

Morphological and Molecular Characterization of *Rotylenchulus borealis* Loof and Oostenbrink, 1962 from Turkey

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Abstract

Reniform nematodes (*Rotylenchulus spp.*) have been reported to be associated with a large number of important products all over the world, ranging from important cereals, vegetables and ornamental plants. In this study, morphologic and molecular characters were used to identify *Rotylenchulus* population obtained from a soybean field in Adana province of Turkey. Nematodes were extracted from the soil using a modified baermann funnel method. The morphological characters and morphometrics of male and immature females were examined and compared with previous studies. For molecular characterisation, DNA was extracted from immature females and the D2-D3 expansion region of the 28S rRNA gene was amplified using primer pair D2A (5'ACA AGTACCGTGAGGGAAAGTTG 3') and D3B (5' TCGGAAGGAACCAGCTACTA 3'). PCR product (780 bp) was sequenced and then compared with sequences of *Rotylenchulus* species available in the GenBank database. The result obtained from morphologic and molecular studies showed that the reniform nematode population was *Rotylenchulus borealis*.

Key words: *Rotylenchulus borealis*, soybean, Reniform nematodes, D2A, D3B.

Rotylenchulus borealis Loof and Oostenbrink, 1962'in morfolojik ve moleküler Karakterizasyonu

Öz

Reniform nematodların (*Rotylenchulus spp.*), tüm dünyada hububattan sebze ve süs bitkilerine kadar birçok önemli ürünlerle ilişkili olduğu rapor edilmiştir. Bu çalışmada, Adana ilinde soya fasulyesi üretimi yapılan bir alandan alınan toprak örneklerinden elde edilen *Rotylenchulus* popülasyonuna ait bir tür, morfolojik ve moleküler yöntemler kullanılarak teşhis edilmiştir. Topraktaki nematodlar, modifiye Baermann huni yöntemi kullanılarak ekstrakte edilmiştir. Tür teşhisi için ilk olarak morfolojik ve morfometrik karakterler ölçülmüş ve önceki çalışmalarla karşılaştırılmıştır. Moleküler karakter özellikleri için, olgunlaşmamış dişilerden DNA izole edilerek ve 28S rRNA geninin D2-D3 genişleme bölgesine ait primer çifti, D2A (5' ACAAGTACCGTGAGGGAAAGTTG 3') ve D3B (5' TCGGAAGGAACCAGCTACTA 3') kullanılarak amplifiye edilmiştir. PCR ürünü (780 bp) dizi analizinin ardından GenBank veri tabanında bulunan *Rotylenchulus* türlerinin dizileriyle karşılaştırıldı. Çalışmada morfolojik, morfometrik ve moleküler olarak incelenen reniform nematod popülasyonu *R. borealis* olarak tespit edilmiştir.

Anahtar kelimeler: *Rotylenchulus borealis*, Soya fasulyesi, Reniform nematodes, D2A, D3B.

Introduction

Reniform nematodes (*Rotylenchulus spp.*) are distributed in tropical, subtropical and warm regions (Liskova, 2002; Wang, 2019). Their

economically great potential as plant pathogens make it especially important (Dasgupta et al., 1968). The genus *Rotylenchulus* comprises 11 valid species: *Rotylenchulus borealis*, *Rotylenchulus clavicaudatus*, *Rotylenchulus eximius*,

Rotylenchulus leptus, *Rotylenchulus macrodoratus*, *Rotylenchulus macrosoma*, *Rotylenchulus macrosomoides*, *Rotylenchulus parvus*, *Rotylenchulus reniformis*, *Rotylenchulus sacchari*, and *Rotylenchulus vitis* (Ortiz et al., 2019). Some of these are distributed worldwide, whereas others have shown a limited distribution (Van Den Berg et al., 2016). *Rotylenchulus* species feed as a semi-endoparasite on the roots of herbaceous and woody plants (Fortuner, 1987). Females are penetrate the root cortex, establish a permanent-feeding site in the root and become immobile. The head region remains in the root whereas the posterior portion protrudes from the root surface and swells during maturation (Wang, 2019). Soybean has an important plant in Turkey both as a nutritional and industrial raw material. More than 100 species of nematodes found in soybean production areas have been reported (Gava et al., 2020). *Rotylenchulus reniformis* is one of the major pests on both *soybean* in the southern United States (Kularathna et al., 2019). In Turkey, *Rotylenchulus macrosomus* was expressed as a plant parasitic nematodes found in soybean fields (Elekcioglu et al., 1994).

Correct identification of plant parasitic nematodes is important at control of nematode in agriculture (Devran and Söğüt, 2009). High intra-species variability makes identification of this nematode group based on morphology a difficult status (Palomares-Rius et al., 2018). Therefore, it is necessary to use molecular identification (Palomares-Rius et al., 2021). Molecular techniques have been successfully used for the molecular diagnostics of species of *Rotylenchulus* (Palomares-Rius et al., 2018). In the present study, morphological and molecular characters were used to identify *Rotylenchulus* population obtained from soybean field in Adana province of Turkey.

