius, De Luca, Liébanas, Landa, Subbotin & Castillo, 2011 are amongst the most well-known plant-parasitic nematodes. The type species, *D. dipsaci*, is distributed worldwide and is a pest of many crops as well as weeds (Skwiercz *et al.*, 2017). *Ditylenchus dipsaci* is a migratory endoparasite that feeds within the parenchyma tissues of more than 500 plant species of Fabaceae, Liliaceae and Chenopodiaceae in most temperate areas of the world (Bridge & Starr, 2007; Tenuta *et al.*, 2014). However, many of the various biological races of this nematode have a limited host range. *Ditylenchus dipsaci* lives mostly as an endoparasite in aerial parts of plants (stems, leaves and flowers) but also attacks bulbs, tubers and rhizomes (Subbotin *et al.*, 2005).

Several species have been recognised from the *D. dipsaci* complex including *D. gigas*, known previously as the giant race of *D. dipsaci* (Vovlas *et al.*, 2011), and *D. weischeri* Chizhov, Borisov & Subbotin, 2010, parasitising the widely distributed weed *Circium arvense* (Chizhov *et al.*, 2010). Some cryptic species of *Ditylenchus* have been identified that show very similar morphological characters but are differentiated based on molecular data (Wendt *et al.*, 1993; Esquibet *et al.*, 1998; Vovlas *et al.*, 2011; Madani & Tenuta, 2018). Morphological similarities of, in particular, juvenile stages with other non-pathogenic soil-inhabiting *Ditylenchus* and tylenchid species make the diagnostics of *D. dipsaci* a difficult task even for experts. Besides precise identification of the species (*e.g.*, in quarantine inspections), knowledge of the local biological race is often desirable, such as when crop rotation measures are considered. As a consequence, a quick and reliable method for diagnosis is required (Subbotin *et al.*, 2005).

Chizhov *et al.* (2010) described a new species, *D. weischeri*, infesting *C. arvense*, in fields, roadsides and ditches near Moscow, Russia. Because, *C. arvense* is widely distributed and persistent perennial weed of temperate climates around the world, correct differentiation of *D.*

 doi: 10.24412/0869-6918-2023-1-51-58
Published online 26 May, 2023

weischeri from *D. dipsaci sensu stricto* is important (Tenuta *et al.*, 2014). In present study, we described *D. weischeri* for the first time from Iran associated with the rhizosphere of weed (*Chenopodium album*) in the potato field in Markazi province.

MATERIAL AND METHODS

Nematode samples. Soil samples were collected from the rhizosphere of weed (*Chenopodium album*) in a potato field in Markazi province, Iran. Nematodes were extracted by the tray method (Whitehead & Hemming, 1965), killed and fixed by hot FPG (4:1:1, formaldehyde: propionic acid: glycerol), processed to anhydrous glycerol, mounted in glycerol on permanent slides using paraffin wax (De Grisse, 1969). Slides were studied using a light microscope, equipped with a Dino-eye microscope eye-piece camera and software Dino Capture version 2.0. Drawings were made by a drawing tube attached to a light microscope and then edited and finalised with Adobe Photoshop 7.0 ME software.

