

First morphological and molecular characterisation of *Hoplolaimus columbus* Sher, 1963 (Tylenchida: Hoplolaimidae) from Iraq

Ahmed Malik Jumaah and Sedighe Azimi

Department of Plant Protection, Faculty of Agriculture, Shahid Chamran University of Ahvaz, Ahvaz, Iran

e-mail: s.azimi@scu.ac.ir

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Summary. The Columbia lance nematode, *Hoplolaimus columbus*, is an important pest that can cause severe damage to a wide host range of agricultural crops. During a survey on the biodiversity of plant-parasitic nematodes in the Misan province, southeast Iraq, *H. columbus* was discovered around the rhizosphere of oleander. The morphological and morphometric data were provided for the recovered species. The phylogenetic relationships of the Iraqi population of *H. columbus* with representatives of the family Hoplolaimidae were reconstructed using partial sequences of the small subunit, D2-D3 expansion segments of the large subunit, and internal transcribed spacer regions of ribosomal DNA based on Bayesian inference. In three inferred SSU, LSU and ITS phylogenies, Iraqi *H. columbus* belonged to the *H. columbus* / *H. seinhorsti* clade. To our knowledge, this is the first report of the species from Iraq.

Key words: lance nematode, ITS, LSU, *Nerium oleander*, molecular, morphometrics, SSU.

The Columbia lance nematode, *Hoplolaimus columbus* Sher, 1963, was first reported in the soil surrounding soybean roots in South Carolina, USA (Sher, 1963). Subsequently, it was collected and identified from cotton in the USA (Gazaway & Armstrong, 1994). Infestations of *H. columbus* can lead to severe damage, ranging from 10-25% in cotton and 10-40% in soybean (Noe, 1993). Cortical parenchyma, endoderm and vascular tissue of the roots can be attacked by the nematode, resulting in stunting and chlorotic tissue in the host. In addition to cotton and soybeans, *H. columbus* is an important pest of corn and has a varied host range, including weeds, vegetables and other crops (Bae *et al.*, 2009a). Population densities of *H. columbus* may increase by 200-400% during a growing season, and therefore, management of the species is essential (Noe, 1993). Due to the significance of *H. columbus* and its negative impact on agricultural production, Ma *et al.* (2021) sequenced the entire genome of this species.

While conducting surveys on the plant-parasitic nematodes in the Misan province, Southeast Iraq, between 2020-2022, the nematode species *Tylenchorhynchus clarus* Allen, 1955, *T. zae* Sethi & Swarup, 1968 (Jumaah & Azimi, 2022a), and *Pratylenchus thornei* Sher & Allen, 1953 (Jumaah

& Azimi, 2022b) were identified. In the present study, *H. columbus* was recovered in this province. According to available literature, this is the first report of the species from Iraq. Therefore, the present study aims to characterise the Iraqi population of *H. columbus* based on morphological and morphometric characteristics. Additionally, molecular data of 18S rDNA, D2-D3 expansion segments of 28S rDNA, and ITS rDNA were used to study the phylogenetic relationships of the recovered species.

MATERIAL AND METHODS

Nematode extraction and morphological observations. Twenty four samples were collected from the rhizosphere of oleander (*Nerium oleander* L.) in the Al-Amarah region (GPS coordinates: 31°47'14.14" N, 47°11'34.02" 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 Grijse (1969). Observations and measurements were conducted using a Leitz SM-LUX light microscope (Leitz Corporation, Wetzlar, Germany) equipped with a drawing tube. The nematode specimens were

photographed using an Olympus DP72 digital camera attached to an Olympus BX51 light microscope (Olympus Corporation, Tokyo, Japan).

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 the 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 15 µl TE buffer. The DNA samples were stored at -20°C until used as a PCR template. 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). Primers for amplification of 18S 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). To amplify the segments mentioned above, the polymerase chain reactions (PCR) were performed as described by Azimi and Abdolkhani (2023). Amplification success was evaluated by electrophoresis on 1% agarose gel. The PCR products were subjected to sequencing using an Applied Biosystems 3500 (ABI) sequencer, Pishgam Corporation, Tehran, Iran. The newly obtained sequences of the studied species were deposited into the GenBank database with accession numbers: OR578531, OR578532 for 18S rDNA, OR578533 for LSU D2-D3 and OR578549 for ITS rDNA.