Material ve Method

Sampling and Nematode Extraction

A plant parasitic nematode survey was conducted in December 2020 and March 2021 in a soybean field in Adana province in Turkey. Soil samples were collected from the rhizosphere of the plants using a hand shovel. Soil samples were taken into plastic bag and transferred to the laboratory for analysis. Samples were stored in refrigerator at 4 °C until nematode extraction process. Nematodes were extracted from 100 cm³ of soil samples with a modified Beermann Funnel method (Hooper, 1986). Extracted nematodes were examined at 40X magnifications under a inverted microscope. When examining the samples, a very dense *Rotylenchulus* spp. was

found. To identification of species, morphological and molecular characters of nematodes were used.

Morphological characterization

Twenty immature female and male were used for morphometric characters. Nematodes were transferred to a drop of pure water on a clean glass slide on the hot plate for 4-6 seconds. Then, samples were immediately examined for morphological characters and morphometric measurements using a camera (Axiocam 105) mounted on the ZEISS primo-vert Light microscope at size of 400X. The following characters were observed and measured from females; body length (L), a (body length/maximum body diameter), b (body length/ distance from anterior to base of esophageal glands), c (body length/ tail length), c' (tail length/ tail diameter at anus), V(% distance of vulva from anterior), DGO, lip height, lip region diameter, stylet length, stylet knob height, stylet knob width, excretory pore from anterior end, distance from anterior to base of esophageal glands, maximum body diameter, tail length, anal body diameter, vulva to posterior end, vulva to anterior end (Fig. 1). In addition to these measurements, spicula length and gubernaculum length were also measured for male. Microsoft excel programme was used to calculate allometrics variables of the females and males. (Fig 2).

The scanning electron microscope (SEM) were used to study nematode morphology. First the nematodes were transferred into vials containing 1 ml of 0.1 M phosphate buffer pH 7.2 and kept for 1 hour in refrigerator at 6°C. At the end of the period, 0.3 ml-0.3 ml-0.4 ml 3 times every 30 minutes, 6% glutaraldehyde was added and kept at 6°C for 24 hours. Later, the nematodes were transferred to phosphate buffer. For cleaning the bodies of nematodes, the petri dish is slightly shaken and buffer is dripped on them. After the cleaning process, 2% Osmium tetroxide was added to cover the nematodes for 12 h at 25°C. After the waiting process, Osmium tetroxide was taken and replaced with a phosphate buffer 5 times every 10 minutes. Then, the nematodes were washed in ethanol series between 10% and 100% and waited 15 minutes at each concentration. It was made ready for imaging by washing 3 times at 100% concentration. Anhydrous nematodes were placed on double-sided carbon conductive tape and coated with gold (20 nm) Automatic Spray Coating. All specimens were examined with a Hitachi SU1510 scanning electron microscope at the Central Research Laboratory, Ordu University.

Molecular characterization

The 28S rDNA D2-D3 region of the large subunit was selected for molecular characterization. The genomic DNA was extracted from females according to the procedure of Pagan et al. (2014). Five specimens were collected into 10 µl of extraction buffer (10 mM Tris HCl, pH 8.8, 1 mM EDTA, 0.1 % Triton X-100 (v/v); 20 mg/ml Proteinase K) in a 1.5 ml eppendorf tube. Sample **Table1**. Nematode species including genbank accession numbers and origin used in the phylogenetic analysis.

Nematode species	Accession Numbers	Country
<i>Rotylenchulus borealis</i>	MW173974	Netherlands
<i>R. borealis</i>	MW173982	Israel
<i>R. borealis</i>	MW173973	Netherlands
<i>R. borealis</i>	MW173977	Israel
<i>R. borealis</i>	MK558206	Belgium
<i>R. borealis</i>	MW173975	Netherlands
<i>R. borealis</i>	MW173970	Netherlands
<i>R. borealis</i>	MW173971	Netherlands
<i>R. borealis</i>	MW173983	Israel
<i>R. borealis</i>	MW173976	Netherlands
<i>R. macrosoma</i>	KT003748	Spain
<i>R. macrosoma</i>	MT084016	Spain
<i>R. macrosoma</i>	MT084017	Serbia
<i>R. macrosoma</i>	KT003751	Spain
<i>R. macrosoma</i>	MK558208	Ethiopia
<i>R. macrosoma</i>	MT775429	India
<i>R. macrosoma</i>	KY992794	Greece
<i>R. macrosoma</i>	MT084014	Greece
<i>R. macrosoma</i>	MT084013	Greece
<i>R. macrosoma</i>	KY992800	Greece
<i>R. reniformis</i>	KP054111	China
<i>R. reniformis</i>	HM131857	China
<i>R. reniformis</i>	KP054116	China
<i>R. reniformis</i>	HM131853	China
<i>R. reniformis</i>	MT160083	China
<i>R. reniformis</i>	KJ721978	China
<i>R. reniformis</i>	KP054091	China
<i>R. reniformis</i>	HM131854	China
<i>R. reniformis</i>	HM131871	China
<i>R. reniformis</i>	MT328542	China
<i>Xiphinema index</i>	HG969307	Hungary