DNA extraction, PCR and sequencing. For molecular analyses, DNA was extracted from a single live nematode specimen, as described by Tanha Maafi *et al.* (2003), and was used as the PCR template. The portion of 18S rRNA gene was amplified as two overlapping fragments, using primer sets 988F (5'-CTC AAA GA TTA AGC CAT GC-3') and 1912R (5'-TTT ACG GTY AGA ACT AGG G-3') for the first fragment, and 1813F (5'-CKG CGY KAG AGG TGA AAT-3') and 2646R (5'-GCT ACC TTG TTA CGA CTT TT-3') for the second fragment (Holterman *et al.*, 2006). The D2-D3 segments of 28S rRNA gene were amplified using the forward D2A (5'-ACA AGT ACC GTG AGG GAA AGT-3') and reverse D3B (5'-TCG GAA GGA ACC AGC TAC TA-3') primers (Nunn, 1992). Each 30 µl amplification reaction mixture contained 15 µl Taq DNA Polymerase 2X Master Mix (Ampliqon), 1 µl of each forward and reverse primers (10 pmol μl^{-1}), 2 µl of DNA template and 11 µl distilled water. Tubes with mixture were placed into a Hybaid Express thermal cycler (Hybaid). The PCR program was as follow: initial denaturation at 95°C for 4 min, followed by 33 cycles of denaturation at 94°C for 30 s, annealing at 54°C for 30 s (18S rRNA gene) and 57°C for 30 s (28S rRNA gene), and extension at 72°C for 90 s. A final extension was performed at 72°C for 10 min. The quality of the PCR products was determined by electrophoresis of 4 µl of each PCR product in 1% agarose gel containing ethidium bromide under ultraviolet light. The PCR products were purified and sequenced in both directions using the same primers with an ABI 3730XL sequencer (Bioneer). The newly obtained sequences were submitted to GenBank under accession numbers: MW965293 for the 18S rRNA gene and MW965295 for the 28S rRNA gene.

Phylogenetic analysis. Phylogenetic relationships of *D. weischeri* with other species of this genus were inferred from the 18S rRNA and D2-D3 expansion fragments of 28S rRNA genes. The newly obtained sequences were edited and aligned with another segments of 18S rRNA or D2-D3 of 28S rRNA gene sequences of *Ditylenchus* species available in GenBank using MUSCLE alignment tool implemented in the MEGA7 (Kumar *et al.*, 2016). The ambiguously aligned parts and divergent regions were known using the online version of Gblocks 0.91b (Castresana, 2000) and were removed from the alignments using MEGA7. The best-fit model of nucleotide substitution for the phylogenetic analysis was statistically selected using jModelTest 2.1.10 (Darriba *et al.*, 2012). Phylogenetic trees were generated with Bayesian Inference (BI) method using MrBayes 3.2.6 (Huelsenbeck & Ronquist, 2001; Ronquist *et al.*, 2012). The analysis under GTR + I + G model was initiated with a random starting tree and run with Markov chain Monte Carlo (MCMC) for 1×10^6 generations. The tree was visualised and saved with FigTree 1.4.3 (Rambaut, 2016) and edited with Adobe® Acrobat® XI Pro 11.0.1.

RESULTS AND DISCUSSION

Ditylenchus weischeri Chizhov, Borisov & Subbotin, 2010 (Iranian population)

Figs 1 & 2

Measurements. See Table 1.

Female. Body straight. Cuticular annuli prominent, 0.8 to 1.1µm width at mid-body. Lateral field with four incisures, delimiting three bands, inner band narrower than outers, 5.0 to 5.4 µm wide occupying 22 to 25% of the corresponding body diameter. Cephalic region flatly rounded, with two annuli, 6.7 to 7.2 µm wide at base and 2.0 to 2.3 µm high, weakly sclerotised. Amphidial apertures inconspicuous. Stylet delicate, conus length about one-third (4.7-5.0 µm, or 44-46%) of the total stylet length; knobs distinct, rounded, 2.3 to 3.0 µm wide. Dorsal pharyngeal gland opening 1.0 µm behind stylet

Table 1. Morphometric data of *Ditylenchus weischeri* from Iran.
All measurements are in µm and in the form: mean ± standard deviation (range).