Phylogenetic analyses. The newly obtained sequences and additional sequences of relevant species were selected after the nucleotide basic local alignment search tool (BLASTn, National Center for Biotechnology Information, Bethesda, Maryland, USA). The sequences were aligned by Clustal X version 2 using the default parameters (Larkin *et al.*, 2007). The outgroup taxa were chosen according to the previous study (Marais *et al.*, 2020). The editing of three alignments was performed manually in the MEGA7 program (Kumar *et al.*, 2016). The base substitution model was selected using MrModeltest2 (Nylander, 2004) based on the Akaike information criteria. A general time reversible model, including among-site rate heterogeneity and estimates of invariant sites (GTR + G + I), was selected for the three datasets.

The 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. The Markov chain Monte Carlo (MCMC) method within a Bayesian framework was used to determine equilibrium distribution and help estimate the posterior probabilities of the phylogenetic trees (Larget & Simon, 1999) using the 50% majority rule. Bayesian posterior probability (BPP) values higher than 0.50 are given on appropriate clades. The output files of the phylogenetic program were visualised using Dendroscope v3.2.8 (Huson & Scornavacca, 2012) and were digitally drawn in CorelDRAW software version 23 (Corel Corporation, Ottawa, Canada).

RESULTS

Iraqi population of *Hoplolaimus columbus* (Fig. 1, Table 1)

Female. Body vermiform, cylindrical, slightly curve to open C-shape after heat fixation. Head hemispherical, with three labial annuli, distinctly set-off from the body by a deep constriction, cephalic framework prominent. Lateral field indistinct, one incisures was hardly visible in some specimens. Stylet strong and large with prominent tulip-shaped knobs, 4.0-4.6 µm high and 5.9-6.2 µm across. The median pharyngeal bulb almost rounded, 20-23 µm wide, with a sclerotised valve, pharyngeal glands overlapping the intestine dorsally with six gland nuclei. Excretory pore two to four annuli anterior to hemizonid and hemizonid about three cuticular annuli long. Two scutella, one anterior to the vulva and the other posterior to the vulva. Reproductive system didelphic-amphidelphic, both genital branches almost equally developed, ovaries straight, epiptygma double, spermatheca absent. Intestine overlaps rectum. Tail short, rounded, with annulated tail terminus.

Male. Not found.

Remarks. The general morphology of the recovered population of the species closely resembles that of the type population from South Carolina (Sher, 1963). Compared with the Taiwanese population reported by Chen *et al.* (2006), the b ratio is lower (9.8-11.4 vs 12.1-16.1), c' ratio is higher (0.9-1.3 vs 0.6-0.8), and o ratio is lower (8.4-10.3 vs 10.5-21.6). Compared with the Indian populations from the rhizosphere of jackfruit, mango and tea reported by Venkadesh *et al.* (2023), no remarkable differences were observed.

Table 1. Morphometrics of *Hoplolaimus columbus* from the Misan Province, Iraq, and comparison with the original description and Taiwanese population.

