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MORPHOLOGY AND MOLECULAR CHARACTERIZATION OF *TYLENCHULUS SEMIPENETRANS* FROM CITRUS ORCHARDS IN NORTHERN IRAN

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Abstract: *Tylenchulus semipenetrans* Cobb, 1913 is among some of the most economically important plant-parasitic nematodes in the world. The nematode has been identified as the causal agent of slow decline. Most studies estimate the yield losses, due to *T. semipenetrans,* to range from 10% to 30%, depending on the level of infection, aggressiveness of the nematode population, soil characteristics, susceptibility of the rootstock, presence of other pathogens and grove management practices. In order to identify the citrus nematode in northern Iran, soil and root samples were collected from infected trees. The second-stage juveniles were isolated from the soils by the tray method. Eggs and females were extracted from roots by the centrifugal-flotation technique. Morphological observations and molecular evidence confirmed this population as *T. semipenetrans*. A phylogenetic tree of *T. semipenetrans* populations was reconstructed based on 28S rRNA gene sequences using RAxML. Morphologically, there is a slight difference between the studied population and the reported populations of *T. semipenetrans* from pomegranate and banana orchards in southern Iran. Phylogenetic analysis showed the close relationship of the *T. semipenetrans* population from northern Iran with other populations of this species. Based on molecular analysis, *Tylenchulus* was identified as a monophyletic group. The phylogenetic position and measurements of *T. semipenetrans* were provided.

Key words: *Citrus sinensis*, 28S rRNA, Iran, phylogeny, slow decline.

Introduction

Iran is the sixth-largest citrus fruit producer in the world, with an annual production of 4.1 million tons (FAO, 2016). Various citrus species are widely

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cultivated in northern and southern parts of Iran, such as sweet orange (*Citrus sinensis* L.), acid lime (*C. aurantifolia* (Christm.) Swingle), sour orange (*C. aurantium* L.), mandarin (*C. reticulata* Blanco), lemon (*C. limon* (L.) Osbeck), and grapefruit (*C. paradisi* Macfad). The main producing regions in Iran are Mazandaran, Fars, Hormozgan, Jiroft and Kahnouj (Espargham et al., 2020). Like many tropical and subtropical crops, citrus is attacked by numerous pests and pathogens. *Citrus* species are susceptible to a large number of destructive diseases caused by fungal, bacterial, and viral plant pathogens, which are continuously emerging and can severely hinder or completely destroy the entire production (Tennant et al., 2009).

Plant-parasitic nematodes are economically important pests that affect many horticultural crops produced in tropical and subtropical areas (Whitehead, 1998). The genus *Tylenchulus* contains four species in the world. Among them, *T. semipenetrans*, the causal agent of "slow decline" of citrus, has a worldwide distribution and causes significant crop losses in all citrus-growing regions of the world (Siddiqi, 1974; Duncan and Cohn, 1990). This nematode was first observed in orange tree in southern California by Cobb in 1912. Crop losses caused by *T. semipenetrans* are estimated to be in the range of 15% to 30% per year (Duncan, 2005). Symptoms of nematode attack often include reduced vigor, chlorosis, leaf fall, dieback, and reduced production and weakened fruit quality (Cohn, 1969). The citrus nematode has been previously reported from citrus orchards in Iran (e.g., Izadpanah and Safarian, 1968; Katcho and Allow, 1969; Abivardi, 1970; Minassian and Moadab, 1970; Sharafeh, 1972; Tanha Maafi and Kheiri, 1991; Tanha Maafi and Damadzadeh, 2008; Rashidifard et al., 2015). Izadpanah and Safarian (1968) first reported *T. semipenetrans* from citrus growing areas in the Ahvaz province. Tanha Maafi and Damadzadeh (2008) revealed that 89% of the soil and root samples of citrus orchards in Mazandaran were infested with citrus nematodes. Additionally, they evaluated the impact of two organophosphates and one carbamate nematicide on citrus nematode under both glasshouse and orchard conditions. Also, several studies were conducted to monitor the population dynamics of *T. semipenetrans* in southern (Sharafeh, 1972; Tanha Maafi and Kheiri, 1991) and northern (Tanha Maafi and Damadzadeh, 2008) parts of Iran. Tanha Maafi et al. (2012) analyzed the phylogenetic relationships within *Tylenchulus* using rRNA gene sequences. Recently, the molecular characterization, phylogenetic position, and seasonal dynamics of *T. semipenetrans* were elucidated in the southern part of Iran (Rashidifard et al., 2015).

The first and most important step in controlling slow decline is to diagnose the disease correctly. Traditionally, the identification of *Tylenchulus* species has been based on a few morphological characters of the male, the mature female, and the second-stage juveniles (Inserra et al., 1988a, b; 1994). However, morphological identification of the J2 of *T. semipenetrans* is difficult due to its small size and

requires taxonomic expertise. In addition, *Tylenchulus* J2 specimens can be easily misidentified with the J2 of some closely related genera (e.g., *Trophotylenchulus* and *Meloidogyne* spp.). Therefore, an accurate and reliable identification procedure for monitoring and diagnostic purposes to distinguish *Tylenchulus* species becomes a very important task. In the past decade, molecular techniques have been developed and applied to identify plant-parasitic nematodes (e.g., Blok et al., 2002; Adam et al., 2007; Park et al., 2009; Liu et al., 2011; Tanha Maafi et al., 2012; Yan et al., 2013; Lin et al., 2016). Recent studies have shown that molecular techniques are more sensitive and accurate tools for the identification of *T. semipenetrans* (Liu et al., 2011).

