Plant Protection (Scientific Journal of Agriculture)
46(4), Winter, 2024

10.22055/ppr.2024.46016.1731

First morphological and molecular characterization of *Psilenchus hilarulus* de Man, 1921 (Nematoda: Psilenchidae) from Iraq

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Received: 11 February 2024 Accepted: 3 April 2024

Abstract

During a survey on the biodiversity of plant-parasitic nematodes in the Misan province, southeast Iraq, a population of *Psilenchus hilarulus* was discovered around the rhizosphere of okra. The study included the analysis of the morphological and morphometric characteristics of the species that were recovered. These characteristics were then compared to those of other populations that have been reported from other locations. The phylogenetic relationships of the Iraqi population of *P. hilarulus* with representatives of tylenchid taxa were reconstructed using the partial sequences of the small subunit (SSU), D2-D3 expansion segments of large subunit (LSU), and internal transcribed spacer (ITS) regions of ribosomal DNA, based on Bayesian inference. In the phylogenetic trees inferred from SSU and LSU sequences, the sequences of genus *Psilenchus* formed a clade separate from the representatives of Tylenchidae and Merliniidae. In the SSU tree, the Iraqi population occupied a placement inside a major clade that includes the sequences assigned to *P. hilarulus*, *P. cucrumerus* and *Psilenchus* sp. In LSU tree, new LSU sequences formed a clade with a major clade that includes sequences assigned to *P. hilarulus*, *P. cucrumerus* and *P. vinciguerrae*. The first ITS sequence of the genus, the ITS rDNA of the Iraqi population of *P. hilarulus*, was utilized to reconstruct and analyze the corresponding phylogenetic tree. This appears to be the initial documentation of *P. hilarulus* emerging from Iraq.

Keywords: ITS rDNA, LSU rDNA, Misan province, phylogeny, SSU rDNA.

Associate editor: M. Pedram (Ph.D.)

Citation: Jumaah, A. M. & Azimi, S. (2024). First morphological and molecular characterization of *Psilenchus hilarulus* de Man, 1921 (Nematoda: Psilenchidae) from Iraq. *Plant Protection (Scientific Journal of Agriculture), 46*(4), 57-69. https://doi.org/10.22055/ppr.2024.46016.1731.

گیاه‌پزشکی (مجله علمی کشاورزی)
جلد ٤٦، شماره ٤، زمستان ١٤٠٢

10.22055/ppr.2024.46016.1731

اولین شناسایی ریخت‌شناسی و مولکولی *Psilenchus hilarulus* de Man, 1921 (Nematoda:Psilenchidae) از عراق

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تاریخ دریافت: ۱۴۰۲/۱۱/۲۲ تاریخ پذیرش: ۱۴۰۳/۰۱/۱۵

چکیده

طی بررسی تنوع نماتدهای انگل گیاهی در استان میسان (جنوب شرقی عراق)، یک جمعیت از گونه *Psilenchus hilarulus* از ریزوسفر گیاه بامیه جداسازی گردید. ویژگیهای ریخت‌شناختی و ریخت‌سنجی برای جمعیت یافت شده تهیه گردید و با برخی از جمعیت‌های گزارش شده این گونه از نقاط مختلف مورد مقایسه قرار گرفت. روابط فیلوژنی جمعیت عراقی گونه *P. hilarulus* با نمایندگانی از گونه‌های tylenchid با استفاده از توالی‌های زیرواحد کوچک RNA ریبوزومی (SSU)، ناحیه D2-D3 از زیرواحد بزرگ RNA ریبوزومی (LSU) و توالی‌های ناحیه بین ژنی (ITS rDNA)، با استفاده از روش بیس (Bayesian inference) بازسازی گردید. در درختان فیلوژنی بازسازی شده با استفاده از ژن‌های SSU و LSU، توالی‌های جنس *Psilenchus* به تنهایی در یک کلاد و جدا از توالی‌های خانواده‌های Tylenchidae و Merliniidae قرار گرفتند. در درخت SSU، جمعیت عراقی گونه *P. hilarulus* متعلق به کلادی بود که شامل توالی‌های تخصیص داده شده به گونه‌های *P. hilarulus*، *P. curcumerus* و *Psilenchus* sp. می‌باشد. در درخت LSU، توالی‌های جدید به‌دست آمده همراه با توالی‌های تخصیص داده شده به گونه‌های *P. hilarulus*، *P. curcumerus* و *P. vinciguerrae* کلاد بزرگی را تشکیل دادند. توالی ناحیه بین ژنی (ITS rDNA) جمعیت عراقی گونه *P. hilarulus* به‌عنوان اولین توالی ناحیه ITS برای این جنس است که درخت فیلوژنی مربوط به آن، بازسازی و مورد بحث قرار گرفت. بر اساس اطلاعات ما، این اولین گزارش از گونه *P. hilarulus* از کشور عراق است.