Characters	Population	Misan Province, Iraq	South Carolina, USA (Sher, 1963)	Dounan, Taiwan (Chen <i>et al.</i> , 2006)
n		10	20	15
L		1438 ± 65 (1330-1510)	1260-1800	1360 ± 80 (1150-1500)
a		29.6 ± 2.0 (28.0-31.8)	30-38	30.4 ± 2.9 (27.7-36.6)
b		10.6 ± 0.9 (9.8-11.4)	9.1-12.4	13.6 ± 1.1 (12.1-16.1)
b'		7.2 ± 0.2 (6.7-7.8)	6.3-9.7	7.1 ± 0.8 (5.9-8.6)
c		48.7 ± 3.2 (42.6-51.2)	39-57	58.7 ± 5.1 (47.5-67.0)
c'		1.1 ± 0.2 (0.9-1.3)	–	0.7 ± 0.1 (0.6-0.8)
V		55.3 ± 3.4 (51.0-59.6)	51-60	55.7 ± 1.7 (53.4-58.4)
Lip region height		8.1 ± 0.2 (7.4-8.3)	–	–
Lip region width		15.4 ± 0.3 (14.9-15.7)	–	–
DGO		6.8 ± 0.3 (5.2-8.4)	–	6.1 ± 1.2 (4.3-9.3)
Styilet length		42.2 ± 1.4 (41.5-45.9)	40-48	42.3 ± 1.0 (40.7-43.3)
o		9.2 ± 0.7 (8.4-10.3)	9-13	14.4 ± 2.8 (10.5-21.6)
Conus		22.2 ± 0.4 (21.5-23.2)	–	–
Medial bulb		62.4 ± 1.7 (58.8-67.2)	–	–
Pharynx		125.3 ± 1.2 (121-127)	–	–
Anterior end to pharyngeal gland base		182.9 ± 4.2 (176-189)	–	–
Excretory pore from anterior end		141.6 ± 2.4 (135-144)	–	125 ± 8 (106-139)
Maximum body width		44.3 ± 1.5 (42.8-46.5)	–	–
Anal body width		33.7 ± 1.3 (32.4-35.8)	–	32 ± 3 (27-37)
Body width at vulva		44.2 ± 1.5 (41.6-47.6)	–	–
Tail annuli		18.9 ± 1.2 (17.0-21.0)	16-22	–
Tail length		27.8 ± 1.8 (25.7-31.4)	–	23 ± 2 (18-28)
Anterior phasmid		37.8 ± 1.8 (35.6-40.0)	34-47	–
Posterior phasmid		84.2 ± 1.6 (82.2-86.6)	80-90	82.3-82.5

Note: All measurements are in µm and in the form: mean ± SD (range).

Males have not been found in most of the studied populations, including the type population and other populations from Taiwan and India, and also for the Iraqi population. The males of *H. columbus* were rarely present. It has been reported for the first time from a soybean field in South Carolina in the ratio of one male to 60 females (Fassuliotis, 1974).

The presently studied population of the species was collected from the rhizosphere of oleander in the Misan province, southeast Iraq. It is herein reported for the first time in Iraq.

Molecular characterisation and phylogenetic relationships. Partial SSU rDNA phylogeny. Two 993 bp long identically aligned sequences of the 18S rDNA were generated for the Iraqi population of *Hoplolaimus columbus* (OR578531, OR578532). The BLASTn search using these sequences revealed they have 99.11% identity with another sequence of the same species (AY912051, Powers *et al.*, unpublished). Sequence variation between the Iraqi population and this sequence was four mismatches in the overlapping region. A total of 37 sequences of