tubes were kept at -20 °C for 1 night. Samples were ground using a glass capillary tube and incubated at 56°C for 1 h and subsequently at 95°C for 10 min. Total 25 µl PCR mixture volume was prepared containing 12,5 µl of DreamTaq Green Master Mix (2X) (Thermo Fisher Scientific), 8,5 µl of DNase/RNase-Free distilled water, 1.5 µl of DNA, and 1.25 µl of each primer. The obtained mixture was used for PCR amplification. PCR reaction was carried out with D2A (5' ACA AGT ACC GTG AGG GAA AGT TG 3') and D3B (5' TCG GAA GGA ACC AGC TAC TA 3') (De Ley et al., 1999). Thermocycling was carried out by using master cycler Veriti (Singapore). The PCR reactions were as follows; 95

°C for 3 min., followed by 40 cycles of 95 °C for 30s, 50 °C for 60s and 72 °C for 1 min; and a final extension step of 72 °C for 7 min.

Fragments of DNA were separated by electrophoresis in 1X Tris Acetate EDTA (TAE) buffer and 1.5% agarose gel stained with Ethidium Bromide, visualized using Er-Biyotek GEN-BOX imagER Fx and photographed. PCR products were sent to STAB VIDA company in Portugal (<https://www.stabvida.com>) for sequence analysis.

In this article, maximum likelihood methods were used for phylogenetic inference. The DNA sequences obtained from this study were edited using Bioedit 7,2,5 programme (Hall, 1999). Consensus sequences were compared with selected other *Rotylenchulus* species sequences obtained from GenBank (NCBI) database using BLAST engine search for sequence homology. Sequences from this study and those retrieved from the GenBank databases (Table 1) were aligned with Clustal W for multiple alignments of 32 nucleotide sequences using MEGA 7 programme (Kumar et al., 2016). The alignment was analysed to get the base substitution model for these sequences. The phylogram was generated running Maximum Likelihood Model (ML) with 1000 bootstrap replicates.

Results and Discussion

The Adana population of reniform nematode studied in this study was identified as *R. borealis* by morphologically, morphometrically and molecularly. In this study, *Rotylenchulus borealis* individuals were detected in the rhizosphere of soybean in high densities (354 nematodes /100 cm³ soil). Sipes and Schmitt (2000), reported that economic threshold for reniform nematode on pineapple is 310 nematodes /250 cm³ soil. Morphological and molecular characters of immature females and males obtained from soil samples were examined.

Morphological characterization

Morphological identification of *Rotylenchulus* spp. immature females is important characteristics such as lip region, stylet, vulva position and tail (Palomares-Rius et. al., 2018). In female, lip region conoid-rounded not set off, finely annulated (Fig. 4C, 4D). Stylet long and well developed. Stylet knobs rounded, sloping posteriorly. Excretory pore is situated opposite middle of isthmus to opposite anterior part of pharyngeal lobe. Pharyngeal glands overlapping intestine laterally and mostly ventrally. Lateral field distinct with three lines (Fig. 4B, 5D). The female reproductive system is amphidelphic with two ovary. The vulva situated near the middle of body

(Fig. 4E). Tail with bluntly rounded terminus, annulation around terminus prominent (Fig. 4A, 4F). In male, similar to immature female except for genital system and a more curved posterior part of body (Fig. 5A, 5B). Tail broadly rounded with

rounded tip (Fig. 5E, 5F, 5G). Gubernaculum and spicules well developed, ventrally arcuate (Fig. 5C).



Figure 1. Photomicrograf of *Rotylenchulus borealis* immature females. A- anterior region (lip, stylet, median bulb, excretory pore) B- whole body, C- posterior region (vulva, tail).