کلیدواژه‌ها: استان میسان، ITS rDNA، LSU rDNA، SSU rDNA، فیلوژنی

دبیر تخصصی: دکتر مجید پدرام

Introduction

Okra (*Abelmoschus esculentus* L. Moench) is a commercial vegetable crop in the tropical, subtropical, and warm temperate regions in the world (Hussain et al., 2015). Some plant-parasitic nematodes, including root-knot nematodes (*Meloidogyne* spp.), *Belonolaimus longicaudatus* Rau, 1958, *Helicotylenchus dihystera* (Cobb, 1893) Sher, 1961, *Hoplolaimus seinhorsti* Luc, 1958 and *Tylenchorhynchus indicus* Siddiqi, 1961 have been associated with the okra in India (Rathour et al., 2006).

The species of genera *Criconema* Hofmaenner & Menzel, 1914, *Helicotylenchus* Steiner, 1945, *Hoplolaimus* Daday, 1905, *Longidorus* Micoletzky, 1922, *Meloidogyne* Goeldi, 1887, *Pratylenchus* Filipjev, 1936, and *Xiphinema* Cobb, 1913, reported from okra in Pakistan and *M. incognita* Kofoid & White, 1919 was the predominant species in all surveyed localities (Hussain et al., 2015). Root-knot nematodes can cause root galling, wilting, and stunted growth in okra (Prajapati et al., 2018). *Rotylenchulus reniformis* Linford & Oliveira, 1940 was reported as an important pest for some okra farms in Nigeria (Claudius-Cole, 2018). *Meloidogyne javanica* (Treub, 1885) Cobb, 1890, *Geocenamus brevidens* (Allen, 1955) Brzeski, 1991, *G. microdorus* (Geraert, 1966) Brzeski, 1991, *G. rugosus* (Siddiqi, 1963) Brzeski, 1991, *Helicotylenchus abunaamai* Siddiqi, 1972, *H. dihystera* (Cobb, 1893) Sher, 1961, *Pratylenchus thornei* Sher & Allen, 1953, *Psilenchus hilarulus* de Man, 1921, *P. vinciguerrae* Brzeski, 1991, and *Tylenchorhynchus elegans* Siddiqi, 1961, were associated with okra in Iran (Pour Ehtesham et al., 2021a, b). Siddiqi (1986, 2000) considered *Psilenchus* under the family Psilenchidae (Paramonov, 1967) Khan, 1969 in superfamily Dolichodoroidea Chitwood in Chitwood & Chitwood, 1950. Geraert & Raski (1987) synonymized the Psilenchinae with Boleodorinae under the family Tylenchidae Örley, 1880. Sturhan & Rahi (1996) placed *Psilenchus* in Psilenchidae under Dolichodoroidea. Geraert (2008), considered the genus *Psilenchus* under Boleodorinae. The phylogenetic inferences using SSU and LSU markers showed *Psilenchus* as a sister taxon with Merliniinae (Subbotin et al., 2006; Palomares-Rius et al., 2009; Carta et al., 2010; Ghaderi et al., 2014; Azimi et al., 2016; Pedram et al., 2018; Amiri Bonab et al., 2021).

During surveys on the plant-parasitic nematodes of Misan province, southeast Iraq, conducted in 2020-2022, the species of genera *Tylenchorhynchus* and *Pratylenchus* were obtained (Jumaah & Azimi, 2022 a, b). In the present study, *Psilenchus hilarulus* was isolated from the rhizosphere of okra in this province. The purpose of this research was to use morphological and morphometric features to describe the population of this species in Iraq. Furthermore, the ITS rDNA, LSU D2-D3, and SSU molecular data were used to examine the evolutionary connections of the recovered species.

Material and methods

Nematode extraction and morphological observations

Twenty-five samples were collected from the rhizosphere of okra in Al-Uzair region (GPS coordinates: 31°18'51.69"N 47°23'59.42"E), Misan province, Iraq. The centrifugal flotation technique (Jenkins, 1964) or the tray method (Whitehead & Hemming, 1965) were used to extract the nematodes from soil samples. The collected specimens were killed in a hot 4% formaldehyde solution, and transferred to anhydrous glycerin, according to De Grisse (1969). The investigations and assessments were carried out utilizing a Leitz SM-LUX light microscope that was augmented with a drawing instrument. Photographs of the nematode specimens were captured with an Olympus BX51 light microscope affixed to an Olympus DP72 digital camera.