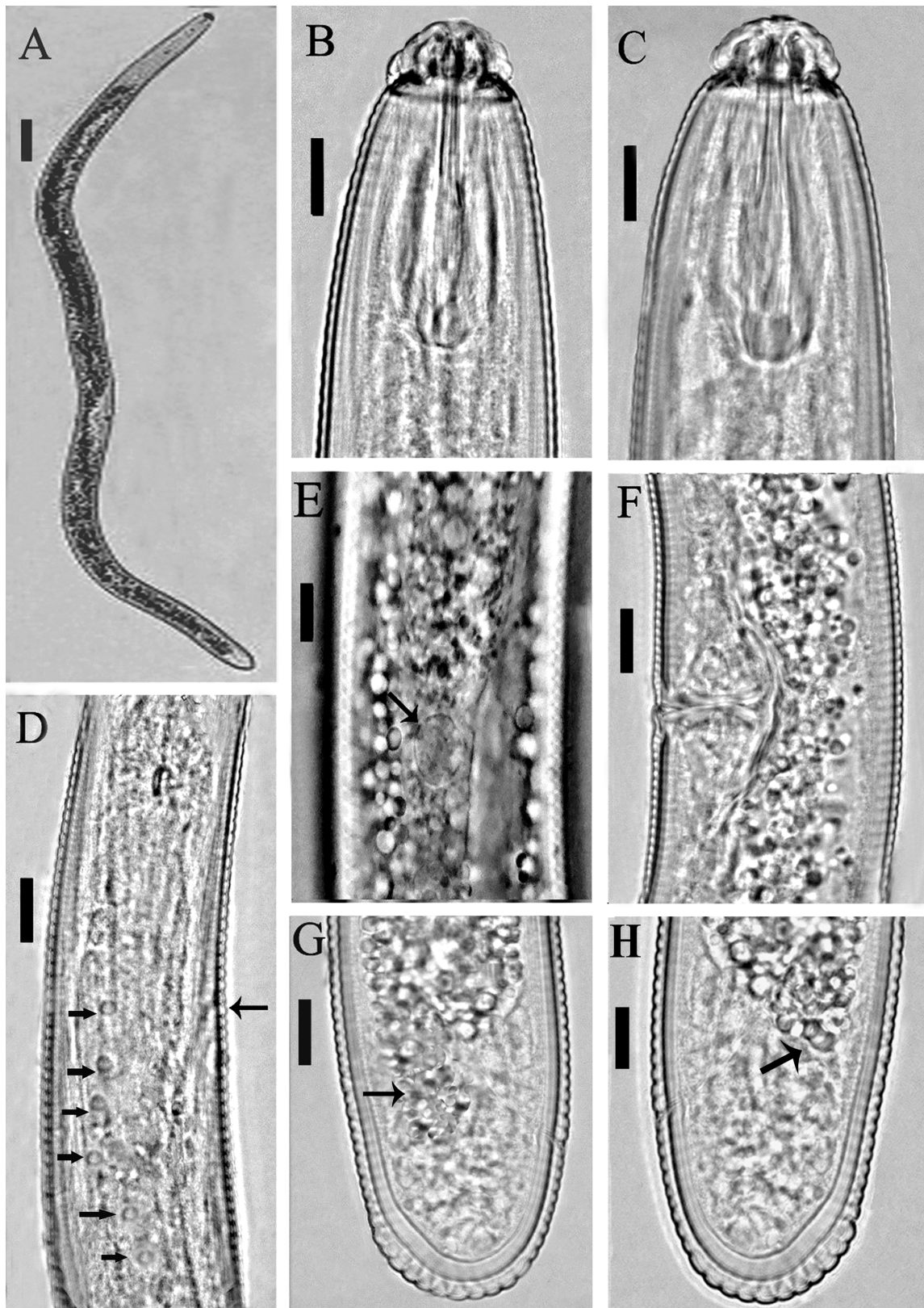


Fig. 1. Light photomicrographs of *Hoplolaimus columbus* female from Iraq. A: Entire body; B, C: Anterior body region; D: Part of the pharynx (the arrows indicate the excretory pore and pharyngeal glands nuclei); E: Anterior part of the body (the arrow indicates the scutella); F: Vulval region; G, H: Posterior body region (the arrows indicate the post rectal sac). Scale bars: A = 50 μ m, B-H = 10 μ m.