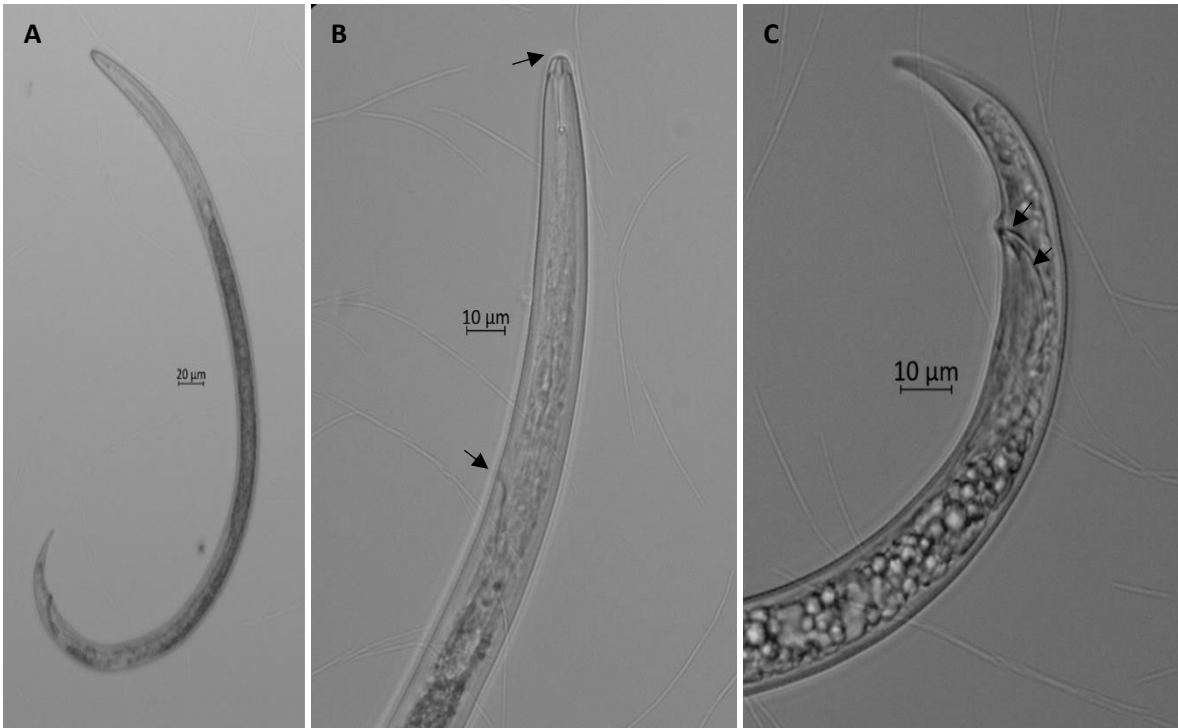


Figure 2. Photomicrograf of *Rotylenchulus borealis* males. A- whole body, B- anterior region (lip, stylet, excretory pore), C- tail (gubernaculum, spicula).

The morphometric characteristics of *R. borealis* male and female in the Turkey soybean population were studied and measured. Morphometric of the immature female and male of *R. borealis* is reported in Table 2 and 3 for this study. Body length in females measured 529.8 μm . When we compare this with the study data of the previous results of Germani (1978), Van Den Berg et al (2003), Liskova et al. (2002) and Tan and Okten (2008), it was larger than other measurements. The stylet length was measured 15.1 μm , it was shorter than the descriptions 18.3 μm reported by Tan and Ökten (2008). However, it was longer than reported of Germani (1978), Liskova et al. (2002) and Van Den Berg et al (2003). Vulva was calculated as 61.0 %. This rate was higher than previously reported by Tan and Okten (2008). However it was lower than reported of Germani (1978), Liskova et al. (2002), Van Den Berg et al. (2003). Robinson et al. (1997) by; Vulva (%) between 55 and 66 morphologically confirms that it is *R. borealis*.

The excretory pore from anterior end measured 95.4 μm , it was longer than the descriptions reported by Liskova et al. (2002) and Van Den Berg et al. (2003). The tail length measured 46.5 μm , it was longer than the measurements reported by Tan and Okten (2008), Liskova et al. (2002) and Van Den Berg et al (2003).

Body length in males was 554.2 μm . When we compare this with the study data of the previous results of Germani (1978), Van Den Berg et al (2003), Liskova et al. (2002) and Tan and Okten (2008), it is larger than other measurements. So et al. (2012) reported that temperature and food plays an important role in body size. The stylet length was 11.7 μm , it was similarly by 11.5 μm than the descriptions reported by Van Den Berg et al (2003) but Germani (1978) and Liskova et al. (2002) was longer. The spicula length was 25.4 μm , it was longer than Germani (1978), Liskova et al. (2002) and Tan and Okten (2008).

Molecular characterization

The nematode population analyzed in this study were identified as *R. borealis*. according to molecular study. The PCR amplification of the 28S

rDNA D2-D3 region of the large subunit of the nematode population yielded single fragment of about 780 bp (Fig. 3). The BLAST analysis of these sequences from Turkey population revealed 98% similarity with the GenBank sequences from *R. borealis* (Accession no: MW173974.1).

According to phylogenetic analysis, the evolutionary relationships of the *R. borealis* shown in Fig 6. The tree is reconstructed from 31 sequences, out of which 10 sequences belong to species of *R. borealis* group, 10 from *R. macrosoma* group, 10 from *R. reniformis* group and *Xiphinema index* as out group taxon. Alignment and phylogenetic analysis of D2-D3 sequences revealed several clades that were separated by varying bootstrap support (BS) values in the Maximum likelihood (ML) analysis.

