Research Article

Study of Plant Parasitic Nematodes and Description of New Record (*Rotylenchus alius*) Associated with Barley (*Hordium vulgare* L.) in Khorasan Razavi Province, Northeast Iran

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Abstract | Surveys were conducted in barley (*Hordium vulgare* L.) fields of Khorasan Razavi Province, Northeast Iran, during 2015-2017. A total of 120 soil and root samples were collected to examine the prevalence of Plant parasitic nematodes. In morphological and morphometrical identification 18 species were recorded belonging to nine genera. Among these species, *Rotylenchus alius* was found as a new record from Iran and *Basiria gracilis*, *Boleodorus thylactus*, *Ditylenchus apus* and *Meloidogyne arenaria* were reported as new host record from barley fields of Iran. *Meloidogyne arenaria*, was selected for detailed investigation at molecular level. Molecular traits on *Meloidogyne arenaria* species done by partial 18s ribosomal DNA primer and sequence results verified the morphometric studies and showed 99 percent resemblance to AB905316 sequence from Japan. In addition, scanning electron microscope (SEM) assay on infected roots by *Meloidogyne arenaria* showed that this species has been able to create gall in barley root and disrupt on cellular metabolism.

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Introduction

Barley (*Hordium vulgare* L.) is one of the most important cereal crops in the world (Czembor and Czembor, 2007). Over 160 million tons of barley produced annually in the world from about 70 million hectares and it ranks fourth among main cereals in the world after wheat, corn, and rice (Akar *et al*., 1999). It is one of the most important agricultural crops in Iran. Barley harvest level in the country has been estimated about 3.2 million tons in the agricultural year, 2011-2012 from Khorasan Razavi Province, Iran (https://www.amar.org.ir/english/Iran-Statistical-Yearbook). One of the growth limiting factors in cereals are plant parasitic nematodes. Globally, many parasitic nematodes have so far been reported from barley *viz*., cereal cyst nematodes (*Heterodera* spp.), root-knot nematodes (*Meloidogyne naasi*), seed gall nematode (*Anguina tritici*), cereal dwarf nematode (*Geocenamus brevidens*), root lesion nematodes (*Pratylenchus* spp.) (Luc *et al*., 2005). This species was also found as a dominant species from barley and wheat fields of Iran (Haji-Hassani *et al*., 2008). In addition, *Criconemella antipolitana* was reported as a dominant species in Esfahan province of Iran from barley fields (Jamali *et al*., 2003). *Heterodera avenae* (Barooti and Alavi, 2003; Tanha *et al*., 2007; Ahmadi and Maafi, 2009), *Anguina scopoli* (Pakniyat and Sahandpoor, 2005), *Heterodera*

latipons (Davis and Venette, 2004), *Anguina agrostis* were reported from Fars Province in barley fields (Tanha, 2005). Several investigations showed that the most prevalent species was *Geocenamus brevidens* via, Harat, Shahrebabak, Zobeideh, Modi-abad cities associated with barley fields in Iran (Barooti and Alavi, 2003; Pakniyat and Sahandpoor, 2005; Haji-Hassani *et al*., 2008; Ahmadi *et al*., 2014).

Although barley is one of the most important cereals in our country, there is no comprehensive study on the variation of plant parasitic nematodes in barley fields especially in Khorasan Razavi Province. The aim of present investigation was to identify plant parasitic nematodes in barley fields of Khorasan Razavi Province of Iran as the major producer in barley (https://www.amar.org.ir/english/Iran-Statistical-Yearbook).

Materials and Methods

Soil and root samples (120) were collected from the rhizospheres of barley along important barley fields in Khorasan Razavi Province, northeast Iran during 2015-2017and after transfer to the laboratory, kept in the refrigerator at 4 °C, until processed. Nematodes were extracted from soil samples by using the Jenkins (1964) method, killed and fixed according to De Grisse (1969). Genera and species were identified based on morphological and morphometerical characters. Perineal pattern of *Meloidogyne arenaria* (Neal, 1889) Chitwood, 1949, the adult females were mounted according to the method of Hartman and Sasser (1985), and the second instar larvae were identified using Jepson (1987) method. Measurements were taken with an ocular micrometer of "Olympus BH2" Model microscope. Drawings were made by drawing tube attached to the microscope and photographed from some of the best-preserved specimens by camera Olympus BX51. To validate the identification of *Meloidogyne* species, molecular analysis was performed based on the Ghaderi *et al*. (2014) method. A single live nematode from pure culture was picked out, then transferred to the small drop of AE buffer (10 mMTris-Cl, 0.5 mM EDTA; pH 9.0) on a clean slide and crushed using a clean slide cover. The suspension was collected by adding 20 µl AE buffer. DNA samples were stored at -20°C until using as PCR templates. The optimal thermo-cycling conditions were adjusted to be: An initial denaturation at 95 °C for 4 min; 35 cycles of amplification (94 °C for 30s; 52 °C for 40s; 72 °C for 80s); followed by a final extension at 72 °C for 10 min Table 1. PCR products were purified and then sequenced by Bioneer Company (South Korea). The phylogenetic tree was drawn by Mega 6 software.

