First molecular characterisation of *Helicotylenchus abunaamai* Siddiqi, 1972 and *H. dihystera* (Cobb, 1893) Sher, 1961 (Tylenchomorpha: Hoplolaimidae) from Iran

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Summary. During a survey on the biodiversity of plant-parasitic nematodes in okra fields of Khuzestan province (southwest Iran), two species, *Helicotylenchus abunaamai* and *H. dihystera*, were recovered and identified. The morphological and morphometric data were provided for the recovered species and their differences with the type and some other populations were discussed. To the best of our knowledge, this is the first report of these two species in okra fields worldwide. In molecular phylogenetic analyses using the D2-D3 expansion segments of large subunit (LSU rDNA) and internal transcribed spacer (ITS rDNA) sequences using Bayesian inference and maximal number of species of the genus, Iranian population of *H. abunaamai* formed a maximally supported clade with *H. caudatus* and *H. digonicus*; and the Iranian population of *H. dihystera* fell into a clade including several isolates of the species. In ITS phylogeny, the Iranian population of *H. abunaamai* formed a maximally supported clade with *H. crenacauda* and an unidentified species of the genus; and the Iranian population of *H. dihystera* fell into a clade including other isolates of the species. The variations of D2-D3 and ITS sequences of *Helicotylenchus dihystera* in comparisons with other available sequences of the species were discussed. *H. abunaamai* was molecularly characterised for the first time and this is the first molecular study of an Iranian population of *H. dihystera*.

Key words: Bayesian inference, D2-D3 expansion segments of LSU rDNA, ITS rDNA, morphometric data, phylogeny, taxonomy.

Spiral nematodes of the genus *Helicotylenchus* Steiner, 1945 are migratory ecto-, or semi-endoparasites of roots of many plants, and are broadly distributed worldwide. The type species of the genus, *H. dihystera* (Cobb, 1893) Sher, 1961, is regarded as a pest for crop plants (Siddiqi, 2000). Identification of *Helicotylenchus* species based solely on morphology frequently remains uncertain, due to common phenotypic plasticity and/or prevalence of cryptic species (Subbotin *et al.*, 2011; Palomares-Rius *et al.*, 2018). Molecular methods however will help better identification and species delimitations in this specious genus (Subbotin *et al.*, 2011, 2015; Palomares-Rius *et al.*, 2018).

Currently 33 species of the genus *Helicotylnchus* have been reported from Iran up to 2016 (Ghaderi *et al.*, 2018). During a survey on the biodiversity of plant-parasitic nematodes in okra fields of Khuzestan province, southwest Iran, two species of *Helicotylenchus* were recovered. The preliminary morphological studies followed by molecular analyses revealed the recovered populations belong to *H. abunaamai* Siddiqi, 1972 and *H. dihystera*. Thus, the present study aimed to characterise the Iranian populations of the two recovered species using traditional and molecular approaches.

MATERIAL AND METHODS

Nematode sampling. Several soil samples were collected from okra fields in Khuzestan province, southwest Iran. The Jenkins (1964) or the tray (Whitehead & Hemming, 1965) methods were used to extract the nematodes from soil samples. The collected specimens were killed in hot 4% formaldehyde solution, transferred to anhydrous

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glycerin according to De Grisse (1969). Observations and measurements were done using a Leitz SM-LUX light microscope equipped with a drawing tube. Some of the specimens were photographed using an Olympus DP72 digital camera attached to an Olympus BX51 light microscope equipped with differential interference contrast (DIC).

DNA extracting, 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, transferred to 3 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. The suspension was collected by adding 20 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 primer D2A (5'-ACA AGT ACC GTG AGG GAA AGT-3') and reverse primer D3B (5'-TCG GAA GGA ACC AGC TAC TA-3') (Nunn, 1992). Primers for amplification of ITS rDNA were forward primer rDNA1 (5'-TTG ATT ACG TCC CTG CCC TTT-3') and reverse primer rDNA1.58S (5'-ACG AGC CGA GTG ATC CAC CG-3') (Subbotin *et al.*, 2000). The 25 µl PCR mixture contained 14.5 µl of distilled water, 3 µl of 10× PCR buffer, 0.5 µl of 10 mM dNTP mixture, 1.5 µl of 50 mM $MgCl_2$, 1 µl of each primer (10 pmol μl^{-1}), 0.5 µl of *Taq* DNA polymerase (Cinna Gen, Tehran, Iran; 5 U μl^{-1}), and 3 µl of DNA template. The thermal cycling program for amplification of both loci was as follows: denaturation at 95°C for 4 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 52°C for 40 s, and extension at 72°C for 80 s. A final extension was performed at 72°C for 10 min. Amplification success was evaluated by electrophoresis on 1% agarose gel. The PCR products were purified using the QIAquick PCR purification kit (Qiagen®) following the manufacturer's protocol and sequenced directly using the PCR primers with an ABI 3730XL sequencer (Bioneer Corporation, South Korea). The newly obtained sequences of the studied species were deposited into the GenBank database (accession numbers MW481640-MW481642 for LSU D2-D3 and MW4880336-MW488039 for ITS rDNA).