So far there has been no comprehensive study on molecular identification or diagnosis of *Tylenchulus* species in the northern part of Iran. Therefore, the aims of the present study were to identify the species of citrus nematode in northern Iran by morphological and molecular methods and to explain the phylogenetic position of the population of *T. semipenetrans* with closely related populations in GenBank.

Material and Methods

Specimens and collections

The samples were collected in the Guilan province. Five trees were selected from each orchard and three samples were collected from each tree. Each soil sample was taken at a depth of 5–30 cm. The samples from each tree were completely mixed and a representative sample of 500 g was prepared. The soil and root samples were transferred to the Nematology laboratory of the University of Guilan and stored at 4°C. The juvenile nematodes were extracted from the soil samples by the centrifugal-flotation technique and the tray method, whereas a centrifugation method (Jenkins, 1964) was used to recover mature females and eggs from the roots. In the present study, *T. semipenetrans* was identified using the descriptions provided by Goodey (1963) and Crozzoli et al. (1998).

DNA extraction, PCR and sequencing

DNA was extracted using the method of Subbotin et al. (2006). Polymerase chain reactions (PCRs) were carried out in a 25-µl reaction mixture, containing 4 µl of master mix, 9 µl of molecular-grade water, 1 µl of each primer and 10 µl of genomic DNA template. The primer pairs D2A (5”-ACAAGTACCGTGAGGGAAAGTTG–3”) and D3B (5”-TCGGAAGGAACCAGCTACTA–3”) were used to amplify ~750-bp fragment of the 28S region (Subbotin et al., 2006). The PCR program consisted of an initial denaturing step at 94°C for 3 min, 37 amplification cycles (94°C for 45s, 56°C for

45s, 72˚C for 60s), and a final step at 72˚C for 6 min. The size of the amplification products was determined using a 1% agarose gel. The PCR product was purified and sequenced in both directions. The sequencing was performed by Bioneer company (South Korea) (http://eng.bioneer.com). The newly obtained sequence data was deposited into the GenBank database (Table 1).

Table 1. The accession numbers of the sequences used in the phylogenetic analysis. The sequence in bold was sequenced in the present study.

Species	GenBank accession number	Origin	Reference
Caloosia longicaudata	GU989627	United States	(Van den Berg et al., 2011)
Coslenchus costatus	DQ328719	Germany	(Subbotin et al., 2006)
Criconema mutabile	AY780954	Venezuela	(Subbotin et al., 2006)
Criconema sp.	AY780952	Italy	(Subbotin et al., 2006)
Criconema sp.	AY780953	Venezuela	(Subbotin et al., 2006)
Criconemoides brevistylus	JQ231185	South Africa	(Van den Berg et al., 2012)
Criconemoides informis	AY780970	Venezuela	(Subbotin et al., 2005)
Criconemoides obtusicaudatus	JQ231186	South Africa	(Van den Berg et al., 2012)
Criconemoides obtusicaudatus	JQ231187	South Africa	(Van den Berg et al., 2012)
Hemicaloosia vagisclera	JQ246423	United States	(Inserra et al., 2013)
Hemicriconemoides alexis	AY780959	Greece	(Subbotin et al., 2005)
Hemicriconemoides gaddi	KC520470	China	(Yang et al., 2013)
Hemicriconemoides ortonwilliamsi	AY780948	Italy	(Subbotin et al., 2005)
Hemicycliophora lutosa	GQ406240	South Africa	(Van den Berg et al., 2010)
Hemicycliophora lutosa	GQ406241	South Africa	(Van den Berg et al., 2010)
Meloidoderita kirjanovae	DQ768428	Italy	(Vovlas et al., 2006)
Ogma civellae	AY780955	Venezuela	(Subbotin et al., 2005)
Paratylenchus aquaticus	KF242240	United States	(Van den Berg et al., 2014)
Paratylenchus aquaticus	KF242239	United States	(Van den Berg et al., 2014)
Paratylenchus bukowinensis	AY780943	Italy	(Subbotin et al., 2005)
Paratylenchus dianthus	KF242229	South Africa	(Van den Berg et al., 2014)
Paratylenchus hamatus	KF242219	United States	(Van den Berg et al., 2014)
Paratylenchus nanus	AY780946	Germany	(Subbotin et al., 2005)
Paratylenchus sp.	AY780944	Italy	(Subbotin et al., 2005)
Paratylenchus sp.	AY780945	United States	(Subbotin et al., 2005)
Paratylenchus straeleni	KF242236	United States	(Van den Berg et al., 2014)
Psilenchus sp.	DQ328716	United States	(Subbotin et al., 2006)
Sphaeronema alni	JQ771954	Czech Republic	(Codejkova and Cermak, 2013)

Continuation of Table 1. The accession numbers of the sequences used in the phylogenetic analysis. The sequence in bold was sequenced in the present study.