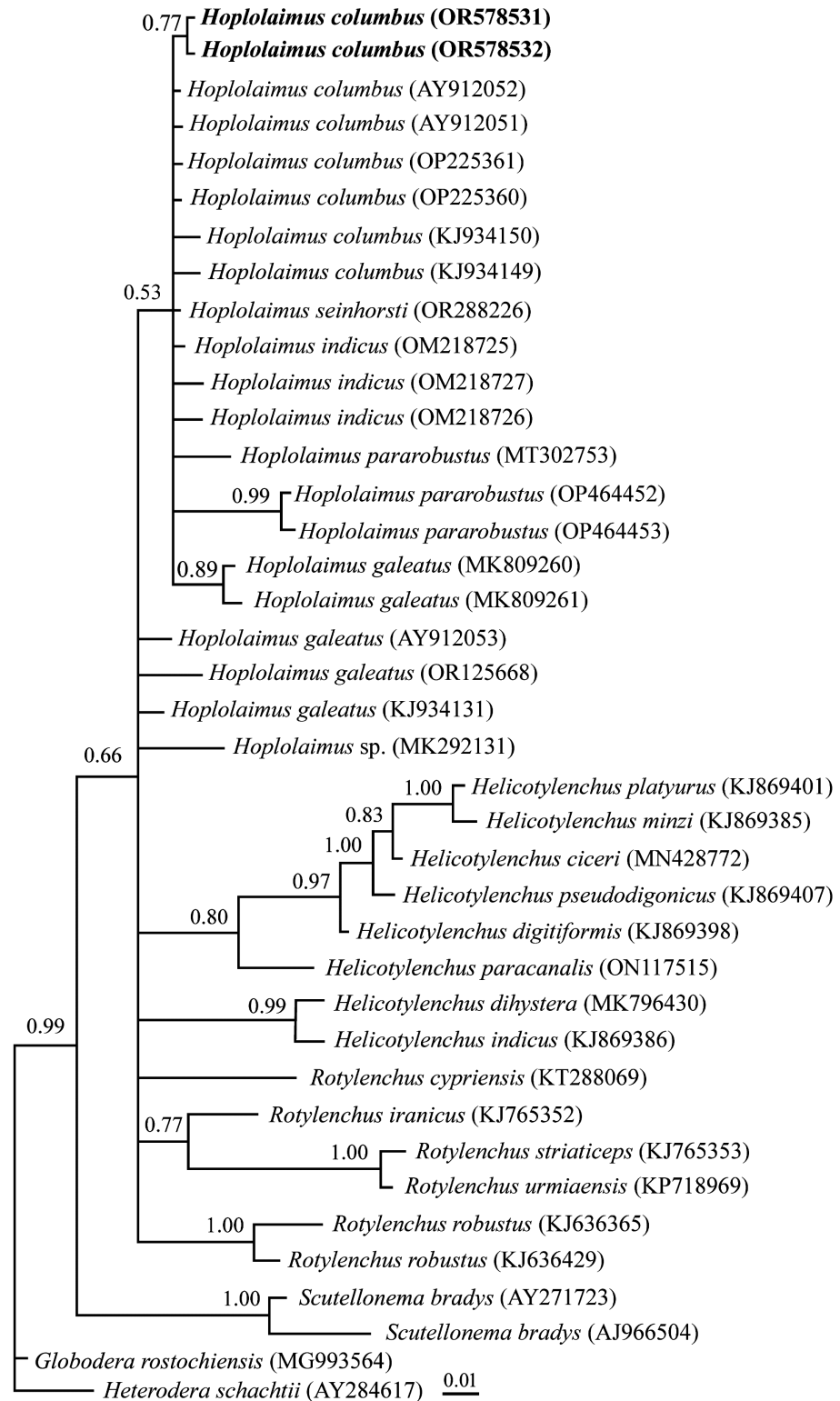


Fig. 2. Bayesian 50% majority rule consensus tree inferred from analysis of the 18S rDNA sequences of Iraqi population of *Hoplolaimus columbus* under the GTR + G + I model. Bayesian posterior probability values of more than 0.50 are given for appropriate clades. New sequences are indicated in bold.