Phylogenetic analyses. The newly obtained sequences of the D2-D3 fragments of LSU rDNA and ITS rDNA and additional sequences of relevant species were selected after a BlastN search. The sequences were aligned by Clustal X2 (http://www.clustal.org/) using the default parameters. The outgroup taxa were chosen according to previous studies (Subbotin *et al.*, 2015; Mwamula *et al.*, 2020). The base substitution model was selected using MrModeltest 2 (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 both (the LSU and ITS) phylogenies. The Bayesian analyses were performed to infer the phylogenetic trees using MrBayes v3.1.2 (Ronquist & Huelsenbeck, 2003), running the chains for two 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 the 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 files of the trees were visualised using Dendroscope V. 3.2.8 (Huson & Scornavacca, 2012) and drawn in CorelDRAW software version 17.

DESCRIPTION

Helicotylenchus abunaamai
Iranian population
(Figs 1 & 2)

Measurements. See Table 1.

Female. Body spirally curved. Cuticle annuli 1.0-1.9 µm wide at mid-body. Lip region hemispherical, continuous with body contour, bearing 4-5 annuli, 3.3-4.5 µm high and 6.2-7.2 µm wide. Outer margins of cephalic framework extending backward for two to three body annuli. Lateral field with four smooth incisures, 3.5-5 µm wide, about 20-25% of body diameter, the two inner lines end on tail in a Y-shaped pattern. Stylet knobs rounded, with flattened to slightly concave anterior surfaces, 4-6 µm across. Median bulb oval, 12.3-14.5 µm long and 8.5-12.5 µm wide, the ventral pharyngeal gland overlaps intestine. Hemizonid two to three body annuli long, one or two annuli anterior to excretory pore. Reproductive system didelphic-amphidelphic, ovary with oocytes arranged in a single row, spermatheca slightly dorsally offset, small, empty, vulva a transverse slit. Tail dorsally more or less concave, nearly ventrally straight to slightly convex, with a small projection, smooth in tip. Phasmids two to five annuli anterior to anus.

Male. Not found.

Remarks. The general morphology of the recovered population of the species fits that illustrated for the type population by Siddiqi (1972).