Characters	Present study		Chizhov *et al.*, 2010	Tenuta *et al.*, 2014
	Female	Male	Fe/Male	Fe/Male
n	12	6	48	20
Total body length	1199 ± 67.8 (1030-1281)	1131 ± 30.3 (1099-1180)	1281-1619	1049-1355
Anterior end to vulva	963 ± 55.5 (817-1025)	–	1132-1308	864-1080
Anterior end to anus	1119 ± 60.8 (974-1188)	1056 ± 28.5 (1026-1098)	–	–
Stylet length	10.8 ± 0.3 (10.2-11.2)	10.9 ± 0.2 (10.5-11.3)	9.0-13.0	8.0-11.5
Conus length	4.9 ± 0.1 (4.7-5)	4.8 ± 0.1 (4.6-5)	–	–
m	45.3 ± 0.6 (43.6-46)	44.6 ± 1.2 (42.4-45.4)	–	–
a	53.9 ± 4.4 (45.7-61)	51.8 ± 1.3 (49.9-53.6)	35.5-60.4	39-56
b	6.1 ± 0.2 (5.5-6.3)	6.1 ± 0.6 (5.6-7.4)	6.3-9.0	6.2-7.2
c	15.2 ± 1.6 (13.6-18.3)	15 ± 0.8 (13.9-16.4)	18.3-21.8	19.0-21.8
c’	6.3 ± 0.6 (5-7.3)	5.0 ± 0.3 (4.6-5.6)	2.9-5.0	3.6-3.9
V	80.2 ± 0.4 (79.3-80.8)	–	81-85	80-82.3
V’	86 ± 0.9 (83.8-87)	–	–	–
Anterior end to median bulb	75.5 ± 2.6 (68-78)	70.5 ± 1.0 (69-72)	65-86	69-77
MB	38.5 ± 1.8 (37-42)	38 ± 3.5 (36-45)	–	–
Anterior end to deirid	146 ± 3.2 (140-150)	138 ± 16.5 (105-150)	–	–
Anterior end to excretory pore	143 ± 4.6 (134-149)	139 ± 4.8 (131-146)	130-150	109-150
Pharynx length	196 ± 7.7 (185-206)	186 ± 13.3 (159-195)	170-214	169-196
Body width	22 ± 0.7 (21-23)	22 ± 0.4 (21-22)	–	–
Vulva body width	19.5 ± 0.4 (19-20)	–	–	–
Distance of vulva to anus	156 ± 11.1 (141-175)	–	–	–
Tail/ distance of vulva to anus	0.4 ± 0 (0.3-0.5)	–	–	–
Anal body width	12.5 ± 0.8 (11-14.5)	15 ± 0.4 (14-16)	–	–
Tail length	79 ± 10.3 (56-94)	75 ± 4.9 (70-82)	–	–
Spicule length	–	21.5 ± 1.0 (20-23)	20-24	23-26
Gubernaculum length	–	9.0 ± 0.7 (8.0-10.0)	6.0-9.0	5.3-7.4
Bursa length	–	41 ± 2.5 (38-45)	48-78	50-56

base. Corpus cylindroid with well develop median bulb, with distinct valve; isthmus as wide as precorpus, nerve ring at mid-isthmus and located at 99 to 112 µm from anterior end; excretory pore at anterior of basal bulb, hemizonid 3 to 4 annuli anterior to excretory pore, located at 128 to 142 µm from anterior end; deirids adjacent to the level of excretory pore, 140 to 150 µm from anterior end. Pharyngeal basal bulb large and cylindrical, 11 to 13 µm wide, 48 to 53 µm in length, in some cases slightly overlapping intestine, maximum 10 µm. Pharyngeal-intestinal valve distinct and large. Vulva a transverse slit, not protruding, without lateral flaps. Vagina width 7.0 to 9.5 µm, 36 to 47% of vulva body diameter. Post-vulval uterine sac (PVUS) length 72 to 125 µm or 3.6 to 6.5 times of vulval body diameter. Spermatheca long, near-rectangular, filled with globular sperms. Ovary outstretched, oocytes arranged in a several rows. Rectum short, length half of anal body diameter. Tail elongate-conoid, beak-lick and pointed at tip.

Male. General morphology similar to that of females. Testis very large, extending to near basal bulb, seldom tips reflexed. Spicules tylenchid-shape, tips pointed. Gubernaculum simple, slightly curved. Bursa adanal, encircled less than half of tail length. Tail elongate conoid, tip pointed.