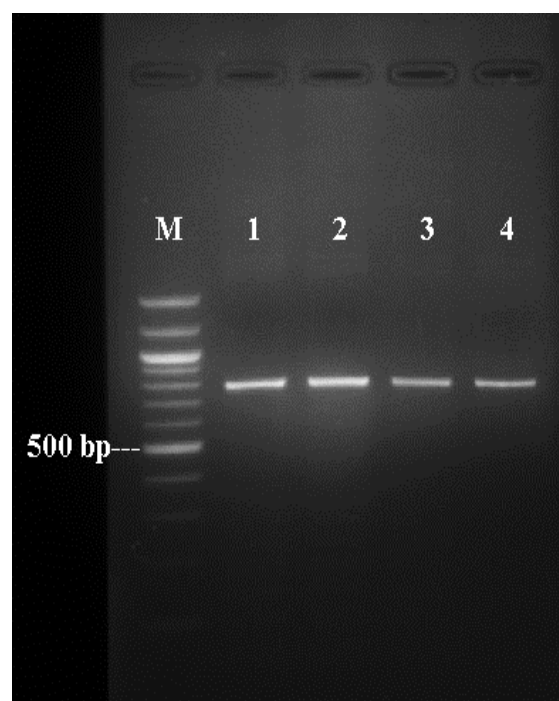


Figure 3. PCR products of *Rotylenchulus borealis* population obtained from soybean field, Turkey using D2A-D3B primer pairs. M= molecular size marker (100 bp DNA ladder), Line 1- 4= samples of *R. borealis*.

Table 2. Morphometrics of *Rotylenchulus borealis* males from a soybean field and comparison with previous study measurements. (All measurements are in μm). (Means \pm standard deviation).

Characteristics	This study n=20		Van Den Berg et al. (2003) n=6		Germani (1978) n=8		Tan and Okten (2008) n=6		Liskova et al. (2002) n=5	
	Mean \pm SD (Min.-Max.)	Mean \pm SD (Min.-Max.)	Mean \pm SD (Min.-Max.)	Mean \pm SD (Min.-Max.)	Mean \pm SD (Min.-Max.)	Mean \pm SD (Min.-Max.)	Mean \pm SD (Min.-Max.)	Mean \pm SD (Min.-Max.)	Mean \pm SD (Min.-Max.)	Mean \pm SD (Min.-Max.)
L	554.2 \pm 30.5 (486.2-599.2)	478 \pm 25.1 (448-513)	480-570	520 \pm 0.016 (470-580)	445 \pm 17.1 (416-459)					
a	32.7 \pm 1.2 (31.0-35.5)	28.4 \pm 3.1 (25-33)	24-36	34.87 \pm 1.57 (30.37-39.71)	31 \pm 1.5 (28.7-32.1)					
b	4.2 \pm 0 (3.7-4.6)	4.3 \pm 0.3 (3.8-4.5)	4.0-5.5	4.14 \pm 0.11 (3.72-4.55)	4.0					
c	13.8 \pm 1 (12.4-16.2)	14 \pm 0.7 (12.7-14.6)	11-18	10.56 \pm 0.80 (8.68-13.76)	14 \pm 0.1 (12.8-15.3)					
c'	3.6 \pm 0.4 (2.9-4.3)	2.9 \pm 0.4 (2.6-3.5)	-	4.7 \pm 0.33 (3.3-5.7)	3.0 \pm 0.3 (2.6-3.5)					
Lip region diameter	5.3 \pm 0.3 (4.8-5.8)	7 \pm 0.6 (6-7)	-	-	-					
Lip region height	4.0 \pm 0.3 (3.5-4.5)	4 \pm 0.4 (3.7-4.4)	-	-	-					
Stylet length	11.7 \pm 0.5 (10.8-12.4)	12 \pm 0.8 (11.5-13)	13-14	-	13 \pm 0.8 (11.5-13.5)					

Table 3. Morphometrics of *Rotylenchulus borealis* females from a soybean field and comparison with previous study measurements. (All measurements are in μm). (Means \pm standard deviation).

Characteristics	This study	Van Den Berg et al. (2003)	Germani (1978)	Tan and Okten (2008)	Liskova et al. (2002)
	n=20 Mean \pm SD (Min.-Max.)	n=8 Mean \pm SD (Min.-Max.)	n=18 (Min.-Max.)	n=12 Mean \pm SD (Min.-Max.)	n=8 Mean \pm SD (Min.-Max.)
L	529.8 \pm 23.0 (486.3-558.0)	426 \pm 18.8 (406-457)	360-550	510 \pm 0.007 (470-550)	428 \pm 18.4 (410-457)
a	31.3 \pm 1.6 (28.8-34.2)	25.5 \pm 2.5 (20.5-28.9)	23-31	34.7 \pm 1.3 (27.3-45.1)	29 \pm 2.3 (7-14.5)
b	3.2 \pm 0 (2.8-3.6)	3 \pm 0.2 (2.8-3.3)	2.0-3.3	3.87 \pm 0.18 (2.9-4.81)	4.2 \pm 0.3 (3.9-4.6)
c	11.5 \pm 1 (8.6-13.4)	13.2 \pm 1 (11.6-14.5)	12.5-17.3	15.05 \pm 0.8 (12.9-20.6)	13.9 \pm 1.4 (12.7-16.7)
c'	4 \pm 0 (4.3-5)	3.4 \pm 0.4 (2.7-3.7)	3-4	3.6 \pm 0.19 (2.6-4.6)	3.4 \pm 0.3 (2.9-3.8)
V (%)	61.0 \pm 1.2 (57.9-63.0)	64 \pm 1.9 (61-67)	57-67	60.5 \pm 0.8 (55-64)	63 \pm 1.3 (62-65)
DGO	2.4 \pm 0.3 (1.9-2.8)	-	-	-	-
Lip region diameter	3.2 \pm 0.3 (2.58-3.6)	7.5 \pm 0.4 (6.5-8)	-	-	-