Results and Discussion

During the present research, 18 species belong to nine genera were identified on the basis of morphological and molecular identification (Table 2). Among these species, *Rotylenchus alius* was reported for the first time from Iran. The nematode fauna identified during the study is listed in Table 2.

Rotylenchus alius Van den Berg, 1986 (Figure 1; Table 3) During the present study, these species were encountered from barley fields of Bajestan county (Southern of Khorasan Razavi Province), as a new record from Iran.

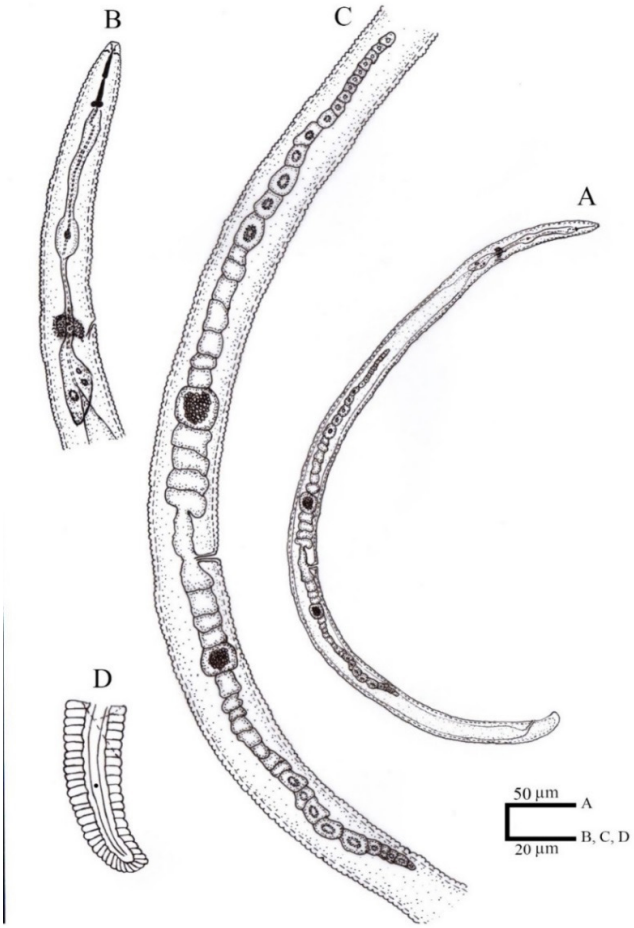

Figure 1: *Rotylenchus alius. A: Whole body; B: Head region with oesophagus; C: Ovary; D: Tail of female; phasmid region.*

Female: Body characteristically curved ventrally upon fixation. Lateral field with four incisures incompletely aerolatedexceptatoesophagealregion.Lipregionconoid with fiveannules, labial framework well developed.

Table 1: *Sequence of 18s rDNA primer for Meloidogyne arenaria.*

Primer name	5′-3′	Gene region	Reference
Forward	GCT TGT CTC AAA GAT TAA GCC	18s	Blaxter in 1998
Reverse	CAT TCT TGG CAA ATG CTT TCG	18s	Blaxter in 1998

Table 2: *Nematode species and collected areas in barley fields of Khorasan Razavi Provinces of Iran.*