DNA extraction, PCR, and sequencing

For molecular analyses, single female specimens were picked out, examined in a drop of distilled water on a temporary slide under light microscope, and transferred to 5 µl of TE buffer (10 mM Tris-Cl, 0.5 mM EDTA; pH 9.0) on a clean slide, and then crushed using a cover slip. Each suspension was collected by adding

10 µl TE buffer. DNA samples were stored at –20°C until used as a PCR template. Primers for amplification of SSU rDNA were: forward SSUF22 (5′- TCC AAG GAA GGC AGC AGG C -3′), and reverse SSUR13 (5′- GGG CAT CAC AGA CCT GTT A -3′) (Dorris et al., 2002). Primers for LSU rDNA D2-D3 amplification were: forward D2A (5′- ACA AGT ACC GTG AGG GAA AGT -3′), and reverse D3B (5′- TCG GAA GGA ACC AGC TAC TA -3′) (Nunn, 1992). Primers for amplification of ITS rDNA were: forward rDNA1 (5′- TTG ATT ACG TCC CTG CCC TTT -3′), and reverse rDNA1.58S (5′- ACG AGC CGA GTG ATC CAC CG -3′) (Subbotin et al., 2000). To amplify the abovementioned loci, the polymerase chain reactions (PCRs) were performed as described by Azimi & Abdolkhani (2023). Amplification success was evaluated by electrophoresis on 1% agarose gel. Sequencing was performed on the PCR products utilizing an Applied Biosystems 3500 (ABI) sequencer manufactured by Pishgam Corporation in Tehran, Iran. The newly obtained sequences of studied species were deposited into the GenBank database with accession numbers: PP204082 for SSU rDNA; PP204084, PP204085 for LSU D2-D3, and PP213273, PP227274 for ITS rDNA.

Phylogenetic analyses

Newly obtained sequences and additional sequences of relevant species were selected using nucleotide basic local alignment search tool (BLASTn). The sequences were aligned by Clustal X version 2 using the default parameters (Larkin et al., 2007). The manual modification of three alignments was executed within the MEGA7 software. The base substitution model was chosen in accordance with the Akaike information criteria using MrModeltest2 (Nylander, 2004). A general time reversible model, including among-site rate heterogeneity, and estimates of invariant sites (GTR + G + I), was selected for three datasets.

Bayesian analysis was performed to infer the phylogenetic trees using MrBayes v3.1.2 (Ronquist & Huelsenbeck, 2003), running the chains for four million generations. After discarding burn-in samples, and evaluating convergence, the remaining samples were retained for further analyses. Utilizing the 50% majority rule, the Markov chain Monte Carlo (MCMC) method within a Bayesian framework was employed to ascertain the equilibrium distribution and assist in the estimation of the posterior probabilities of the phylogenetic trees (Larget & Simon, 1999). Bayesian posterior probability (BPP) values higher than 0.50 are given on appropriate clades. The output files of phylogenetic program were visualized using Dendroscope v3.2.8 (Huson & Scornavacca, 2012) which were digitally drawn in CorelDRAW software version 23.

Results

Iraqi population of *Psilenchus hilarulus*

(Figure 1, Table 1)

Female

Body vermiform, ventrally arcuate following heat fixation. Cuticle annuli 0.9-1.1 µm wide at mid-body. Lateral field with four incisures, 5.5-7.0 µm wide, not areolated. Lip region smooth, continuous with the body, its cephalic framework weak. Stylet fine, without basal knobs. Procorpus cylindroid, median pharyngeal bulb oval, isthmus narrower than procorpus, pharyngeal bulb elongate pyriform**,** 6-7 µm wide and 16.5-18.0 µm long, offset from the intestine, intestine simple, rectum functional and post-anal intestinal sac absent. Excretory pore at the level of the anterior part of the pharyngeal bulb. Reproductive system didelphic-amphidelphic, spermatheca axial, elongate, filled with spheroid sperm, vulva a transverse slit lacking flap or epiptygma. Tail elongate, regularly tapering, with clavate terminus. Phasmids located in the proximal half of the tail.

Male

General morphology is similar to that of female except for character states associated with sexual differences. Spicules tylenchoid, slightly ventrally curved, gubernaculum simple, arcuate, bursa 39.8-42.0 µm long. Tail elongate, regularly tapering, with clavate terminus.

Table 1. Morphometrics of *Psilenchus hilarulus* de Man, 1921 from Misan province, Iraq. All measurements are in µm and in the form: mean ± s.d. (range).