Voucher specimens. Twelve females and six males are deposited in the nematode collection of the Department of Plant Protection, Faculty of Agriculture, Islamic Azad University of Tehran Varamin-Pishva Branch, Iran.

Habitat and locality. Soil samples were collected from the rhizosphere of weed, *Chenopodium album*, in potato farm in Markazi province, by the first author in February 2020. The geographical position of the sampling site (GPS) coordinates: 34°36’36.91” N, 49°31’27.97” E.

Morphological remarks. The Iranian population of *D. weischeri* morphologically is similar to the original description by Chizhov *et al.* (2010) but differs in some morphometrical characters such as having shorter female body length (1030-1281 *vs* 1371-1619 µm), longer a ratio (45.7-61.0 *vs* 35.5-44.8), b ratio (5.5-6.3 *vs* 7.0-9.0), c ratio (13.6-18.3 *vs* 18.3-28.1), c' ratio (5.0-7.3 *vs* 2.9-4.8) and slightly shorter PVUS (72-125 *vs* 101-150 µm) but resembles a Canadian population (Tenuta *et al.*, 2014).

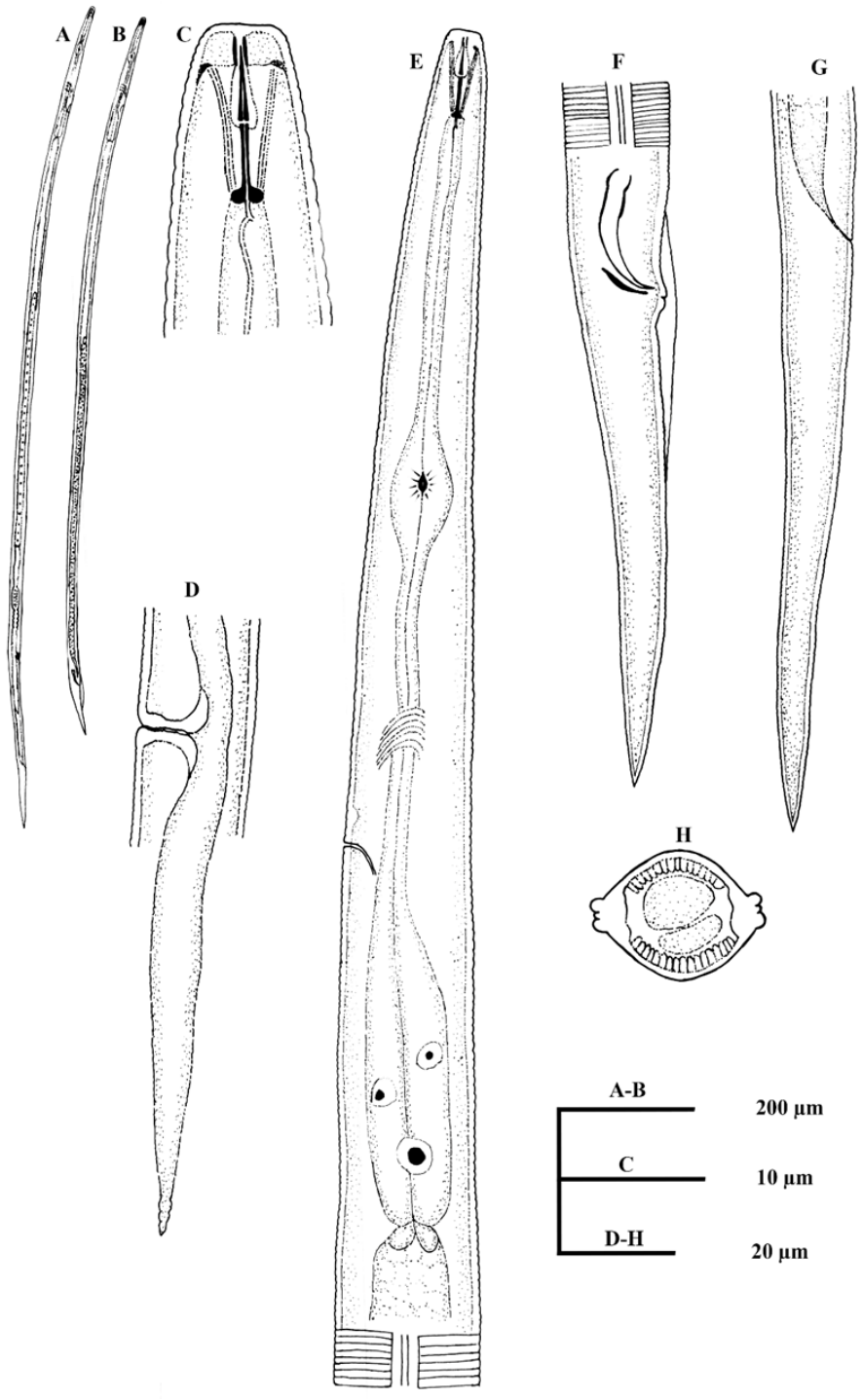

Fig. 1. Line drawing of the Iranian population of *Ditylenchus weischeri*. Female (A, C-E, G-H) and male (B & F). A & B: Entire body; C: Anterior end; D: Vulva region and PUS; E: Pharyngeal region; F & G: Tail; H: Cross section at mid-body.