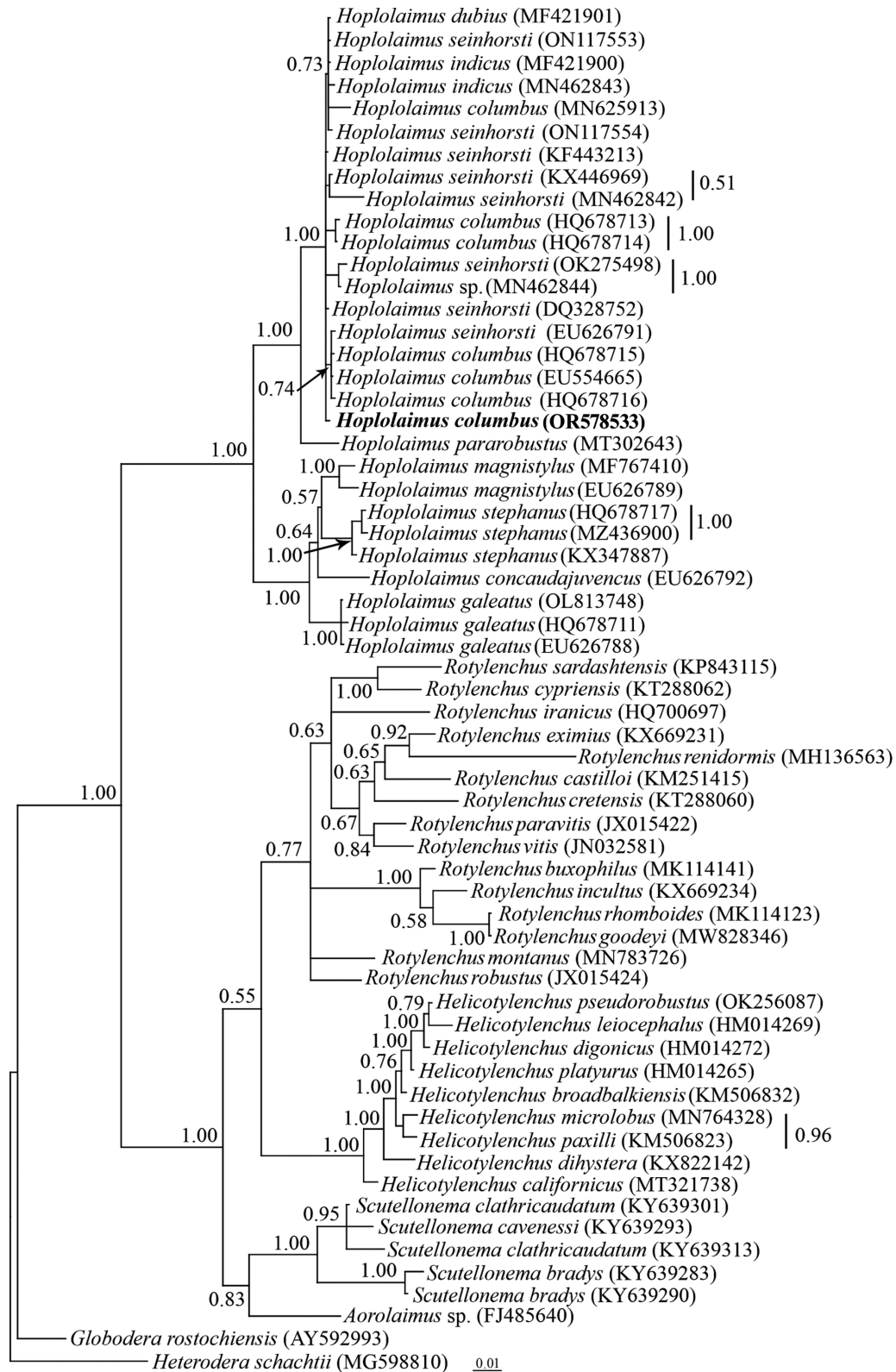


Fig. 3. Bayesian 50% majority rule consensus tree inferred from analysis of the D2-D3 domain of the 28S rDNA sequence of Iraqi population of *Hoplolaimus columbus* under the GTR + G + I model. Bayesian posterior probability values of more than 0.50 are given for appropriate clades. New sequence is indicated in bold.