Characters	*Psilenchus hilarulus*	
	Females	Males
n	8	4
L	816.2±54.7 (721-872)	810±2.7 (802-818)
a	43.2±1.2 (42.4-44.6)	44.2±1.2 (43.8-45.6)
b	7.9±0.3 (7.4-8.4)	7.4±0.3 (7.2-7.8)
c	7.7±0.3 (7.4-8.1)	8.0±0.2 (7.8-8.4)
c´	10.4±0.9 (9.8-11.4)	11.6±0.6 (10.9-12.4)
V	46.7±0.6 (45.9-47.4)	-
V´	52.7±1.4 (51.8-54.2)	-
Stylet length	13.4±0.9 (12.4-14.7)	11.7±0.3 (11.2-12.0)
MB	56.9±2.3 (52.2-59.2)	56.3±2.0 (53.8-58.4)
Anterior end to excretory pore	81.1±3.4 (72.2-84.6)	82.3±2.4 (79.3-84.9)
Pharynx	102.2±3.4 (98.2-106.2)	105.8±3.8 (102.2-109.7)
Lip region-vulva	378.3±27.7 (349.6-406.8)	-
Vulva-anus	332.6±26.8 (302.5-359.7)	-
Tail length	104.2±9.8 (92.3-114.5)	100.4±1.2 (99.4-102.5)
T/VA	0.3±0.1 (0.2-0.4)	-
Maximum body width	19.5±0.6 (18.8-20.8)	-
Vulval body width	18.8±1.2 (17.2-20.4)	-
Anal body width	9.8±0.6 (9.2-10.5)	8.6±0.4 (8.3-9.2)
Spicules	-	22.2±0.8 (21.4-23.2)
Gubernaculum	-	7.6±0.4 (7.1-8.2)

Remarks

Compared to the morphometric data ranges given for the species by Geraert (2008)**,** Iraqi population has a slightly shorter body (721-872 *vs* 890-1150 µm), shorter pharynx (98.2-106.2 *vs* 130-157 µm), and shorter tail (92.3-114.5 *vs* 117-160 µm). Compared to the Argentinian population of *P. hilarulus* reported by Marcelo (1996), the body length, stylet length, spicules and gubernaculum length are shorter (721-872 *vs* 1007-1400, 12.4-14.7 *vs* 15-18, 21.4-23.2 *vs* 27-33, and 7.1-8.2 *vs* 10-13 µm, respectively). Compared with the Iranian populations reported by Alvani et al., 2015, Eisvand et al., 2019 and Pour Ehtesham et al., 2021a, the body and tail length are shorter (721-872 *vs* 887.5-1224.5 and 92.3-114.5 *vs* 125-148 µm, respectively), V´ ratio is lower (51.8-54.2 *vs* 54.7-59.3), and the excretory pore from the anterior end is shorter (72.2-84.6 *vs* 96.9-128.7 µm). *P. hilarulus* is herein reported for the first time from Iraq.

Molecular characterization and phylogenetic relationships

Partial SSU rDNA phylogeny

The sequencing of the SSU rDNA of the Iraqi population of *P. hilarulus* yielded a partial sequence with 944 bp long (PP204082). This sequence's 99.89% similarity with other sequences of the same species (MK639401-MK639403) from China was found using BLAST search utilizing this sequence. One mismatch in the overlapped area was the sequence variance between these sequences and the Iraqi population. A total of 58 sequences of tylenchid taxa and two sequences of aphelenchid taxa as outgroups (EU306347 and HQ218323) were used for SSU phylogeny. This dataset comprised 1733 total characters. The phylogenetic tree inferred using this dataset is presented in Figure 2. The newly generated sequence of the Iraqi population of *P. hilarulus* formed a clade with other sequences of this species, *P. curcumerus* Rahaman, Ahmed & Jairajpuri, 1994, and *P. hilarus* Siddiqi, 1963 with maximal support (BPP = 1.00.

D2-D3 fragment of LSU rDNA phylogeny

To reconstruct the LSU rDNA tree, newly obtained 692, and 730 nt long partial sequences of D2-D3 region with accession numbers PP204084 and PP204085 were used. Both sequences were identical in overlapping regions and differed only in length. BLAST search using these sequences showed that they have 97.69% and 97.81% identity with another sequence assigned to *P. hilarulus* from China (MW716285). The sequence variation between the Iraqi population and this sequence was 16 mismatches in the overlap region. LSU phylogeny was performed using 47 tylenchid sequences and two aphelenchid sequences as outgroups (DQ328683 and DQ328684). This dataset comprised 816 total characters. The phylogenetic tree inferred using this dataset is presented in Figure 3. The major clade, including the newly generated sequences of the Iraqi population of species, also includes *P. curcumerus* and *P. vinciguerrae* Brzeski, 1991 with maximal support (BPP = 1.00).