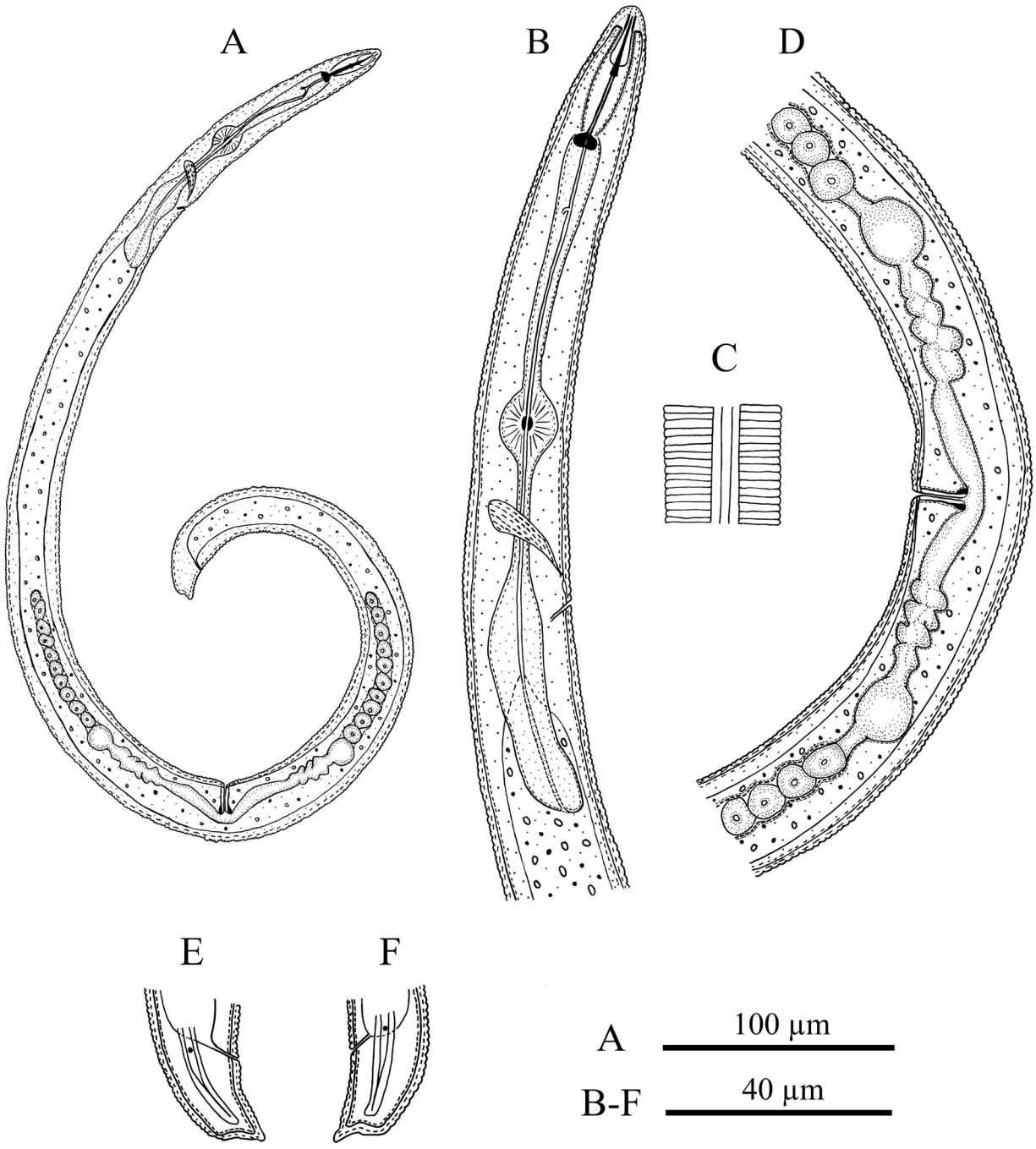

Fig. 1. Line drawings of *Helicotylenchus abunaamai* Siddiqi, 1972 from Khuzestan province, Iran. A: Entire body; B: Pharynx; C: Lateral field at mid-body; D: Part of reproductive system; E & F: Tail.