Trophotylenchulus floridensis	JN112254	United States	(Tanha Maafi et al. 2012)
Trophotylenchulus floridensis	JN112253	United States	(Tanha Maafi et al., 2012)
Tylenchulus furcus	JN112257	South Africa	(Tanha Maafi et al., 2012)
Tylenchulus furcus	JN112258	South Africa	(Tanha Maafi et al., 2012)
Tylenchulus graminis	JN112259	United States	(Tanha Maafi et al., 2012)
Tylenchulus graminis	JN112260	United States	(Tanha Maafi et al., 2012)
Tylenchulus musicola	JN112247	Iran	(Tanha Maafi et al., 2012)
Tylenchulus musicola	JN112248	Iran	(Tanha Maafi et al., 2012)
Tylenchulus palustris	JN112255	United States	(Tanha Maafi et al., 2012)
Tylenchulus semipenetrans	AY780972	Egypt	(Subbotin et al., 2005)
Tylenchulus semipenetrans	JN112249	United States	(Tanha Maafi et al., 2012)
Tylenchulus semipenetrans	JN112250	United States	(Tanha Maafi et al., 2012)
Tylenchulus semipenetrans	JN112251	South Africa	(Tanha Maafi et al., 2012)
Tylenchulus semipenetrans	JN112252	United States	(Tanha Maafi et al., 2012)
Tylenchulus semipenetrans	FJ969710	Korea	(Park et al., 2009)
Tylenchulus semipenetrans	FJ969711	Korea	(Park et al., 2009)
Tylenchulus semipenetrans	FJ969712	Korea	(Park et al., 2009)
Tylenchulus semipenetrans	FJ969713	Korea	(Park et al., 2009)
Tylenchulus semipenetrans	FJ969714	Korea	(Park et al., 2009)
Tylenchulus semipenetrans	FJ969715	Korea	(Park et al., 2009)
Tylenchulus semipenetrans	KJ577615	Iran	(Rashidifard et al., 2015)
Tylenchulus semipenetrans	KM598333	Iran	(Rashidifard et al., 2015)
Tylenchulus semipenetrans	KM598334	Iran	(Rashidifard et al., 2015)
Tylenchulus semipenetrans	KM598335	Iran	(Rashidifard et al., 2015)
Tylenchulus semipenetrans	**?**	**Iran**	**Present study**
Xenocriconemella macrodora	AY780960	Italy	(Subbotin et al., 2005)

Phylogenetic analysis

All sequences were aligned in the MAFFT v.7 online servers (http://mafft.cbrc.jp/ alignment/server/; Katoh et al., 2019) and concatenated for phylogenetic analysis, with *Coslenchus costatus* and *Psilenchus* sp. added as outgroups. Maximum likelihood (ML) analysis was performed with RAxML (Stamatakis, 2006) as implemented in raxmlGUl 1.3 (Silvestro and Michalak, 2012), using the ML + rapid bootstrap setting and the GTRGAMMA substitution model with 1000 bootstrap replicates. Bootstrap support values above 50% are given in Figure 2.

Results and Discussion

Morphological characteristics

In adult females (Figure 1, Table 2), the body was 349–406 µm long, proximally elongated and irregular, distal half swollen. The thickness of the cuticle in the middle of the body was 5–9 µm. The stylet was 12–20 µm long with rounded knobs. The dorsal esophageal gland Orifice (DEGO) was 4–8 µm below the stylet knobs. The size of the Post-Vulval Section Cavity (PVSC) reached 3–4 µm. The length of the the post-vulval sac was 14–28 µm. The width of the post-vulva section (PVSW) was 11–18 micrometers. The basal bulb was oval, 14–24 µm long and 14–25 µm wide. The excretory pore was located at 69–78% of body length. The reproductive system was monodelphic. Eggs were ovoid, with sizes ranging from 33 to 67 µm. The tail was curved towards the abdomen.

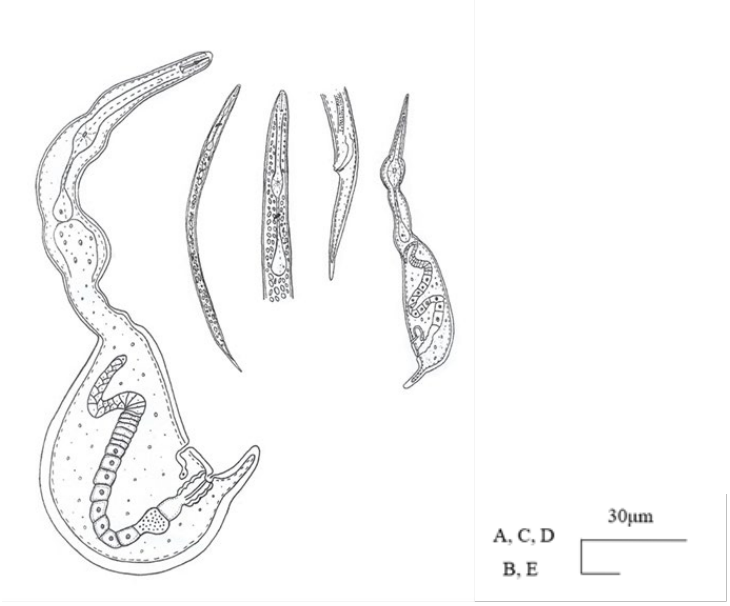

Figure 1. *Tylenchulus semipentrans*, A: Entire body of female; B: Entire body of second-stage juvenile; C: Anterior body portion of juvenile; D: Posterior body portion of male; E: Young female; (Scale bars: A–D: 30µm.)