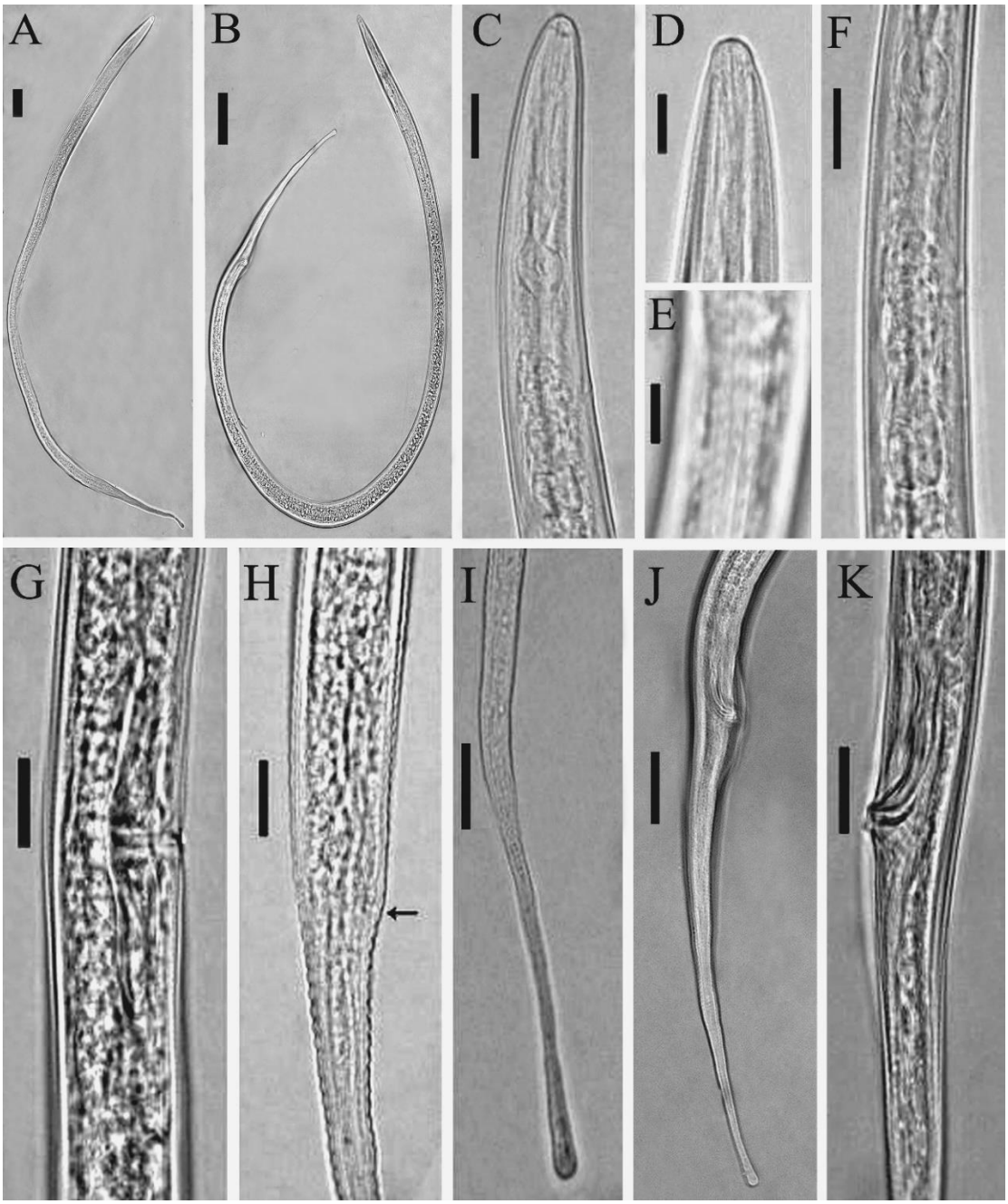

Figure 1. Light photomicrographs of *Psilenchus hilarulus* de Man, 1921 from Iraq. A, C-I: Female. B, J&K: Male. A, B: Entire body; C, D: Anterior body region; E: Lateral field at mid-body, F: Pharynx; G: Vulval region; H-K: Posterior body region (the arrows indicate the anus). Scale bars: A, B = 50 µm; C-K: 10 µm.