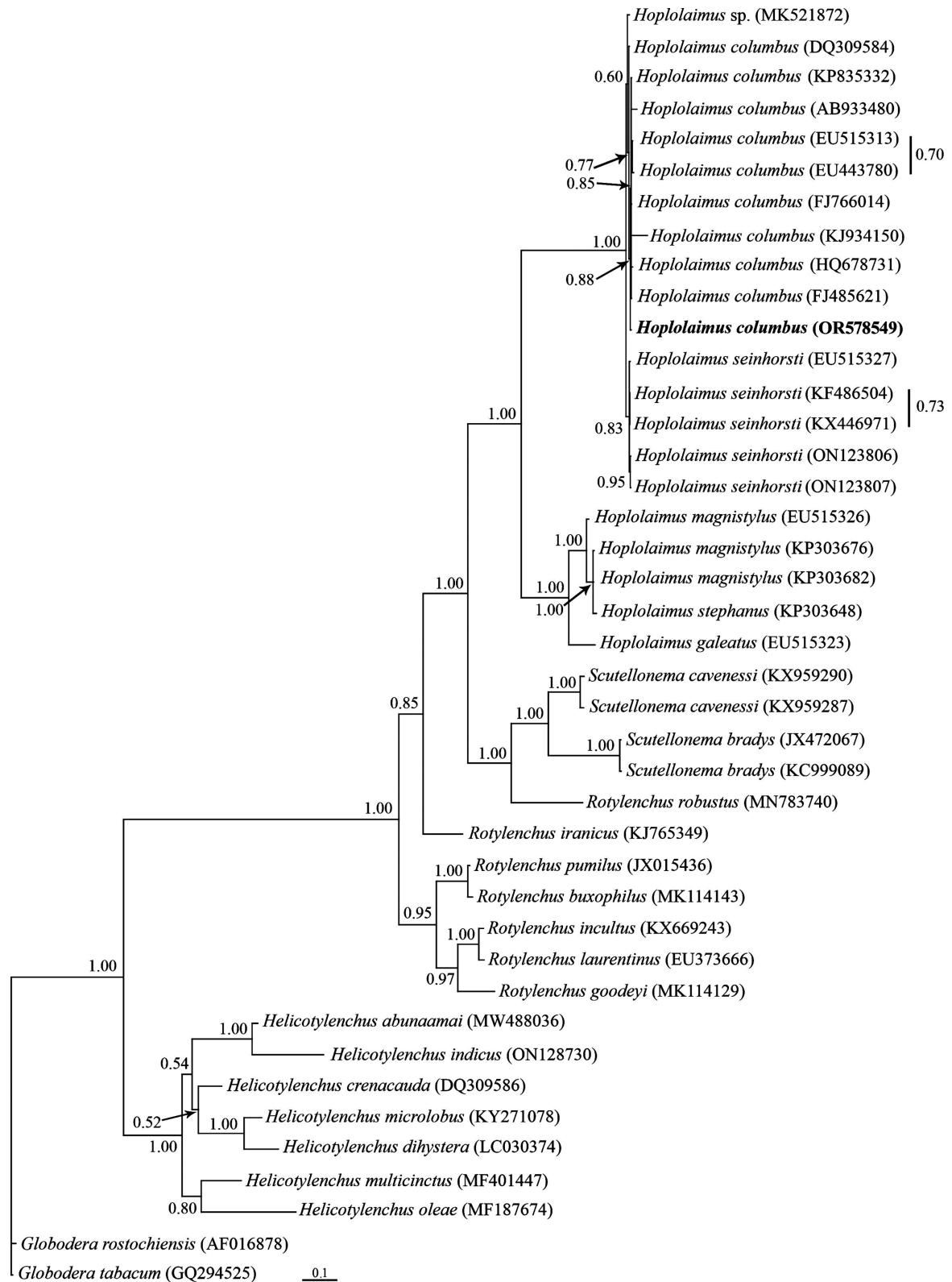


Fig. 4. Bayesian 50% majority rule consensus tree inferred from analysis of the ITS rDNA sequence of Iraqi population of *Hoplolaimus columbus* under the GTR + G + I model. Bayesian posterior probability values of more than 0.50 are given for appropriate clades. New sequence is indicated in bold.

the family Hoplolaimidae and two sequences of the genus *Globodera* Skarbilovich, 1959 and *Heterodera* Schmidt, 1871 as outgroups (MG993564 and AY284617) were used for SSU phylogeny. This dataset comprised 1754 total characters. The phylogenetic tree inferred using this dataset is presented in Figure 2. In this tree, the newly generated sequences of the Iraqi population of *H. columbus* formed a clade with other sequences of this species, *H. seinhorsti* Luc, 1958, *H. indicus* Sher 1963, *H. pararobustus* (Schuurmans Stekhoven & Teunissen, 1938) Sher, 1963, and *H. galeatus* (Cobb, 1913) Thorne, 1935 with low support (BPP = 0.53).