In males (Figure 1, Table 3), the body was "J" shaped, 326–399 µm long. The esophagus and the stylet were weakly developed. The stylet was 7–9 µm long with rounded knobs. Excretory pores were located in 20–23% of the body length. The gubernaculum was simple and 4.2–5 µm long.

In second-stage juveniles (Figure 1, Table 3), bodies were 302–333 µm long. The stylet was 12–16 µm long with rounded knobs. The excretory pore was located at 47–54% of the body length and 118–148 µm to the genital primordium. The deirid was not visible. The genital primordium had 3 cells and was 60–67% of the distance from the anterior end.

De Man ratios obtained were: female: a = 3.7 ± 1.1; male: a = 29.6 ± 1.4, b = 4.2 ± 0.6, c = 8.7 ± 0.5; second-stage juvenile: a = 22 ± 0.5, b = 2.9 ± 9.2, c = 6.4 ± 0.8.

Table 2. Measurements of females of *Tylenchulus semipenetrans* Cobb, 1913 collected from the Guilan province. All measurements are in µm.

Province	Chabahar (Tanha Maafi et al., 2012)	Shahdad (Rashidifard et al., 2015)	Present study
Number	12 ♀♀	6 ♀♀	5 ♀♀
L	287 ± 32.6 (240–370)	273.9± 21.6 (245–295)	267 ± 11 (254–292)
a	4.8 ± 1.4 (3.7–8.2)	3.8 ± 1.2 (2.7–5.2)	3.7 ± 1.1 (2.8–4.9)
Stylet	10 ± 1.2 (8–12)	14.3 ± 6.1 (10–22)	13.8 ± 5.3 (12–20)
DEGO	4.0 ± 0.8 (3–5)	5 ± 1.9 (4–8)	4.5 ± 1.7 (4–8)
Anterior end to median bulb	46 ± 7.7 (35–55)	27 ± 2.9 (25–31)	26 ± 2.2 (25–29)
Median bulb length	15.3 ± 2.4 (12–19)	18 ± 4.7 (15–24)	19 ± 3.4 (16–22)
Median bulb width		14.4 ± 3.7 (11–19)	13.3 ± 2.6 (12–19)
Pharynx length	94 ± 15.2 (66–112)	131.6 ± 3.2 (129–135)	130.4 ± 3.3 (128–134)
Basal bulb length	21.5 ± 3.4 (15–27)	20 ± 2.9 (13–25)	20.1 ± 2.4 (14–24)
Basal bulb width	12.6 ± 2.0 (10–16)	18 ± 3.9 (13–26)	19 ± 2.3 (14–25)
Anterior end to excretory pore	222 ± 37.7 (150–280)	205.2 ± 30.3 (162–230)	210 ± 22.5 (173–228)
Excretory pore from anterior end as % of body length	77.4 ± 8.8 (61.2–93.3)	72±5.7 (66.5–78)	71 ± 5.1 (69–78)
Vulva-excretory pore distance	14.1 ± 3.3 (10–20)	17.2 ± 3.4 (13–22)	17.2 ± 3.4 (13–22)
Post-vulva section width (PVSW)	11.0 ± 1.4 (9–14)	13 ± 3.1 (10–18)	14 ± 2.2 (11–18)
Post-vulva section length (PVSL)	27 ± 6.0 (17–36)	19.4 ± 5.9 (13–31)	18.3 ± 4.2 (14–28)
Post-vulva section cavity (PVSC)	6.6 ± 0.7 (6–8)	4.2 ± 0.9 (4–5)	3.9 ± 0.8 (3–4)
Swollen posterior body length	-	178 ± 21.2 (154–211)	167 ± 18.2 (160–209)
Swollen posterior body as % of total body length	45.6–54.0	57.5 ± 3.7 (55–63)	53.2 ± 3.1 (50–60)
Body width at vulva	23 ± 3.8 (15–29)	47 ± 8.6 (37–65)	42 ± 1.9 (38–62)
Body width at mid-body	64 ± 16.8 (30–80)	79.5 ± 13.5 (55–97)	73 ± 8.8 (60–94)
Cuticle thickness at mid-body	4.2 ± 0.8 (3–5)	5.4 ± 1.2 (4–9)	5.2 ± 1.3 (5–9)

Remarks: The measured characters were generally similar to reported values for *T. semipenetrans* from Iran (Rashidifard et al., 2015, Tanha Maafi et al., 2012) (Tables 2 and 3). The differences between the studied traits and the population

reported from the south of Iran were as follows. The values of stylet length, DEGO, median bulb length, and body width were lower in the Chabahar population.

Table 3. Measurements of second-stage juveniles (J2) and males of *Tylenchulus semipenetrans* Cobb, 1913 collected from the Guilan province.