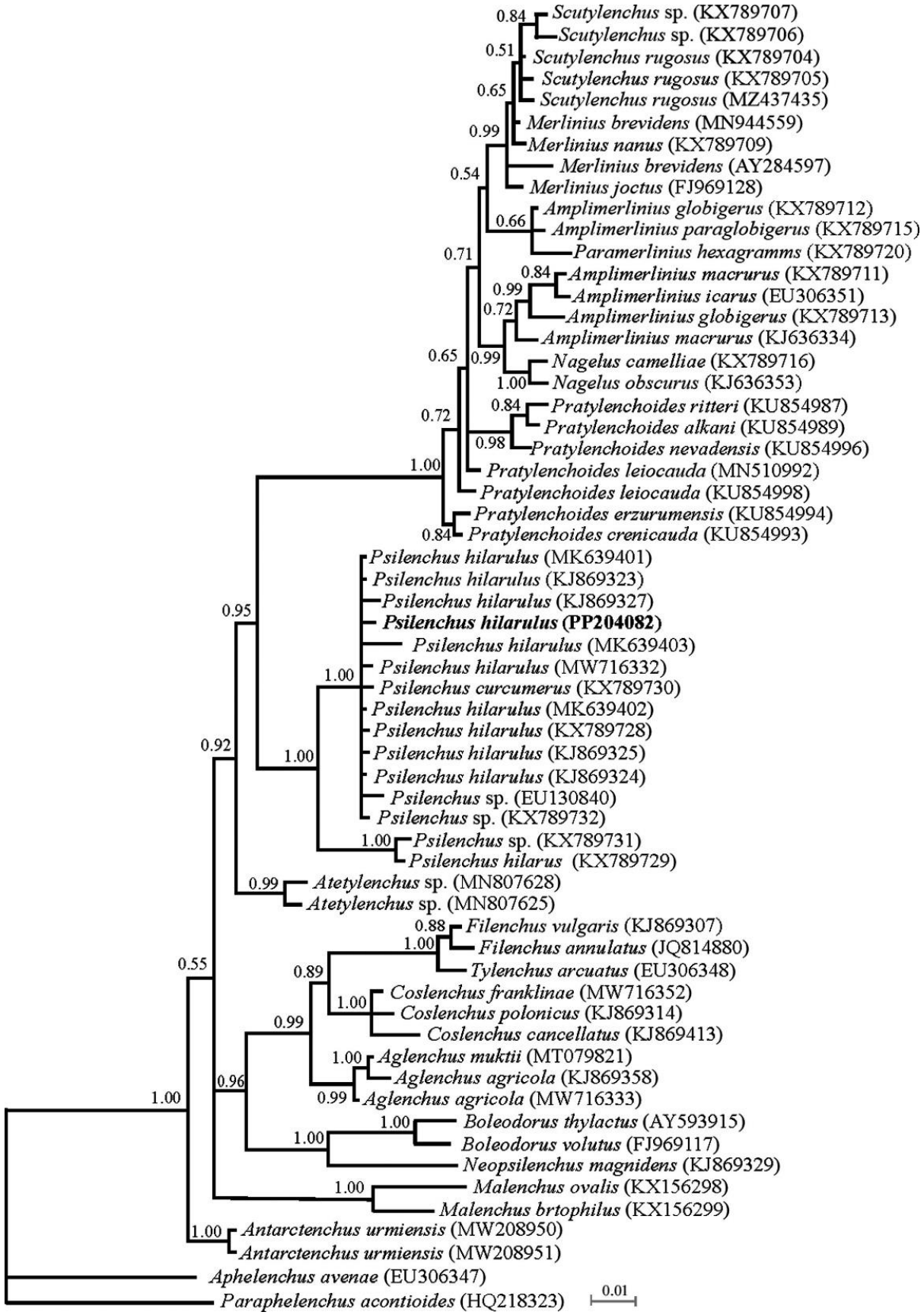

Figure 2. Bayesian 50% majority rule consensus tree inferred from analysis of the SSU rDNA sequences of Iraqi population of *Psilenchus hilarulus* de Man, 1921 under the GTR + G + I model. Bayesian posterior probability values of more than 0.50 are given for appropriate clades. New sequence is indicated in bold.