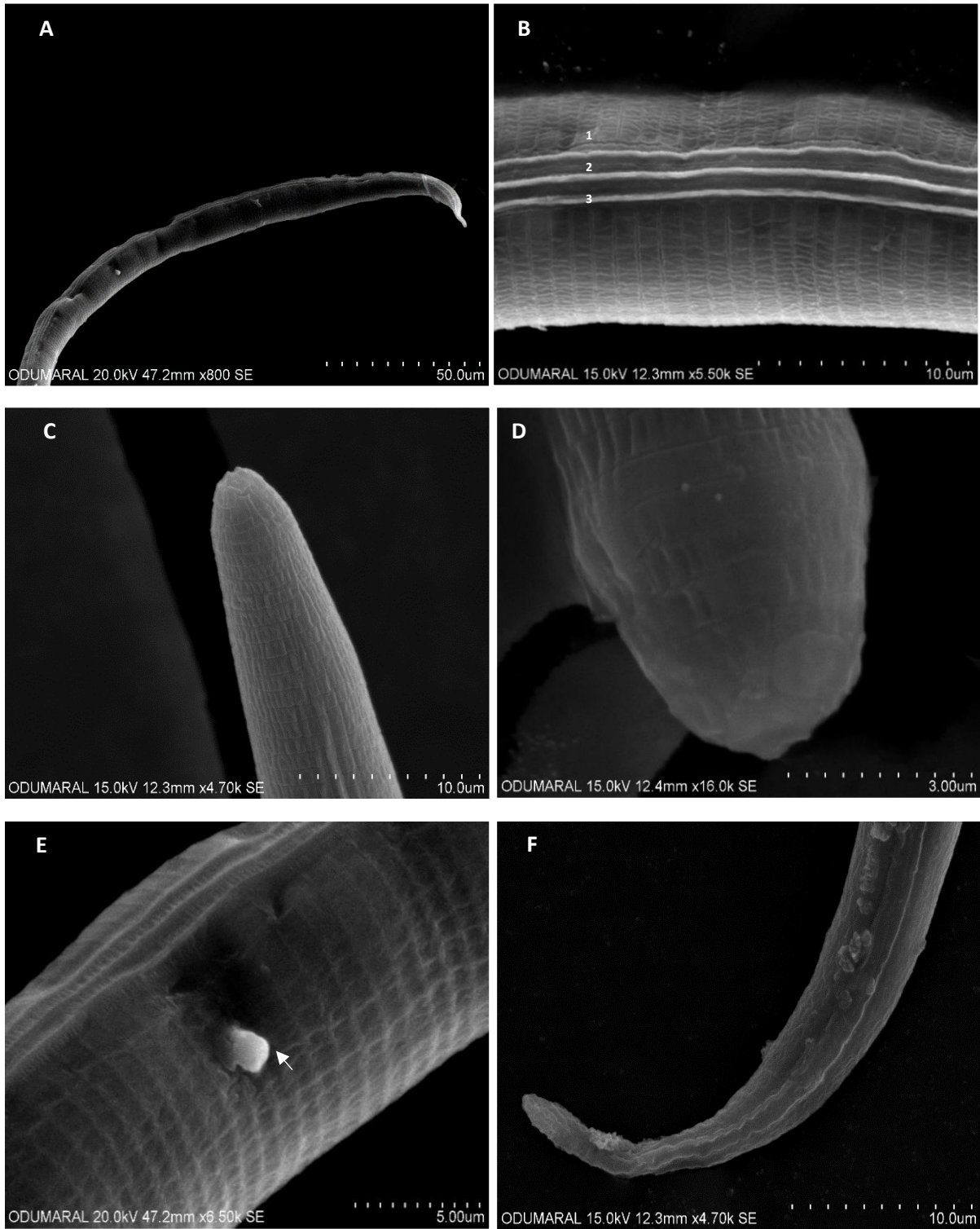


Figure 4. SEM photomicrographs of *Rotylenchulus borealis* immature female: (A) posterior region; (B) lateral field in middle body region showing lateral line; (C) anterior region; (D) head region; (E) vulva; (F) tail region.