S.No.	Nematode species	Collection area
1	*Aphelenchus avenae* (Bastian, 1865)	Mashhad, Neyshabour, Golbahar, Torbat heydariye, Gonabad
2	*Basiria gracilis* (Thorne, 1949; Siddiqi, 1963)	Mashhad, Feizabad, Chenaran, Quchan
3	*Basiria graminophila* (Siddiqi, 1959)	Torbat heydariye, Fariman
4	*Boleodorus thylactus* (Thorne, 1941)	Mashhad, Torbat heydariye
5	*Ditylenchus apus* (Brzeski, 1991)	Neyshabour
6	*Ditylenchus medians* (Thorne and Malek, 1968) Fortuner and Maggenti, 1987	Quchan, Neyshabour, Kalat nadery
7	*Geocenamus brevidens* (Allen, 1955) Brzeski, 1991	Most areas
8	*Geocenamus dobroticus* (Budurova *et al*., 1996)	Bajestan, Kashmar, Golbahar, Quchan, Daregaz
9	*Geocenamous graminicola* (Kirjanova, 1951)	Mashhad, Torbat jam, Tayebad
10	*Geocenamus rugosus* (Siddiqi, 1963)	Mashhad, Khaf, Torbat jam and Heydariye
11	*Geocenamus tartuensis* (Krall, 1959; Brzeski, 1991)	Khaf
12	*Geocenamus tenuidens* (Thorne and Malek, 1968)	Torbat jam, Fariman
13	*Meloidogyne arenaria* (Chitwood, 1949)*	Neyshabour
14	*Pratylenchus thornei* (Sher and Allen, 1953)	Most areas
15	*Pratylenchus neglectus* (Filipjev and Stekhoven, 1941)	Most areas
16	*Pratylenchus penetrans* (Cobb, 1917) Filipjev and Stekhoven, 1941	Most areas
17	*Rotylenchus alius* (Van Den Berg, 1986)**	Bajestan county
18	*Zygotylenchus guevarai* (Tobar-Jiménez, 1963)	Mashhad, Tayebad, Torbat jam, Bajestan, Fiezabad

**First isolation in barley fields of Iran; **First report from Iran nematode fauna.*

Stylet slender, stylet knobs flattened. Conus of stylet shorter than telenchium (47.05%). Median bulb ovate to long ovate, excretory pore opposite posterior part of oesophagus. Terminal bulb dorsally overlaps. Hemizonid 1.5 annules long and two annules anterior to excretory pore. Spermatheca round and filled with sperms. Phasmids situated three annules posterior to annus. Tail 117.5-25µm long having 11-13 annules, ventrally curved with a ventral point.

Male: Not found.

General description and morphometric measurements of this species (Table 3) closely fit to the original description of *Rotylenchus alius* by Van den Berg (1986). However, the (a) parameter was bigger (27.33-29 vs. 25.4-27.4).

Meloidogyne arenaria (Neal, 1889) Chitwood, 1949

Morphometric analyses: The magnitude of morphometric characters is influenced by several factors and can show significant differences not only between species but also among populations within a species (Doucet and Cagnolo, 1998; Carneiro *et al*., 2008; Skantar *et al*., 2008). Morphometrics of female, male and juveniles of under studied population were compared with original description of Chitwood (1949) and two other description from Iran (Table 4).

Female: Species of *Meloidogyne* can be identified based on female adult morphology, including head structures, perineal patterns, and stylet (Eisenback *et al*., 1980). The perineal pattern is a valuable morphological feature for identification of the species in genus *Meloidogyne* (Chitwood, 1949; Eisenback *et al*., 1980; Hirschmann, 1985). Three difference type of perineal patterns was seen in population under study. Adults with semi protuberance at the end of the body, pyriform, style 575-710 µm, lateral field existed but not clear, conus dorsally curved with range of 12-19 µm.

Table 3: *Morphometric characters of the Iranian population of Rotylenchus alius and their comparison with type population (Van den Berg, 1986). All measurements are in µm and in the form: mean ± S.D. (range).*

Characters	Khorasan Razavi region (Bajestan county)	Van Den Berg, 1986
	Female	Female
Number	8	3
L	881±84.95 (770-990)	600-900
A	28.22±0.68 (27.33-29)	25.4-27.4
B	5.65±0.32 (5.22-5.92)	5-6.8
b'	5.42±0.56 (4.66-5.91)	5.4-7.2
C	40.55±4.5 (32.8-44.61)	33.4-42.1
c'	1.15±0.12 (1-1.25)	1.1-1.4
V	59.80±1.66 (57.47-62.19)	59-60
v'	61.63±2.31 (58.82-65.4)	-
Stylet	28.83± 0.75 (28-30)	26.5-31.3
Pharynx length	142.58±5.96 (133-150)	-
S.E-pore	128.67±1.97 (125-130)	103-121
DGO	16±1.9 (13-18)	11.1-21.6
Mb	52.59±0.78 (51.47-53.25)	-
Head-vulva	526.67±45.02 (470-590)	-
Vulva-anus	333.33±41.64 (282.5-375)	-
Width at vulva	31.25±3.06 (27.5-35)	-
Tail length	22±3.39 (17.5-25)	16.9-25.7
Width at anus	19.08±1.24 (17.5-20)	-
G1	299.42±18.56 (285-325)	-
G2	235±27.88 (187.5-265)	

Second stage larvae: Body length range 410-512 µm with13-16.5 µm body width, the average stylet length 13.33 µm (12-15), more or less similar length given by and near to studied population with Garcia and Sanchez-Puerta (2012) but it is greater than Chitwood (10 µm). Lateral field with four ridges. The rectum undilated as reported earlier (Esser *et al.*, 1976; Garcia and Sanchez-Puerta, 2012). Tail tip pointed to rounded 50-64.5 µm in length. The alpha (body length/the maximum of body width) 32.42 µm and gamma (Body length/tail length) 8.2 µm.