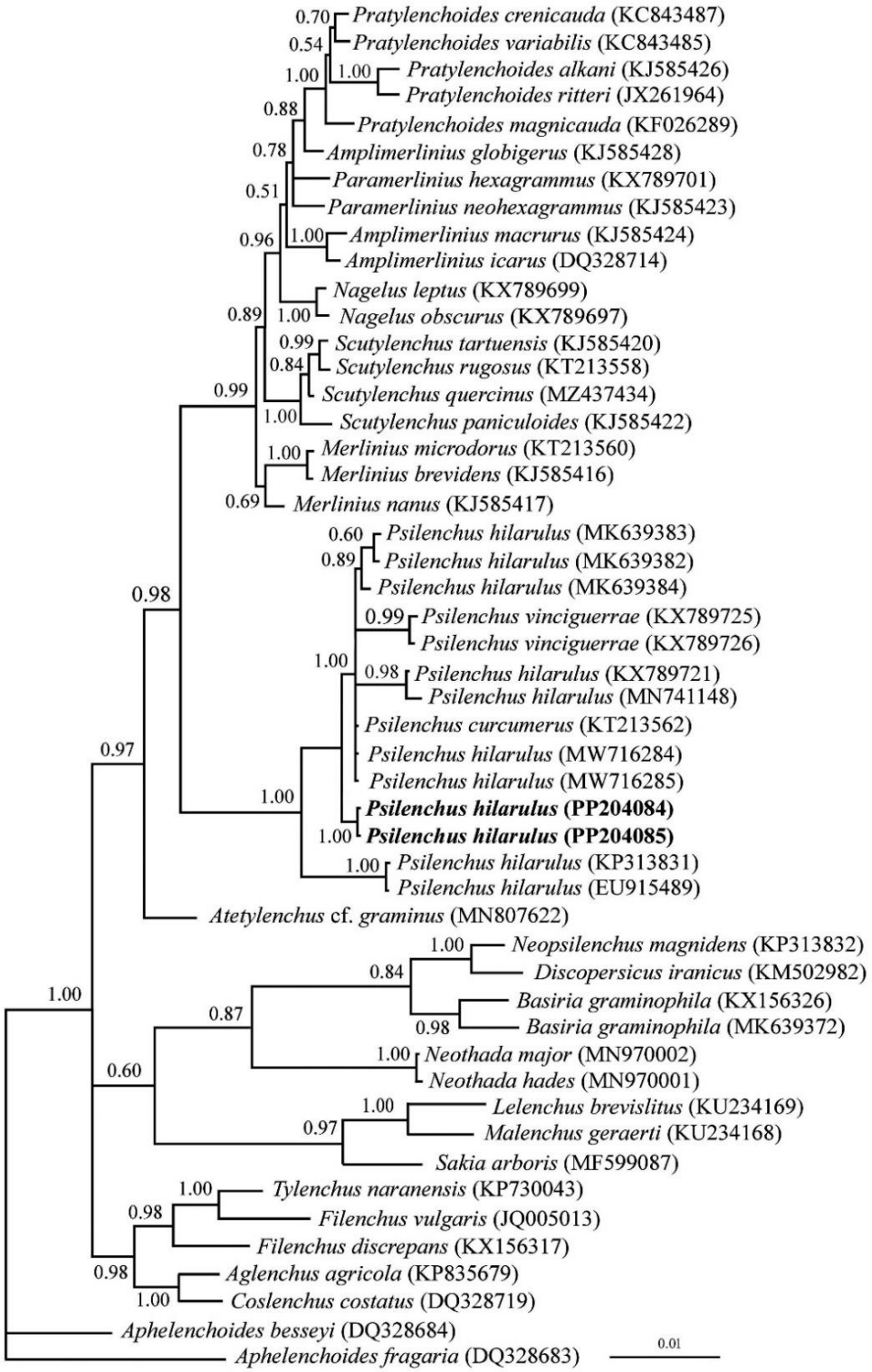

Figure 3. Bayesian 50% majority rule consensus tree inferred from analysis of the D2-D3 domain of the LSU rDNA sequence of Iraqi population of *Psilenchus hilarulus* de Man, 1921 under the GTR + G + I model. Bayesian posterior probability values of more than 0.50 are given for appropriate clades. New sequences are indicated in bold.

Partial ITS rDNA phylogeny

Two 508 and 503 bp long partial sequences of ITS rDNA (PP213273, PP227274) were obtained for Iraqi population. Both sequences were identical and differ only in length. An identification BLAST search employing these sequences returned 95.95% and 95.80% similarity with *Anguina wevelli* (Van den Berg,

1985) Siddiqi, 2000 (AF363109) from South Africa, with 130 mismatches and 32 gaps in the overlapping region, respectively. For ITS phylogeny, a total of 29 sequences originating from tylenchid taxa and two sequences from aphelenchid taxa served as outgroups (KM657966 and JX683685).

This dataset comprised 1054 total characters. The phylogenetic tree inferred using this dataset is presented in Figure 4. So far, no sequence data of the genus *Psilenchus* for the ITS rDNA region was recorded in the GenBank database. The sequences of the ITS rDNA region of genus *Psilenchus* were provided for the first time.

Discussion

The aims of the present study were the morphological, and molecular characterization of the recovered population of *Psilenchus hilarulus* from Iraq, which is a new record for the country. The minor morphometric differences of recovered population compared to other populations, as already discussed, are explained by intraspecies variations resulted from differences in geographical distribution regions.