Province	Chabahar (Tanha Maafi et al., 2012)		Shahdad (Rashidifard et al., 2015)		Present study	
Number	31 J2	5 ♂♂	5 J2	6 ♂♂	6 J2	4 ♂♂
Body length	306 ± 13.8 (278–334)	310 ± 19.2 (286–326)	323.6 ± 13.7 (309–345)	328.1 ± 21.8 (305–355)	322 ± 10.6 (302–333)	372 ± 17.9 (326–399)
a	30.1 ± 1.6 (27.7–33.4)	27.2 ± 3.8 (23.8–32.5)	27.3 ± 2.3 (20.9–27.1)	32.1 ± 2 (29.5–33.6)	22 ± 0.5 (21–22.2)	29.6 ± 1.4 (27.1–30)
b	3.5 ± 0.2 (3.2–4.0)	3.5 ± 0.2 (3.3–3.7)	3.3 ± 0.3 (3.1–4)	4.4 ± 0.8 (3.3–5.8)	2.9 ± 9.2 (2.8–3)	4.2 ± 0.6 (4.2–4.6)
c	-	8.1 ± 0.1 (8.0–8.2)	7.5 ± 1.3 (6–9.2)	7.3 ± 0.6 (6.4–7.8)	6.4 ± 0.8 (6–6.7)	8.7 ± 0.5 (8.1–8.8)
Stylet	11.1 ± 0.6 (10–12)	8.5 ± 0.6 (8–9)	13.4 ± 2.9 (12–19)	8.8 ± 2.1 (7–11)	14 ± 2.1 (12–16)	8 ± 0.8 (7–9)
Anterior end to median bulb	43.6 ± 2.5 (38–48)	36.0 ± 1.4 (35–37)	43 ± 6.8 (37–54)	36.5 ± 7.4 (27–46)	47 ± 1.9 (45–55)	38 ± 1.2 (33–45)
Pharynx length	87 ± 4.3 (78–100)	89 ± 1.5 (87–90)	96.1 ± 7.4 (85–105)	73.1 ± 5.2 (68–82)	108 ± 3.4 (98–112)	88 ± 6.4 (70–90)
Anterior end to hemizonid	65 ± 4.3 (57–71)	57 ± 6.7 (50–65)	69.6 ± 4.8 (66–77)	73.7 ± 4 (69–78)	82 ± 1.3 (80–88)	80 ± 4.3 (77–83)
Anterior end to excretory pore	169 ± 8.4 (148–184)	174 ± 4.0 (170–178)	169.6 ± 5.4 (163–177)	171.5 ± 10.6 (163–182)	84 ± 0.8 (80–86)	77 ± 2.1 (75–79)
Anterior end to nerve ring	-	-	75.2 ± 8.5 (66–88)	49.7 ± 12 (32–63)	75 ± 1.5 (68–77)	65 ± 1.8 (59–69)
Excretory pore to genital primordium	28.5 ± 6.8 (15–40)	-	46.7 ± 13.5 (37–70)	-	127 ± 10.3 (118–148)	-
Anterior end to genital primordium	198 ± 9.9 (179–217)	-	216.3 ± 16.1 (200–243)	-	199 ± 9.8 (185–212)	-
Genital primordium to posterior end	109.2 ± 7.6 (90–124)	-	134.6 ± 9.9 (124–151)	-	125 ± 1.9 (122–130)	-
Maximum body diameter	10.1 ± 0.3 (10–11)	11.5 ± 1.0 (10–12)	13.7 ± 1 (13–15)	10.9 ± 1.3 (10–13)	14.5 ± 0.5 (14–15)	12.5 ± 0.6 (12–13.5)
Excretory pore from anterior end as % of body length	55.3 ± 2.0 (49.5–58.4)	54.9 ± 3.4 (52.1–58.7)	52.4 ± 1.4 (50–54)	17.7 ± 6.2 (13–28)	50 ± 3.1 (47–54)	22.2 ± 1.5 (20–23)
Genital primordium (%)	64.7 ± 2.0 (61–70)	-	66.7 ± 2.3 (65–70)		65 ± 1.9 (60–67)	-
Spicules	-	15.7 ± 2.1 (14–18)		20 ± 1.1 (19–22)	-	21.3 ± 0.5 (21–22)
Gubernaculum	-	4.5 ± 0.7 (4–5)		4 ± 0.3 (3–4)	-	4.4 ± 0.3 (4.2–5)
Tail	-	38.3 ± 2.4 (35–40)	43.9 ± 7.8 (33–54)	44.8 ± 5.8 (39–53)	50.3 ± 2.2 (45–55)	42.5 ± 2.3 (40–45)

Molecular phylogenetic analysis

The present study confirmed the occurrence of *T. semipenetrans* in the main citrus growing areas of the Guilan province. The best ML tree (lnL = -9636.915433) obtained by RAxML is shown in Figure 2. Of the 775 nucleotide characters of the matrix, 372 were parsimony informative. Phylogenetic analysis revealed the existence of five major clades including:

I) *Caloosia longicaudata* (Loos, 1948), *Hemicaloosia vagisclera* (Inserra et al., 2013), *Criconemoides* spp., *Hemicycliophora lutosa* (Loof and Heyns, 1969), *Criconema* spp., *Ogma civellae* (Steiner, 1949; Raski and Luc, 1987), *Hemicriconemoides* spp., *Xenocriconemella macrodora* (Taylor, 1936; De Grisse and Loof, 1965);

II) *Paratylenchus* spp;

III) *Trophotylenchulus floridensis* (Raski, 1957), and T*ylenchulus* spp;

IV) *Sphaeronema alni* (Turkina and Chizhov, 1986);

V) *Meloidoderita kirjanovae* (Pogosjan, 1966).