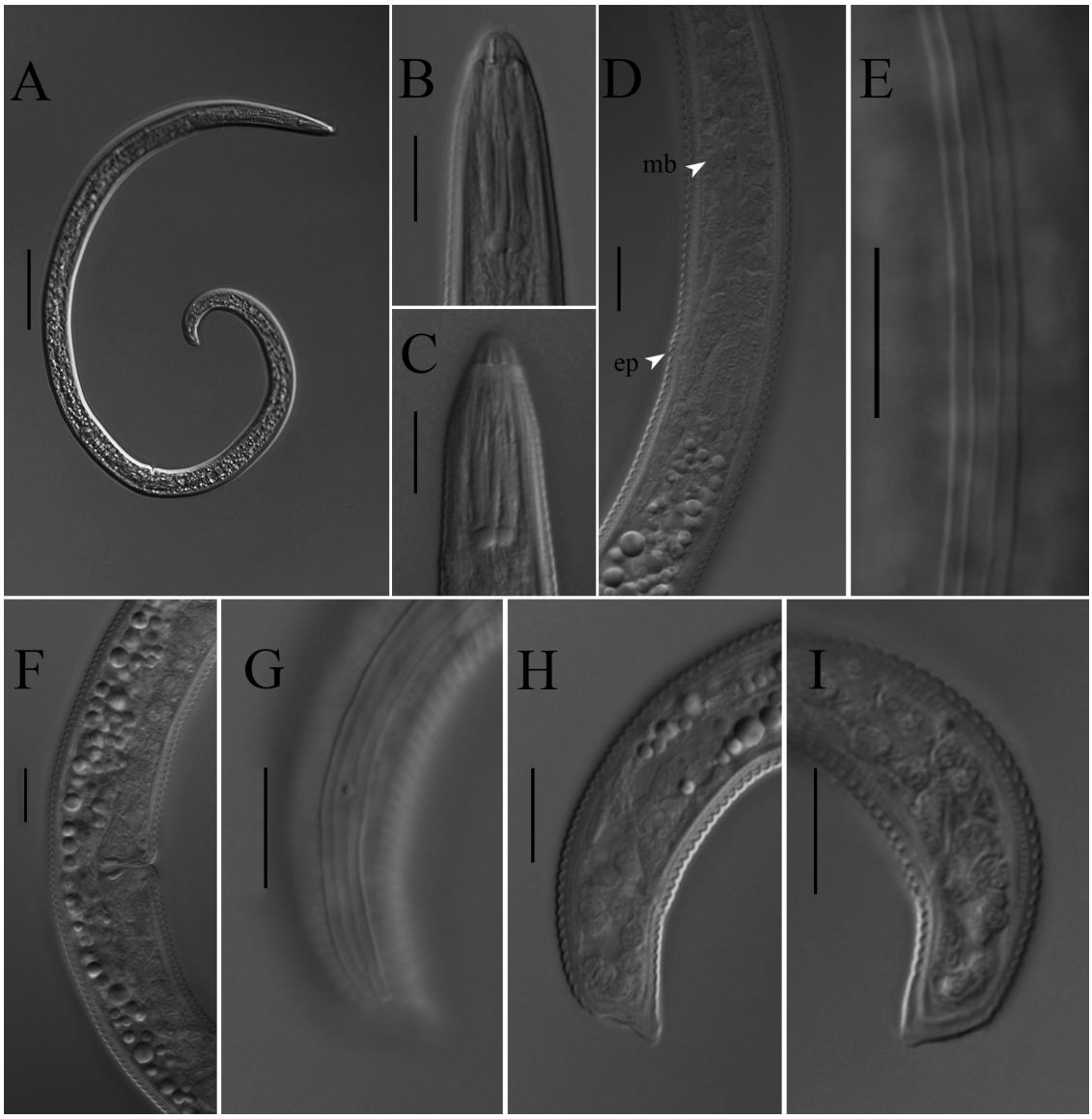

Fig. 2. Light photomicrographs of *Helicotylenchus abunaamai* Siddiqi, 1972 from Khuzestan province, Iran. A: Entire body; B & C: Anterior body region; D: Part of pharyngeal region (arrows showing position of median bulb and excretory pore); E: Lateral field at mid-body; F: Vulval region; G: Lateral field at tail and phasmid; H & I: Tail. Scale bars: A = 50 µm, B-I = 10 µm.

However, the stylet is slightly longer compared to that of the type population (22.5-25.3 *vs* 21-22 µm). Compared to the population reported by Sauer & Winoto (1975), it has higher c ratio (41.1-53.3 *vs* 31-40). From the population reported by Firoza & Maqbool (1991), it has a slightly longer body (612-691 *vs* 480-600 µm). Compared to the population reported by Kepenekci (2002), it has a slightly longer stylet (22.5-25.3 *vs* 20-22 µm). From the population reported by Kashi & Karegar (2014), it has a higher c ratio (41.1-53.3 *vs* 24.2-33.7), lower c' ratio (0.8-1.3 *vs* 1.4-1.9) and shorter tail (11.7-16.8 *vs* 20.0-29.1 µm) and compared to the population reported by Eisvand *et al.* (2019), it has a slightly longer body (612-691 *vs* 515-611 µm) and stylet (22.5-25.3 *vs* 18.0-21.5 µm).

Variation in tail morphology of the species has been already observed among populations of the species. A ventrally convex and dorsally concave (Siddiqi, 1972), dorsally curved and ventrally almost straight or variously shape from trapezoid to conical (Kashi & Karegar, 2014), generally dorsally less convex and ventrally nearly straight as well as dorsally and ventrally tapering (Mizukubo *et al.*, 1993), have been observed. Fine striations in the dorsal section of the tail have been seen in some populations too.

Table 1. Morphometrics of females of *Helicotylenchus abunaamai* Siddiqi, 1972 and *Helicotylenchus dihystera* (Cobb, 1893) Sher, 1961 from Khuzestan province, Iran. All measurements are in µm and in the form: mean ± s.d. (range).