Male: Body length 1340-1352 µm, body ventrally curved, the mean spicules length 31.75±1.06. Pharynx length 167.5- 170 µm. Overall, all measurements of this population were consistent with original description by Chitwood, 1949.

Populations of root-knot nematodes *Meloidogyne arenaria* species have been well documented, by Mahdikhani *et al.* (2003) and Sajjadi and Assemi (2016) from Iran, however, there is no information about the presence of root-knot nematodes in the barley fields of Iran. Morphometric results showed that all measurements were consistent with the original description given by Chitwood (1949) with few variations and differences, can be seen in the present population and earlier studies of Mahdikhani *et al.* (2003) and Sajjadi and Assemi (2016). These differences can be attributed to the intra species variations due to geography, host type, root structure and vegetation season of host.

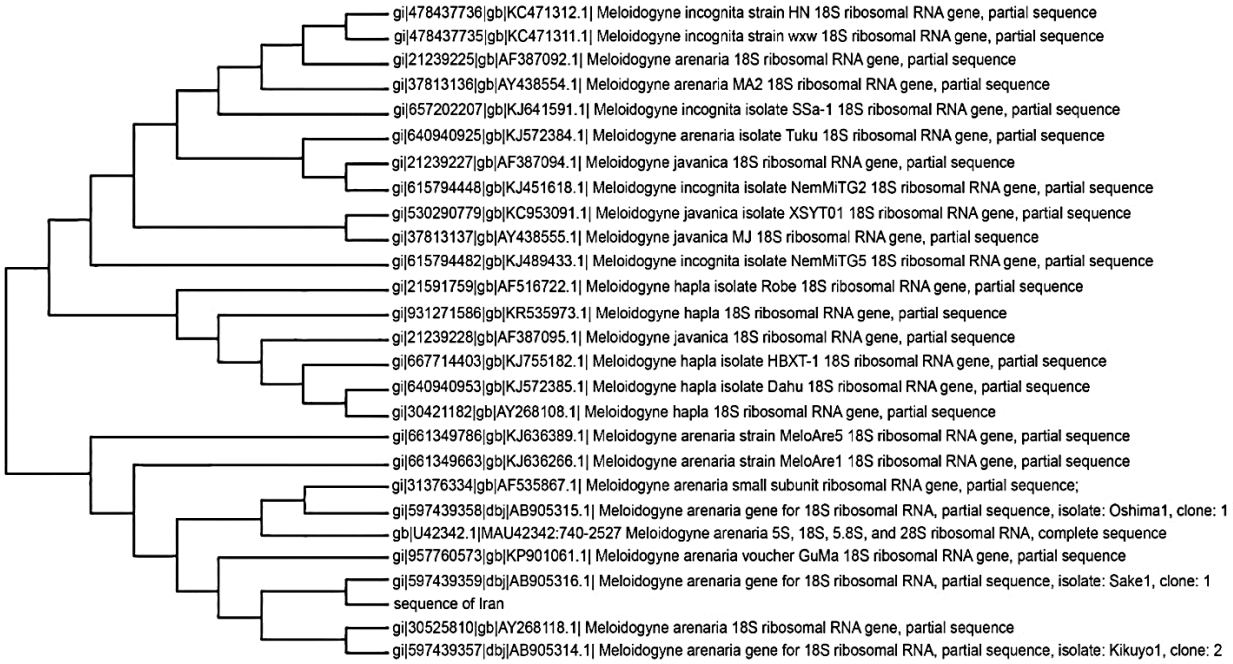

Figure 2: *Phylogenetic tree (Bootstrap type) for Iranian population of Meloidogyne arenaria.*

Table 4: *Morphometrics characters of Meloidogyne arenaria from Iranian population and their comparison with the type population (Chitwood, 1949) and other population from Iran. All measurements are in µm and in the form: mean ± S.D. (range).*