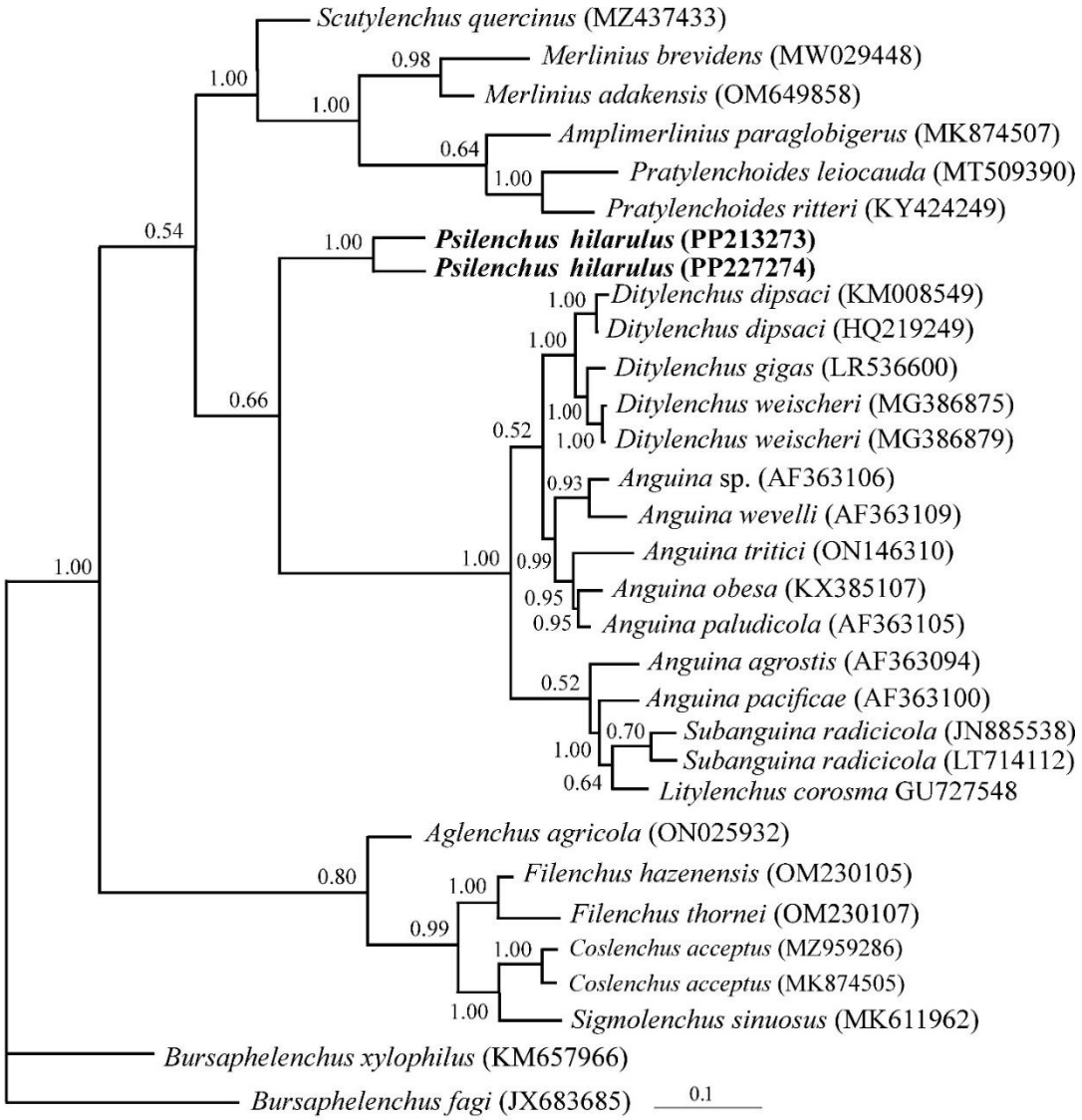

Figure 4. Bayesian 50% majority rule consensus tree inferred from analysis of the ITS rDNA sequence of Iraqi population of *Psilenchus hilarulus* de Man, 1921 under the GTR + G + I model. Bayesian posterior probability values of more than 0.50 are given for appropriate clades. New sequences are indicated in bold.

In the phylogenetic tree using SSU and LSU rDNA sequences, all currently available sequences of genus *Psilenchus* formed a clade. This clade was separate from the clade of Tylenchidae and Merliniidae Siddiqi, 1971 sequences. These findings, as noted by other writers as well (Palomares-Rius et al., 2009; Azimi et al., 2016; Pedram et al., 2018; Amiri Bonab et al., 2021), are consistent with Siddiqi's (1986, 2000) taxonomic framework, which suggests the separate family Psilenchidae as housing the genus.

So far, 21 valid species were described under the genus *Psilenchus* (Geraert, 2008), but the available sequences of genus in the GenBank database are very few. The topotype specimens of eldest species were not sequenced. In SSU phylogeny, sequences of the Iraqi population of *P. hilarulus* belonged to *P. hilarulus/P. curcumerus* clade. In LSU phylogeny, the currently available sequences of *P. hilarulus* did not form a clade. Sequences of the topotype specimens in these situations are required to confirm the identity of these isolates before classifying sequence differences as intraspecies variations or determining the complex nature of the species, in line with earlier arguments (Lazarova et al., 2019; Monemi et al., 2022).

Acknowledgments

The authors thank the Research Council of Shahid Chamran University of Ahvaz, Iran (Grant no. SCU.AP1402.638) for financial supports.

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