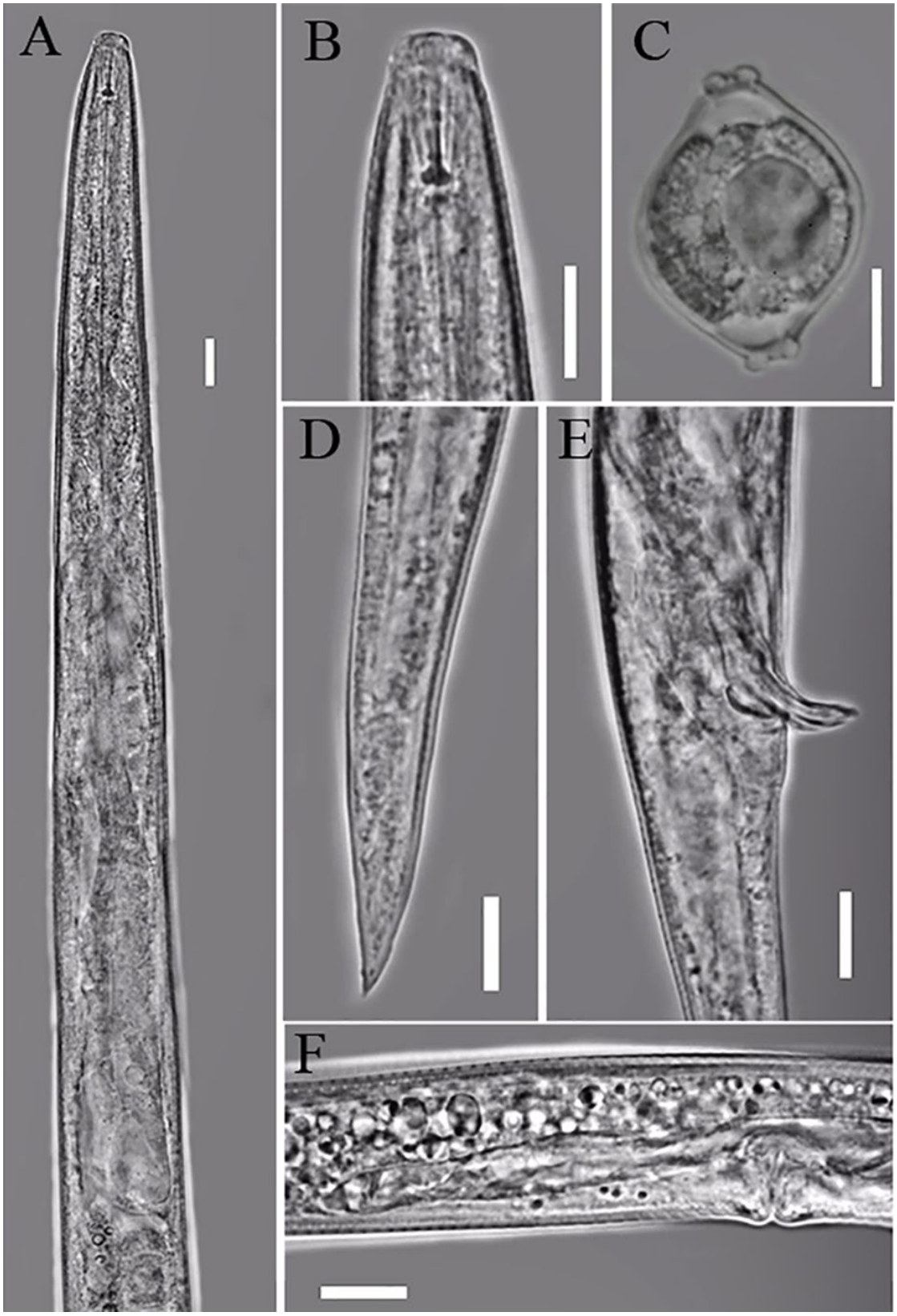

Fig. 2. Light photomicrographs of the Iranian population of *Ditylenchus weischeri*. Female (A-D, F) and male (E). A: Pharyngeal region; B: Anterior end; C: Cross section at mid-body; D & E: Posterior end; F: Vulva region and PUS. Scale-bars: A-F = 10 µm.