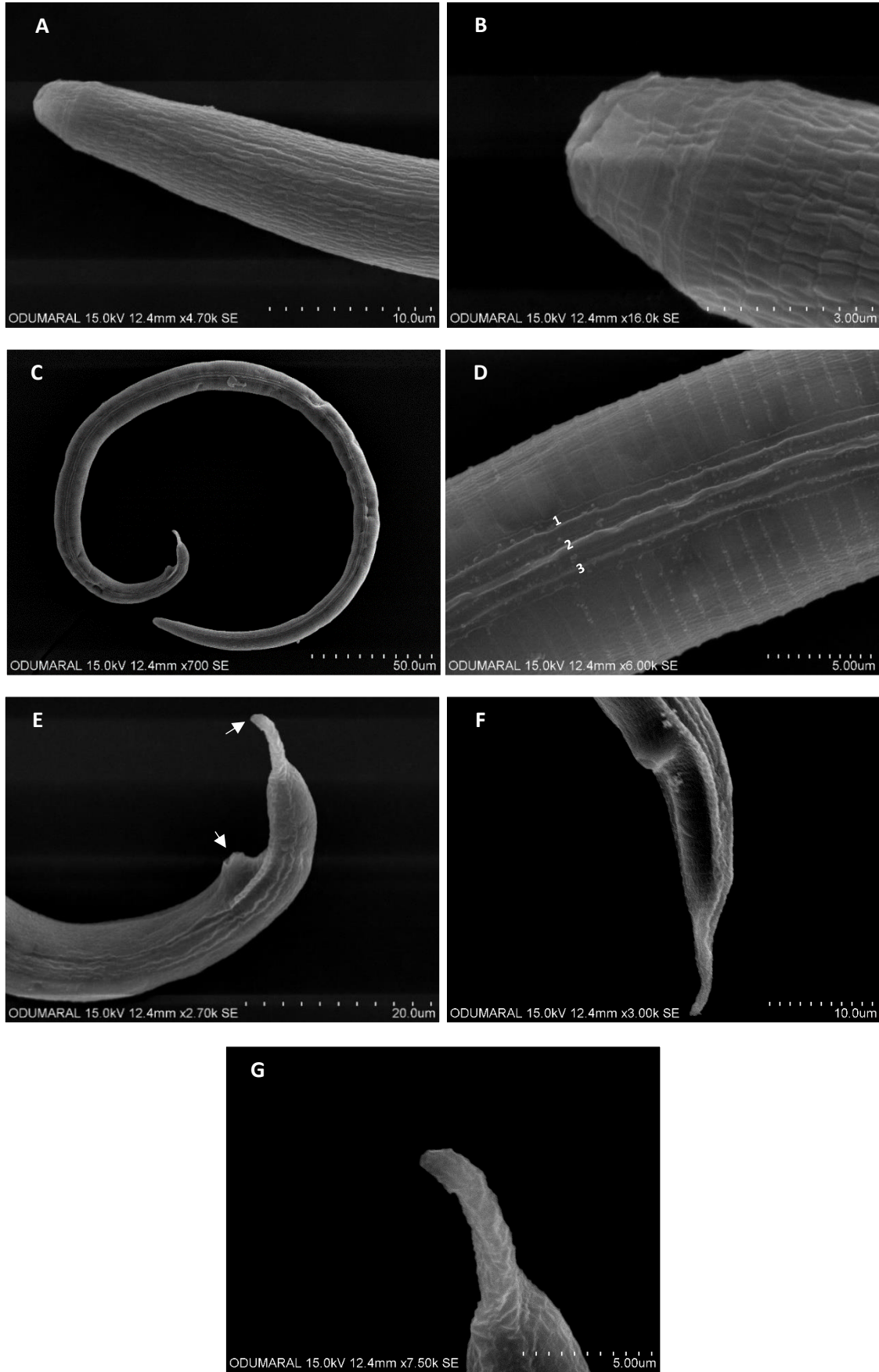


Figure 5. SEM photomicrographs of *Rotylenchulus borealis* male: (A) anterior region; (B) head region; (C) whole body; (D) lateral field in middle body region showing lateral line; (E) tail region and cloaca; (F) tail region; (G) tail terminus.