As in previous studies, *Tylenchulus* was found to be a monophyletic group and all species were included within the strongly supported clade (83%) (e.g., Subbotin et al., 2005; Tanha Maafi et al., 2012). Our analysis supports the taxonomic status of *Trophotylenchulus* Raski, 1957 as a separate taxon from *Tylenchulus*. This result is similar to the analysis of Tanha Maafi et al. (2012) based on molecular data. *Trophotylenchulus* was also united with *Tylenchulus* as a sister group, confirming another recent study (Rashidifard et al., 2015). Our sequence of *T. semipenetrans* was clustered with other *T. semipenetrans* sequences from GenBank with maximum support (99%), which is in agreement with other previous studies (e.g., Tanha Maafi et al., 2012; Rashidifard et al., 2015). Further phylogenetic studies are needed in order to provide a clearer idea of the generic relationships within *Tylenchuloidea.*

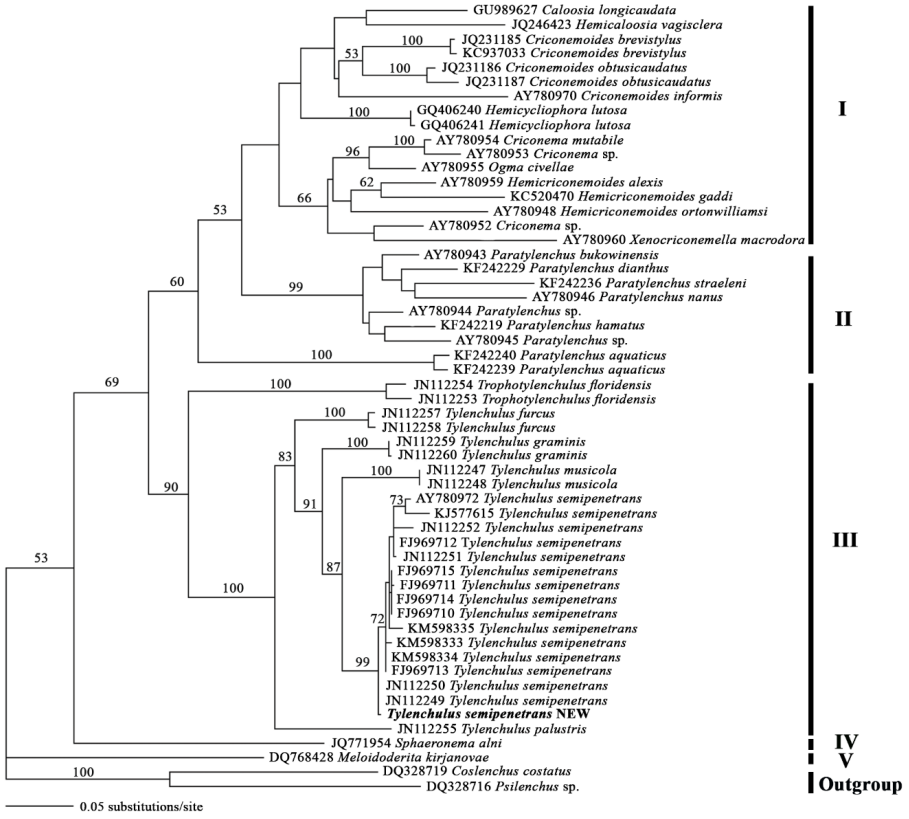

Figure 2. The phylogram of the best ML trees (lnL = -9636.915433) revealed by RAxML from an analysis of the 28S rDNA region.
The sequence in bold is new from Iran.

Conclusion

Morphologically, there is a slight difference between the studied population and the reported populations of *T. semipenetrans* from pomegranate and banana orchards in southern Iran. These characters include stylet length, DEGO, median bulb length and body width. The minimum morphological differences were observed in the Shahdad population of pomegranates.

Phylogenetic analysis using 28S rDNA showed the close relationship of the *T. semipenetrans* population from northern Iran with other populations of this species. The most similarity was observed in the JN112249 and JN112250 populations. This result indicates that *Tylenchulus* forms a monophyletic group.

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References

Abivardi, C. (1970). Occurrence of *Paratylenchus hamatus* on citrus in Iran and its sensitivity to two nematicides under laboratory condition. *Plant Disease Reporter*, *54* (12), 1085-1088.

Adam, M.A.M., Phillips, M.S., & Blok, V.C. (2007). Molecular diagnostic key for identification of single juveniles of seven common and economically important species of root-knot (*Meloidogyne* spp.). *Plant Pathology*, *56*, 190-197.

Blok, V.C., Wishart, J., Fargette, M., Berthier, K., & Phillips, M.S. (2002). Mitochondrial DNA differences distinguishing *Meloidogyne mayaguensis* from the major species of tropical root-knot nematodes. *Nematology*, *4*, 773-781.

Cohn, E. (1969). The citrus nematode (*Tylenchulus semipenetrans* Cobb) as a pest of Citrus in Israel. *Proceedings First International Citrus Symposium*, *2*, 1013-1017.

Crozzoli, R., Lamberti, F., Greco, N., & Rivas, D. (1998). Nematodes fitoparasiticos associados con los citricos en Venezuela. *Nematologica Mediterranea*, *26*, 31-58.

Duncan, L.W. (2005). Nematode parasites of citrus. In M. Luc, R. A. Sikora & J. Bridge (Eds.), *Plant Parasitic Nematodes in Subtropical and Tropical Agriculture*. (pp. 437-466). Wallingford, UK, CABI Publishing.