D2-D3 fragment of LSU rDNA phylogeny. The sequencing of the D2-D3 region of the Iraqi population yielded a partial sequence with 686 bp long (OR578533). The BLAST search using this sequence revealed it has 99.85% identity with another sequence of the same species (HQ678716, South Carolina, USA; Ma *et al.*, 2011). Sequence variation between the Iraqi population and this sequence was two mismatches and one gap in the overlapping region. Fifty-nine sequences of the family Hoplolaimidae and two sequences of the genus *Globodera* and *Heterodera* as outgroups (AY592993 and MG598810) were used. This dataset comprised 1118 total characters. The phylogenetic tree inferred using this dataset is presented in Figure 3. The major clade, including the newly generated sequence of the Iraqi population of the species, also includes *H. seinhorsti*, *H. indicus* and *H. dubius* Chaturvedi, Singh & Khera, 1979 with high support (BPP = 1.00).

Partial ITS rDNA phylogeny. A 736 bp long partial sequence of the ITS rDNA (OR578549) was obtained for the Iraqi population. A BLAST search using this sequence revealed it has 99.18% identity (six mismatches in the overlapping region) with another sequence of the same species (DQ309584, Taiwan; Chen *et al.*, 2006). Thirty-nine sequences of the family Hoplolaimidae and two sequences of the genus *Globodera* as outgroups (AF016878 and GQ294525) were used for ITS phylogeny. This dataset comprised 1456 total characters. The phylogenetic tree inferred using this dataset is presented in Figure 4. The major clade, including the newly generated sequence of the Iraqi population of the species, also includes *H. seinhorsti* with high support (BPP = 1.00).

DISCUSSION

The objectives of the present study were the morphological and molecular characterisation of the recovered population of *Hoplolaimus columbus*

from Iraq, which is a new record for the country. In three inferred SSU, LSU and ITS phylogenies, Iraqi *H. columbus* belonged to the *H. columbus* / *H. seinhorsti* clade. Topologies of these trees are congruent to those published by other authors (Bae *et al.*, 2009b; Ma *et al.*, 2019; Marais *et al.*, 2020; Olajide *et al.*, 2023).

In the phylogenetic tree inferred from the 18S rRNA gene, three accession numbers (OM218725-OM218727), were deposited into the GenBank database as *Hoplolaimus indicus* (Afzali *et al.*, unpublished), however, Olajide *et al.* (2023), based on the phylogenetic and species delimitation outcomes concluded these sequences belong to *H. columbus*. Although morphological data of these specimens are not available, we identified them as *Hoplolaimus indicus* in the tree.

Considering the economic importance of *H. columbus*, more studies are needed regarding the sampling of different agricultural crops for the existence of the *Hoplolaimus* species and studying pathogenicity, and threshold levels for estimating crop damage of *H. columbus* in Iraq.

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A.M. Jumaah and S. Azimi. Морфологическая и молекулярная характеристика *Hoplolaimus columbus* Sher, 1963 (Tylenchida: Hoplolaimidae) из Ирака.

Резюме. Колумбийская ланцетная нематода, *Hoplolaimus columbus*, является важным вредителем, который может нанести серьезный ущерб широкому кругу сельскохозяйственных культур. В ходе изучения биологического разнообразия фитопаразитических нематод в провинции Мисан (юго-восточный Ирак) *H. columbus* был обнаружен в ризосфере олеандра. Для обнаруженного вида приведены морфологические и морфометрические данные. Филогенетические взаимоотношения иракской популяции *H. columbus* с представителями семейства Hoplolaimidae были реконструированы с использованием частичных последовательностей малой субъединицы (SSU), последовательностей D2-D3 сегмента большой субъединицы (LSU) и внутренних транскрибируемых спейсерных (ITS) участков рРНК на основе Байесова анализа. В трех филогениях, построенных по результатам анализа последовательностей SSU, LSU и ITS участка, иракский *H. columbus* принадлежал к кладе *H. columbus* / *H. seinhorsti*. По мнению авторов, это первое сообщение о данном виде из Ирака.