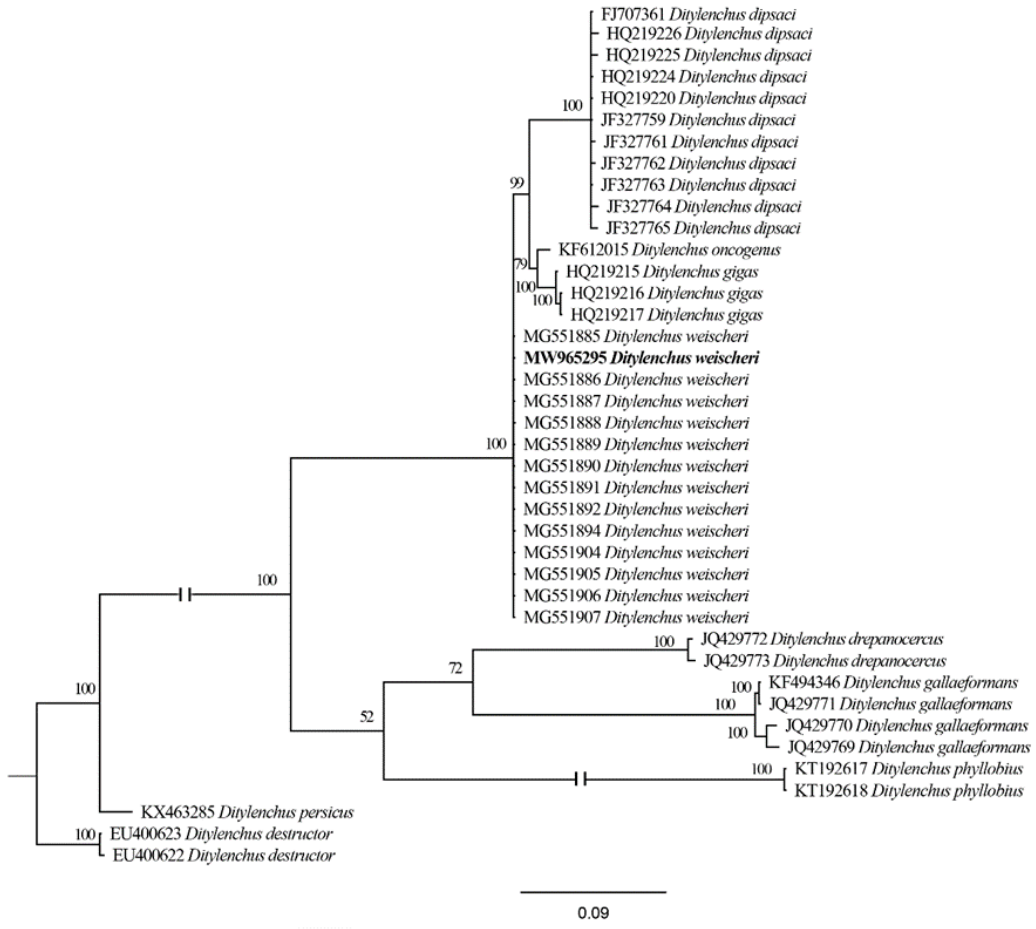

Fig. 3. Phylogenetic relationships of *Ditylenchus weischeri* with representatives of the genus *Ditylenchus*. Bayesian 50% majority rule consensus tree as inferred from D2-D3 expansion segments of 28S rRNA gene sequence alignment under under the GTR + I + G model. Posterior probabilities greater than 50 are given for appropriate clades. Newly obtained sequences in this study are shown in bold. Scale bar = expected changes per site.

The Iranian population was isolated from soil, whereas Russian and Canadian populations were isolated from stems and leaves, which indicates that morphological characters may vary by host and accessibility of nutrient source.

Molecular phylogenetic study. One new D2-D3 28S rRNA gene sequence was obtained in the present study (MW965295). This sequence is identical to those of several isolates of *D. weischeri* (MG551885-1892; MG551894-1898; MG551902-1907 and MG551910) using BlastN search in NCBI. The D2-D3 of 28S rRNA gene sequences alignment contained 36 taxa, the new sequence and three sequences of *D. destructor* (EU400623 and EU400622) and *D. persicus* (KX463285) considered as outgroup taxa. The 50% majority rule consensus phylogenetic tree generated from the D2-D3 of 28S rRNA gene alignment by BI analysis under GTR + I + G model is presented in Figure 3. In the D2-D3 of the 28S rRNA gene tree the Iranian population of *D. weischeri* clustered in a strongly supported clade with several isolates of *D. weischeri*. Sequence of Iranian *D. weischeri* was different in maximum 26 bp (4.1%) from those *D. dipsaci*.

One new 18S rRNA gene sequence was obtained in the present study (MW965293). This sequence showed a 99.93% similarity values to those of the isolates of *D. dipsaci* (MK292125) and *D. weischeri* (MG383839, MG383840, MG383847 and MG383848) using BlastN search in NCBI. The 18S rRNA gene sequences alignment contained 32 taxa, the sequence of Iranian population of *D. weischeri* and *Coslenchus persicus* Hosseinvand, Eskandari & Ghaderi, 2020 (MT073109) considered as outgroup taxa and was 1640 bp in length after removing ambiguously aligned regions. The 50% majority rule consensus phylogenetic tree generated from the 18S rRNA gene alignment by BI analysis under