Duncan, L.W., & Cohn, E. (1990). Nematode parasites of citrus. In M. Luc, R.A. Sikora & J. Bridge (Eds.), *Plant parasitic nematodes in subtropical and tropical agriculture*. (pp. 321-346). Wallingford, UK, CABI Publishing.

Espargham, N., Mohammadi, H., & Gramaje, D. (2020). A survey of trunk disease pathogens within Citrus trees in Iran. *Plants*, *9* (6), 754.

FAO. (2016). Citrus Fruit Fresh and Processed Statistical Bulletin. Available online: http://fao.org/3/a-i8092e.pdf (accessed in May 2020).

Goodey, J.B. (1963). *Soil and freshwater nematodes*. New York: John Wiley and Sons Inc.

Inserra, R.N., Esser, R.P., & O'bannon, J.H. (1988a). Identification of *Tylenchulus* species from Florida. Nematology Circular 153, Florida Department of Agriculture and Consumer Services, Division of Plant Industry.

Inserra, R.N., Vovlas, N., & Di Vito, M. (1994). Identification of second-stage juveniles of *Tylenchulus* spp. on the basis of posterior morphology. *Nematropica*, *24*, 25-33.

Inserra, R.N., Vovlas, N., O'bannon, J.H., & Esser, R.P. (1988b). *Tylenchulus graminis* n. sp. and *T. palustris* n. sp. (Tylenchulidae) from native flora of Florida, with notes on *T. semipenetrans* and *T. furcus*. *Journal of Nematology*, *20*, 266-287.

Inserra, R.N., Stanley, J.D., Troccoli, A., Chitambar, J. & Subbotin, S.A. (2013). *Hemicaloosia vagisclera* sp. n. (Nematoda: Caloosiidae) from *Bermuda grass* in Florida and its phylogenetic relationships with other criconematids. *Nematology, 15*, 23-29.

Izadpanah, K., & Safarian, A. (1968). Possible role of citrus nematode, *Tylenchulus semipenetrans*, in the citrus decline in southern Iran. *Proceedings of the first national congress of plant medicine of Iran*, (pp. 40-41).

Jenkins, W.R. (1964). A Rapid Centrifugal-Flotation Technique for Separating Nematodes from Soil. *Plant Disease Reporter*, 48, 692.

Katcho, Z.A., & Allow, J.M. (1969). Citrus root nematode in Iran. *Plant Disease Reporter*, 53, 84.

Katoh, K., Rozewicki, J., & Yamada, K.D. (2019). MAFFT online service: multiple sequence alignment, interactive sequence choice and visualization. *Briefings in Bioinformatics*, *20* (4), 1160-1166.

Lin, B., Wang, H., Zhuo, K., & Liao, J. (2016). Loop-mediated isothermal amplification for the detection of *Tylenchulus semipenetrans* in soil. *Plant Disease*, *100*, 877- 883.

Liu, G.K., Chen, J., Xiao, S., Zhang, S.S., & Pan, D.M. (2011). Development of species-specific PCR primers and sensitive detection of the *Tylenchulus semipenetrans* in China. *Agricultural Sciences in China*, *10*, 252-258.

Minassian, V., & Moadab, H. (1970). The occurrence and distribution of the citrus root nematode, *Tylenchulus semipenetrans* Cobb, in Khuzestan, Iran. *Iranian Journal of Plant Pathology*, *6* (2), 25-28.

Park, B.Y., Park, S.N., Lee, J, K., & Bae, C.H. (2009). Morphometric and genetic variability among *Tylenchulus semipenetrans* populations from citrus growing area in Korea. *Plant Pathology Journal*, *25*, 236-240.

Rashidifard, M., Shokoohi, E., Hoseinipour, A., & Jamali, S. (2015). Distribution, morphology, seasonal dynamics, and molecular characterization of *Tylenchulus semipenetrans* from citrus orchards in southern Iran. *Biologia*, *70* (6), 771-781.

Sharafeh, M. (1972). A preliminary study on population dynamics of the citrus nematode, *Tylenchulus semipenetrans* Cobb, in Khafr an important citrus growing region of Fars. *Entomologie et Phytopathologie Appliquées*, *33*, 9-14.

Siddiqi, M.R. (1974). *Tylenchulus semipenetrans*, CIH descriptions of plant-parasitic nematodes. CAB International Institute of Parasitology, London. Set. 3, No. 34.

Silvestro, D., & Michalak, I. (2012). raxmlGUI: a graphical front-end for RAxML. *Organisms Diversity and Evolution*, *12*, 335-337.

Stamatakis, A. (2006). RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. *Bioinformatics*, *22*, 2688-2690.

Subbotin, S.A., Vovlas, N., Crozzoli, R., Sturhan, D., Lamberti, F., Moens, M., & Baldwin, J.G. (2005). Phylogeny of Criconematina Siddiqi, 1980 (Nematoda: Tylenchida) based on morphology and D2-D3 expansion segments of the 28S-rRNA gene sequences with application of a secondary structure model. *Nematology*, *7* (6), 927-944.