Character	*Helicotylenchus abunaamai*	*Helicotylenchus dihystera*
n	10	10
L	646.2 ± 21.4 (612-691)	666.9 ± 87.6 (526-764)
a	30.2 ± 3.1 (25.8-37.2)	28.1 ± 2.1 (25.2-30.7)
b	6.8 ± 0.6 (5.8-7.6)	5.4 ± 2.1 (4.2-6.2)
b’	4.5 ± 0.2 (4.2-4.9)	4.3 ± 0.5 (3.6-5.1)
c	50.8 ± 4.5 (41.1-53.3)	38.9 ± 3.2 (33.7-44.0)
c’	1.1 ± 0.2 (0.8-1.3)	1.2 ± 0.1 (1.0-1.3)
V	62.1 ± 1.8 (59.1-65.2)	64.1 ± 2.6 (60.5-68.5)
Stylet length	24.0 ± 0.9 (22.5-25.3)	27.0 ± 0.8 (25.7-28)
Conus length	11.0 ± 0.5 (10.0-11.7)	12.7 ± 0.5 (12.1-13.7)
m	45.8 ± 1.2 (43.9-47.6)	47.1 ± 1.1 (45.8-48.9)
DGO	11.0 ± 0.5 (10.0-11.6)	14.1 ± 1.3 (13.0-16.3)
O	46.2 ± 1.7 (43.9-48.7)	52.4 ± 3.7 (48.7-58.2)
Pharynx length	99.8 ± 9.1 (91-119)	124.8 ± 12.7 (107.3-143.0)
Pharyngeal glands	143.3 ± 6.3 (136.5-154.7)	156.4 ± 8.5 (143-167)
Excretory pore from anterior end	105.1 ± 4.3 (99.5-111.8)	119.9 ± 9.5 (104.0-133.3)
MB	72.8 ± 3.1 (65.7-76.6)	83.6 ± 0.5 (74.1-89.7)
Body width	22.0 ± 2.2 (17.6-25.0)	23.8 ± 2.4 (19.5-26.0)
Lip region-vulva	402.3 ± 11.4 (386-424)	426.2 ± 49.6 (343-475)
Vulval body width	21.4 ± 2.1 (17.6-24.7)	22.9 ± 2 (19.5-26.0)
Anal body width	12.3 ± 1.1 (11.1-14.3)	14.9 ± 1.5 (12.4-16.9)
Vulva-anus distance	230.4 ± 15.9 (207-255)	222.9 ± 40.4 (153-270)
Tail length	14.0 ± 2.0 (11.7-16.8)	17.3 ± 2.8 (13.0-20.8)
Tail annuli	8.6 ± 1.8 (6-11)	14.9 ± 1.5 (11-17)
Phasmids from tail terminus	17.4 ± 1.8 (15.6-20.0)	23.2 ± 3.4 (17.6-26.0)

Helicotylenchus abunaamai has already been reported in association with sugarcane (Kashi & Karegar, 2014), citrus, sour orange, lemon and tangerine (Eisvand *et al.*, 2019), in Khuzestan province, southwest Iran. In the present study, it was recovered from the rhizospheric soil of okra in the vicinity of Karun (GPS coordinates: 31°13’48.9” N, 48°38’1.0” E) city, Khuzestan province, southwest Iran.

Helicotylenchus dihystera
Iranian population
(Figs 3 & 4)

Measurements. See Table 1.

Female. Body spirally curved. Cuticle annuli 1.3-2 µm wide at mid-body. Lip region hemispherical, not offset, bearing 5-6 annuli, 3.2-4.2 µm high and 5.9-7.8 µm wide. Outer margins of cephalic framework extending back about three body annuli long. Lateral field with four incisures, crenate at beginning, smooth towards the tail, 4-5 µm wide, about 20-25% of body diameter, inner two lines end on tail in a V-, or Y-shaped pattern. Stylet knobs rounded, anteriorly indented, 4.5-6.3 µm across. Median bulb oval, 12.5-15.5 µm long and 8.5-12 µm wide, pharyngeal glands with longer ventral overlap. Hemizonid three body annuli long, one to two annuli anterior to excretory pore. Reproductive system didelphic-amphidelphic, ovary with oocytes arranged in one row, spermatheca non-offset, large, empty, vulva a transverse slit. Tail subcylindrical, dorsally convex, ventrally almost straight, without projection, with a rounded terminus. Phasmids distinct, located four to seven annuli anterior to anal level.