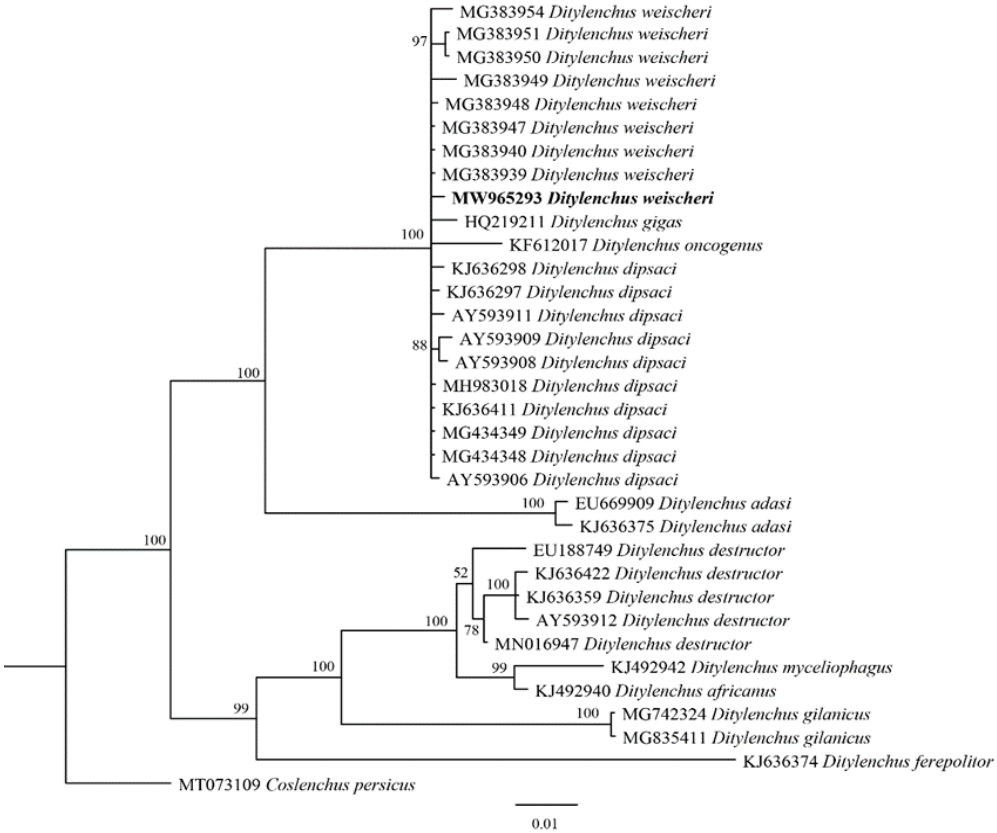

Fig. 4. Phylogenetic relationships of *Ditylenchus weischeri* with representatives of the genus *Ditylenchus*. Bayesian 50% majority rule consensus tree as inferred from partial 18S rRNA gene sequence alignment under the GTR + I + G model. Posterior probabilities greater than 50 are given for appropriate clades. Newly obtained sequences in this study are shown in bold. Scale bar = expected changes per site.

GTR + I + G model is presented in Figure 4. In the 18S rRNA gene tree, Iranian sample occupied a clade with several isolates of *D. weischeri* and *D. dipsaci*. The Iranian sequence differed by 0-6 bp (0-0.2%) from those of other *D. weischeri* sequences and by 1-5 bp (0.01-0.2%) from those of *D. dipsaci*. The 18S rRNA gene was not able to distinguish between species of *D. dipsaci* group.

This study identified the species of *Ditylenchus*, associated with rhizosphere of weeds in potato fields and was identified as *D. weischeri* based on morphologic and molecular characters. Moreover, it seems the feeding habit and the host, where species of *Ditylenchus* was isolated, impact their morphometric characters and highlights the role of molecular data for correct identification of species with complex morphology.

ACKNOWLEDGEMENTS

The authors thank Dr Manouchehr Hosseinvand (Department of Plant Protection, Faculty of Agriculture, University of Zanjan, Iran), Dr Sergei A. Subbotin (CDFA, USA), and respected editor for valuable comments to improve the article.

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Резюме. В ризосфере сорняков на картофельном поле в провинции Маркази обнаружен *Ditylenchus weischeri*. Приводится морфологическое описание и анализ нуклеотидных последовательностей 18S рРНК и 28S рРНК этих нематод, которые оказались на 99.7-100% и 99.9-100% идентичными таковым других изолятов *D. weischeri*.