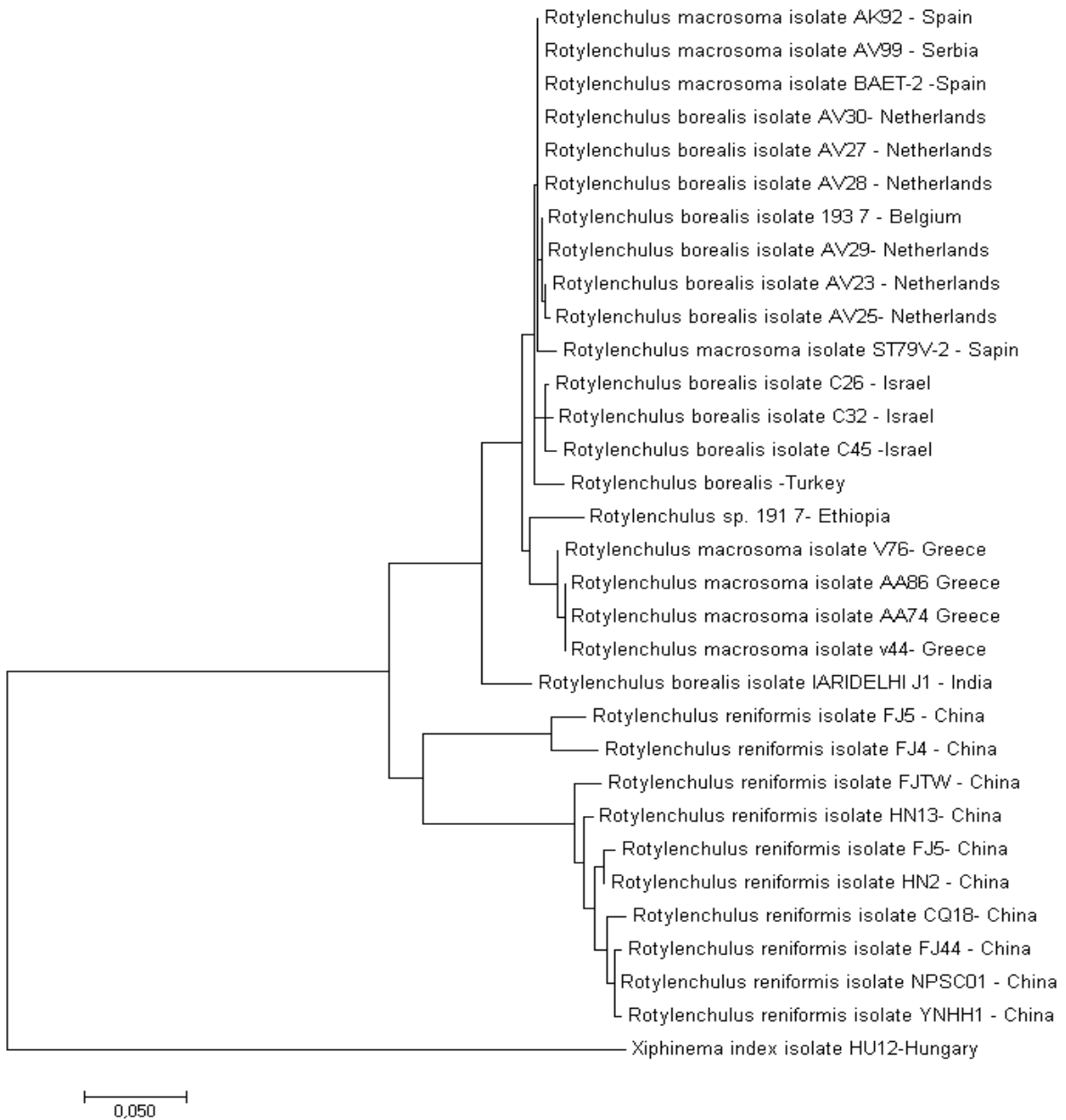


Figure 6. Maximum likelihood (ML) phylogenetic tree of *Rotylenchulus borealis*, inferred from D2 expansion segment of LSU rDNA. The analysis was using 1000 bootstrap replicates. *Rotylenchulus borealis* (Turkey) obtained from this study. *Xiphinema index* sequences were used as out group for the construction of phylogram.

Rotylenchulus borealis is one of the important species from reniform nematodes. This species has been reported from European countries; Estonia, France, Germany, Italy, Spain, Slovak Republic, Turkey and Netherlands (Dasgupta et al., 1968; Germershausen and Gunther, 1984; Ryss, 1992; Liskova, 2002; Tan and Okten, 2008). However it has also been reported in West Africa, Cameroon, Kenya, Rwanda and South Africa (Van Den Berg et al., 2003). Until now, *R. borealis* was detected on *Arachis hypogea*, *Phaseolus vulgaris*,

Zea mays, *Pisum sativum*, *Solanum tuberosum*, *Sorghum bicolor*, *Ipomoea batatas*, *Citrus* spp. *Cucumis melo*, *Chenopodium album*, *Gossypium hirsutum*, *Grass* spp., *Musa cavendishii*, *Vitis* spp. (Bello, 1972; Germani, 1978; Desgupta et al., 1968; Loof and Oostenbrink, 1962; Robinson et al., 1997; Tan and Okten, 2008). However, the nematode hasn't been found in soybean-grown areas. Among the six reniform species identified from different plants in Turkey, this species was reported for the

first time on melon (*Cucumis melo* L.) (Tan and Okten, 2008).

Conclusion

Soybean production is important for Turkey and cultivated areas increases especially in recent years. The important plant parasitic nematodes have been identified in soybean areas worldwide, and host selection of the species varies. The correct diagnosis of nematode species is essential to choose adequate control methods. It is also important to distinguish between the different species of nematodes occurring associated with a specific crop to make decisions on appropriate control measures. Therefore, morphological and molecular characters were used to identify *Rotylenchulus* species from a soybean field in Turkey. All the results confirmed that this species is *R. borealis*.

Conflict of Interest Declaration: The authors have no conflict of interest concerned to this work.

Contribution Rate Statement Summary: The authors declare that they have contributed equally to the article.

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