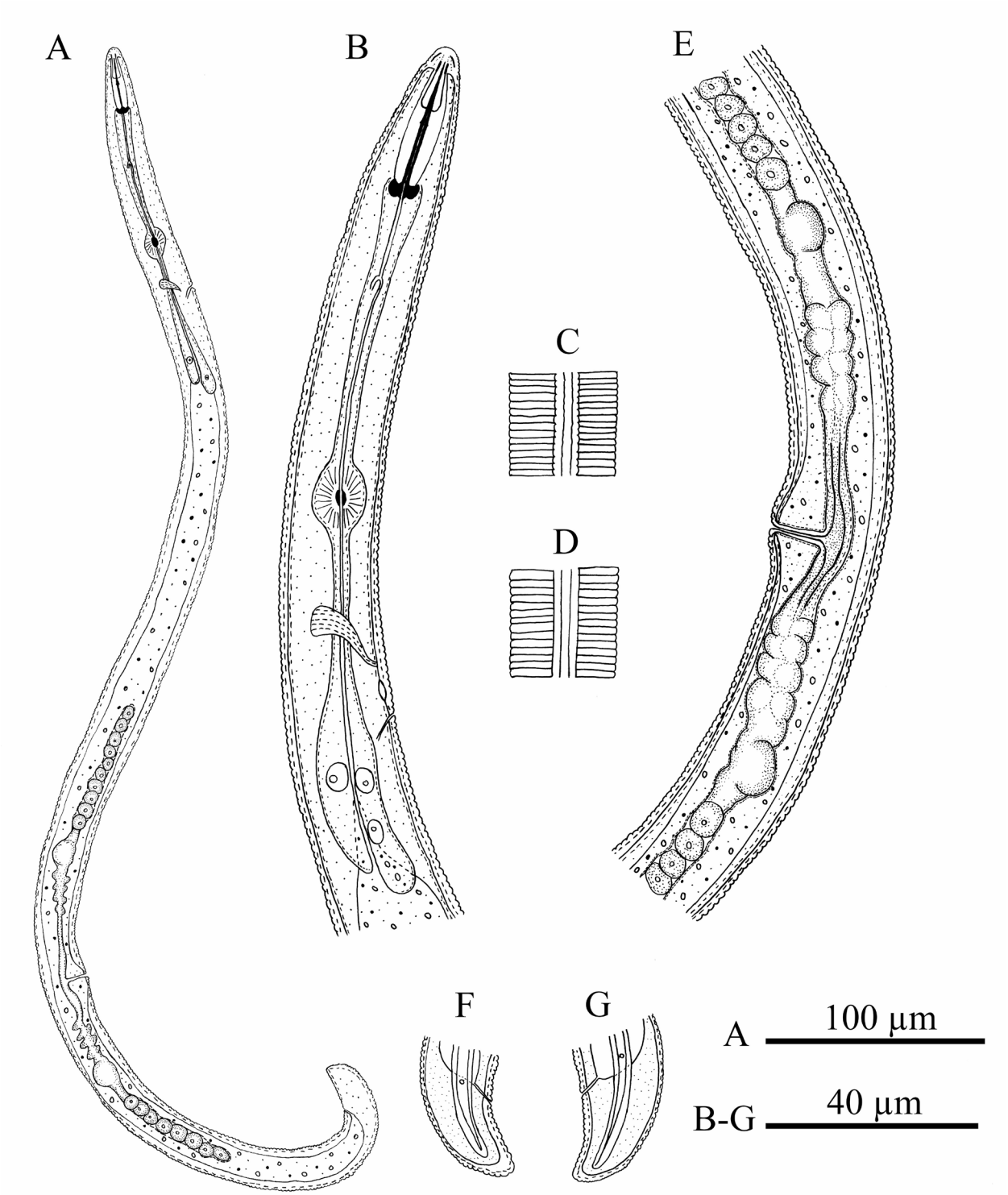

Fig. 3. Line drawings of *Helicotylenchus dihystera* (Cobb, 1893) Sher, 1961 from Khuzestan province, Iran. A: Entire body; B: Pharynx; C: Lateral field at pharyngeal region; D: Lateral field at mid-body; E: Part of reproductive system; F & G: Tail.

Male. Not found.

Remarks. The general morphology of the recovered population of the species closely resembles that of the type, and other populations of the species (Sher, 1961, 1966; Sauer & Winoto, 1975; Marais, 2001; Marais *et al.*, 2005).