Subbotin, S.A., Sturhan, D., Chizhov, V.N., Vovlas, N., & Baldwin, J.G. (2006). Phylogenetic analysis of Tylenchida Thorne, 1949 as inferred from D2 and D3 expansion fragments of the 28S rRNA gene sequences. *Nematology*, *8* (3), 455-474.

Tanha Maafi, Z., & Kheiri, A. (1991). Citrus nematode (*Tylenchulus semipenetrans*) in Hormozgan province. *Iranian Journal of Plant Pathology*, *27*, 31-42.

Tanha Maafi, Z., & Damadzadeh, M. (2008). Incidence and control of the citrus nematode *Tylenchulus semipenetrans* Cobb, in the north of Iran. *Nematology*, *10* (1), 113-122.

Tanha Maafi, Z., Amani, M., Stanley, J.D., Inserra, R.N., Van den Berg, E., & Subbotin, S.A. (2012). Description of *Tylenchulus musicola* sp. n. (Nematoda: Tylenchulidae) from banana in Iran with molecular phylogeny and characterization of species of *Tylenchulus* Cobb, 1913. *Nematology*, *14* (3), 353-369.

Tennant, P.F., Robinson, D., Fisher, L., Bennett, S.M., Hutton, D., Coates-Beckford, P., & McLaughlin, W. (2009). Disease and pests of Citrus (*Citrus* spp.). *Tree and Forestry Science and Biotechnology*, *3*, 81-107.

Van den Berg, E., Subbotin, S.A., & Tiedt, L.R. (2010). Morphological and molecular characterisation of *Hemicycliophora lutosa* Loof & Heyns, 1969 and H. typica de Man, 1921 from South Africa (Nematoda: Hemicycliophoridae). *Nematology, 12* (2), 303-308.

Van den Berg E., Tiedt, L.R., & Subbotin, S.A. (2011). Morphological and molecular characterization of *Caloosia longicaudata* (Loos, 1948) Siddiqi & Goodey, 1963 (Nematoda: Caloosiidae) from Maui, the Hawaiian Islands with notes on some species of the genus. *Nematology, 13* (4), 381-393.

Van den Berg E., Tiedt L.R., & Subbotin, S.A. (2012). Morphological and molecular characterisation of *Criconemoides brevistylus* Singh & Khera, 1976 and *C. obtusicaudatus* Heyns, 1962 from South Africa (Nematoda: Criconematidae) with first description of a male of *C. obtusicaudatus* and proposal of new synonyms. *Nematology, 14* (8), 961-976.

Van den Berg, E., Tiedt, L.R., & Subbotin, S A. (2014). Morphological and molecular characterisation of several *Paratylenchus* Micoletzky, 1922 (Tylenchida: Paratylenchidae) species from South Africa and USA, together with some taxonomic notes. *Nematology, 16* (3), 323-358.

Vovlas, N., Landa, B.B., Liébanas, G., Handoo, Z.A., Subbotin, S.A., & Castillo, P. (2006). Characterization of the cystoid nematode *Meloidoderita kirjanovae* (Nemata: Sphaeronematidae) from Southern Italy. *Nematolology, 38* (3), 376-382.

Whitehead, A.G. (1998). *Plant nematode control*. CAB Interna-tional, Wallingford, UK.

Yang, G., Smiley, R.W., Okubara, P.A., Skantar, A.M., & Reardon, C.L. (2013). Developing a real-time PCR assay for detection and quantification of *Pratylenchus neglectus* in soil. *Plant Disease*, *97*, 757-764.

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MORFOLOGIJA I MOLEKULARNA KARAKTERIZACIJA *TYLENCHULUS SEMIPENETRANS* IZ VOĆNJAKA AGRUMA U SEVERNOM IRANU

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R e z i m e

Tylenchulus semipenetrans Cobb, 1913 spada među neke od ekonomski najvažnijih biljnih parazitskih nematoda na svetu. Nematoda je identifikovana kao uzročnik sporog propadanja citrusa. Većina studija procenjuje da se gubici prinosa, usled prisustva *T. semipenetrans* kreću od 10% do 30%, u zavisnosti od nivoa zaraze, agresivnosti populacije nematoda, karakteristika zemljišta, osetljivosti podloge, prisustva drugih patogena i proizvodne prakse upravljanja voćnjakom. Da bi se identifikovala citrusna nematoda u severnom Iranu, sa zaraženih stabala su prikupljeni uzorci zemljišta i korena. Juvenili drugog stadijuma izolovani su iz zemljišta metodom sita. Jaja i ženke su izdvojene iz korena centrifugalno-flotacionom tehnikom. Morfološkim i molekularnim analizama potvrđeno je prisustvo populacije *T. semipenetrans*. Filogenetsko stablo populacija *T. semipenetrans* je rekonstruisano na osnovu sekvenci gena 28S rRNK korišćenjem RAxML. Morfološki, postoji mala razlika između proučavane populacije i već opisanih populacija *T. semipenetrans* iz voćnjaka nara i banana iz južnog Irana. Filogenetska analiza je pokazala blisku vezu populacije *T. semipenetrans* iz severnog Irana sa drugim populacijama ove vrste. Na osnovu molekularne analize, *Tylenchulus* je identifikovan kao monofiletička grupa. Pružene su informacije o filogenetskom položaju i sličnosti populacija *T. semipenetrans*.

Ključne reči: *Citrus sinensis*, 28S rRNA, Iran, filogenija, sporo propadanje citrusa.

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