Characters	Population under study*			Chitwood, 1949			Mahdikhani et al. (2003)			Sajjadi and Assemi, 2016		
	Female	Male	Juveniles	Female	Male	Juveniles	Female	Male	Juveniles	Female	Male	Juveniles
Number	10	5	20	-	-	-	20	10	20	20	30	30
Body length	655±43.9 (575-710)	1346±8.5 (1340-1352)	464.7±35.2 (410-512)	510-1000	1270-2000	450-490	892 (770-1020)	1528 (1080-1800)	435 (400-480)	741±52 (601-986)	1720±223 (978-2279)	504±34 (391-606)
Body width	403.44±78.45 (315-510)	30.25±2.47 (28.5-32)	14.39±1.08 (13-16.5)	400-600	-	-	-	-	-	448±41.6 (334-626)	38.8±2.9 (35-40)	17±1.8 (15-20)
a	-	57.9±2.3 (56.3-59.5)	32.4±3.1 (26.4-37.9)	-	44-65	26-32	-	41 (31-43)	36 (33-39)	-	45.8±9/7 (35.2-56.1)	29.6±5.1 (26.7-30.8)
b	-	-	8.2±0.8 (6.8-9.7)	-	11-16	7.2-7.8-	-	-	6.8 (6.5-7.8)	-	-	5.6±0.7 (4.6-5.9)
Testis	-	578±2.8 (576-580)	-	-	-	-	-	-	-	-	-	-
Stylet knob width	4.6±0.7 (3.5-5.5)	-	-	4-5	4-5	-	-	-	-	3.9±0.52 (2.1-4.5)	-	-
Stylet length	15.7±1.9 (12-19)	22.7±0.3 (22.5-23)	13.3±1.1 (12-15)	14-16	20-24	10	16 (15-17)	24 (22-28)	11.2 (10-12)	15.2±3.8 (12-17)	23±3.4 (22-28)	11.2±2.4 (10-12)
Pharynx length	-	168.7±1.8 (167.5-170)	-	-	-	-	-	-	-	-	-	-
S.E-pore	-	-	85.7±6.6 (75-95.5)	-	-	-	-	-	-	-	-	-
DGO	5.2±0.6 (4.5-6)	-	3.2±0.4 (2.5-4)	4-6	4-7	3	-	-	-	4.7±0.85 (3.1-6.7)	-	3.7±0.44 (3-5)
Mb	76.2±4 (68-82)	72±2.8 (70-74)	-	-	-	-	-	-	-	86.2±4.7 (80-92)	-	-
Vulva width	19.7±2.7 (15-23)	-	-	-	-	-	22.5 (22-26)	-	-	29.2±2.4 (24-37)	-	-
Vulva-anus	13.4±0.8 (12-15)	-	-	-	-	-	14.5 (13-15)	-	-	20.8±1.7 (18-22)	-	-
Tail length	-	-	56.94±4.94 (50-64.5)	-	-	-	-	-	-	-	-	46.2±2.7 (40-49)
Spicule length	-	31.75±1.06 (31-32.5)	-	-	31-34	-	-	25 (21-28)	-	-	32±5.2 (27-39)	-

**Khorasan Razavi province, Neyshabour region. a: Body length/body width; b: Body length/esophagus length; S.E-pore: Excretory pore to head; DGO: Dorsal gland orifice; Mb: Position of median bulb center as a percentage of the pharynx length.*

Molecular analyses result

Molecular examination of 18S rDNA ribosomal domain in *Meloidogyne arenaria* verified the morphometric studies. Sequence of 18S ribosomal gene (Sequence of Iran) showed 99 percent resemblance to AB905316 from Japan. For phylogenetic tree, the evolutionary history inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The bootstrap consensus tree inferred from 500 replicates (Felsenstein, 1985) is taken to represent the evolutionary history of the taxa analyzed (Saitou and Nei, 1987). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates collapsed. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al*., 2004) and are in the units of the number of base substitutions per site. The analysis involved 30 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were 579 positions in the final dataset (Figure 2). Evolutionary analyses were conducted in MEGA6 (Tamura *et al*., 2013).

Novelty Statement

In this study, Rotylenchus alius was found as a new record from Iran and Basiria gracilis, Boleodorus thylactus, Ditylenchus apus and Meloidogyne arenaria were reported as new host record from barley fields of Iran.

Author's Contribution

M. Shahi-Bajestani and E. Mahdikhani-Moghadam: Study concept and design.
M. Shahi-Bajestani: Drawing, analysis and interpretation of data.
M. Shahi-Bajestani: Drafting of the manuscript.
E. Mahdikhani-Moghadam, R. Aghnoum and H. Rohani: Critical revision of the manuscript for important intellectual content.

Conflict of interest

The authors have declared no conflict of interest.

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