Helicotylenchus dihystera has been reported from various plants in different regions of Iran, but no full description, illustration and molecular data were provided for the reported populations. Thus, the current study supports the prevalence of *H. dihystera* in Iran based on the both morphological and molecular data. The presently studied population was recovered from the rhizosphere of okra in the vicinity of Masjed Soleyman city (GPS coordinates: 31°57'57.9" N, 49°17'25.4" E), Khuzestan province, southwest Iran.

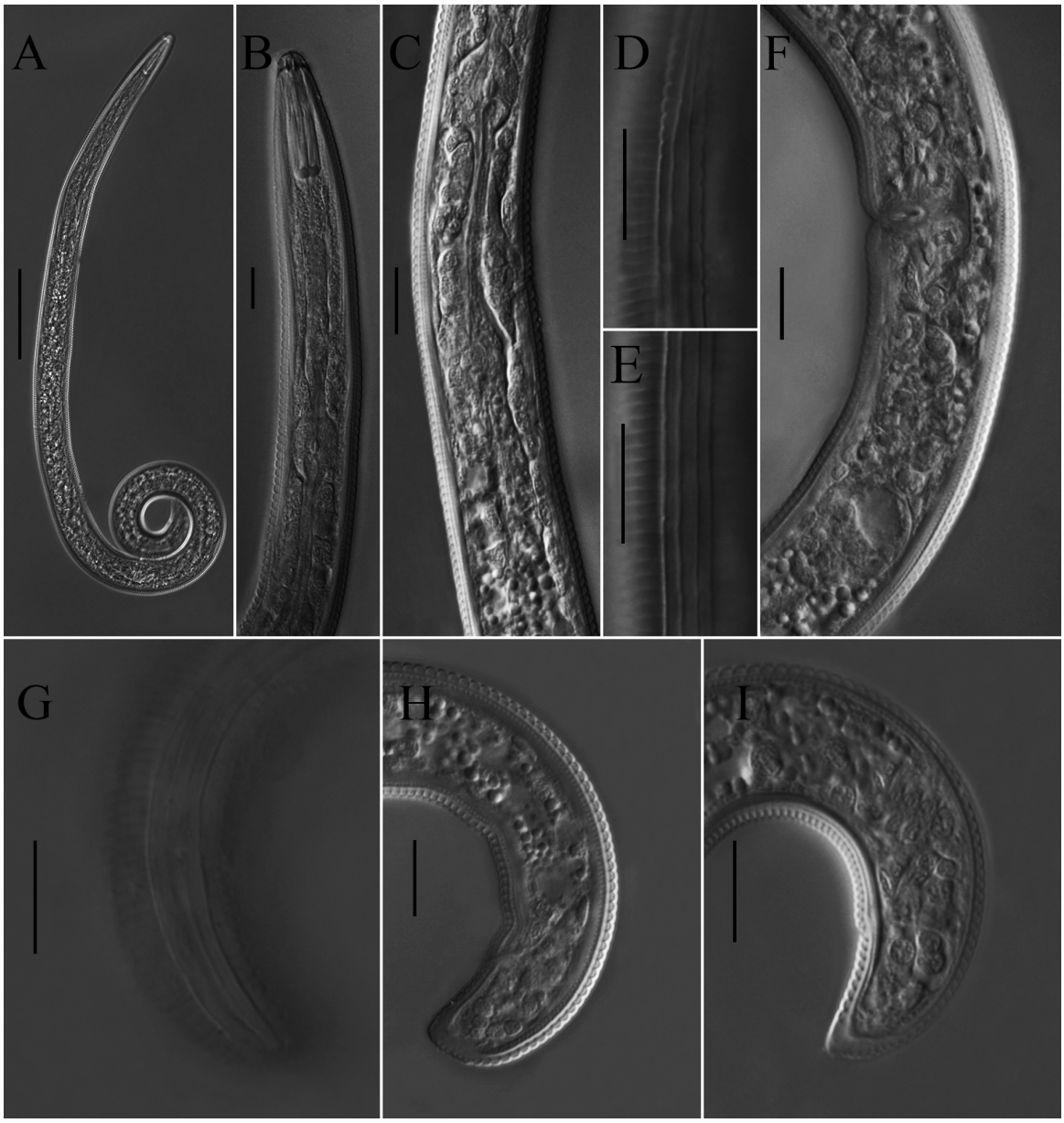

Fig. 4. Light photomicrographs of *Helicotylenchus dihystera* (Cobb, 1893) Sher, 1961 from Khuzestan province, Iran. A: Entire body; B: Anterior part of pharynx; C: Posterior part of pharynx; D: Lateral field at pharyngeal region; E: Lateral field at mid-body; F: Vulval region; G: Lateral field at tail and phasmid; H & I: Tail. Scale bars: A = 50 µm, B-I = 10 µm.

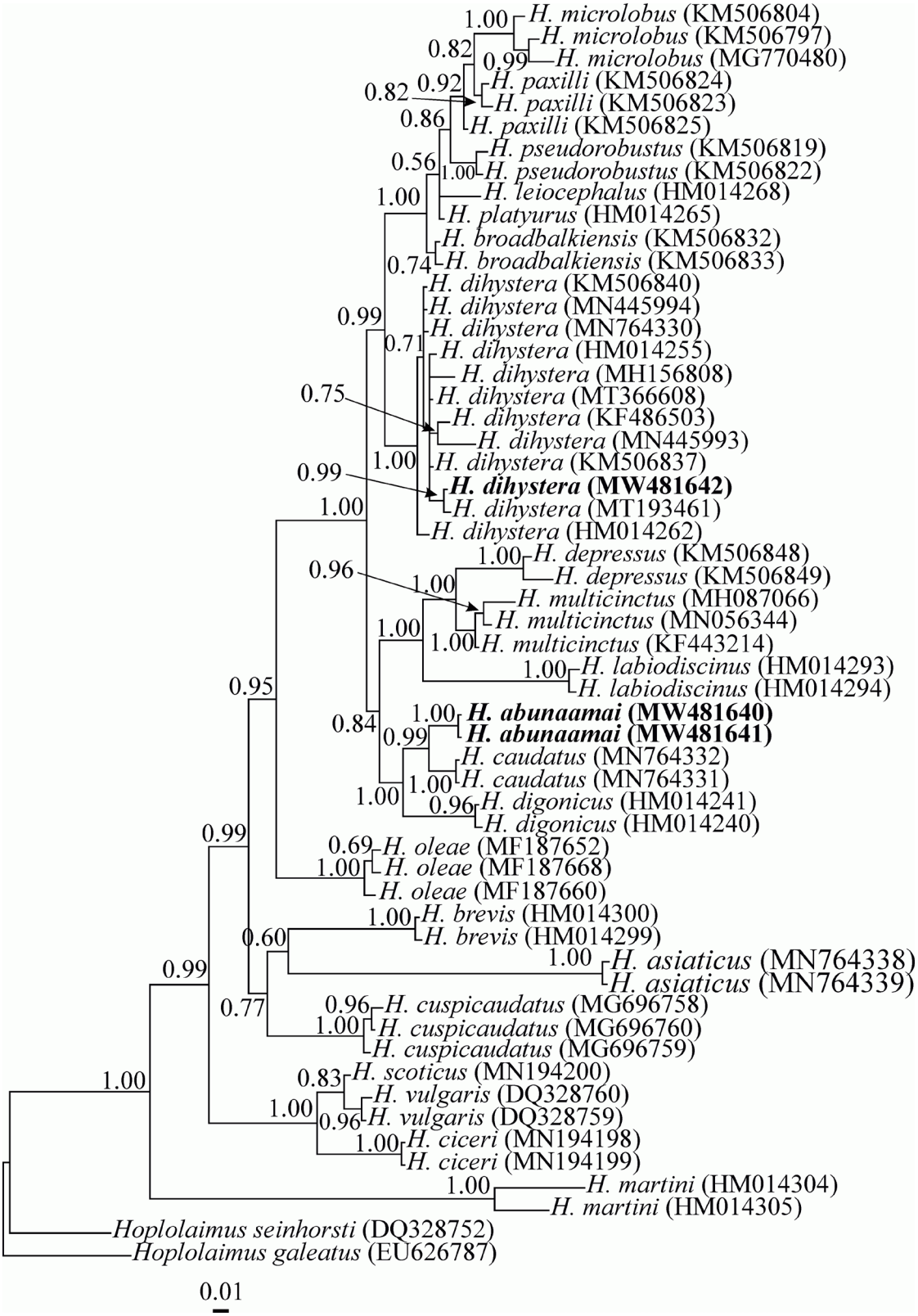

Fig. 5. Bayesian 50% majority rule consensus tree inferred using D2-D3 sequences of the LSU rDNA of *Helicotylenchus abunaamai* and *H. dihystera* under the GTR + G + I model. Bayesian posterior probability values more than 0.50 are given for appropriate clades. Newly generated sequences are indicated in bold.

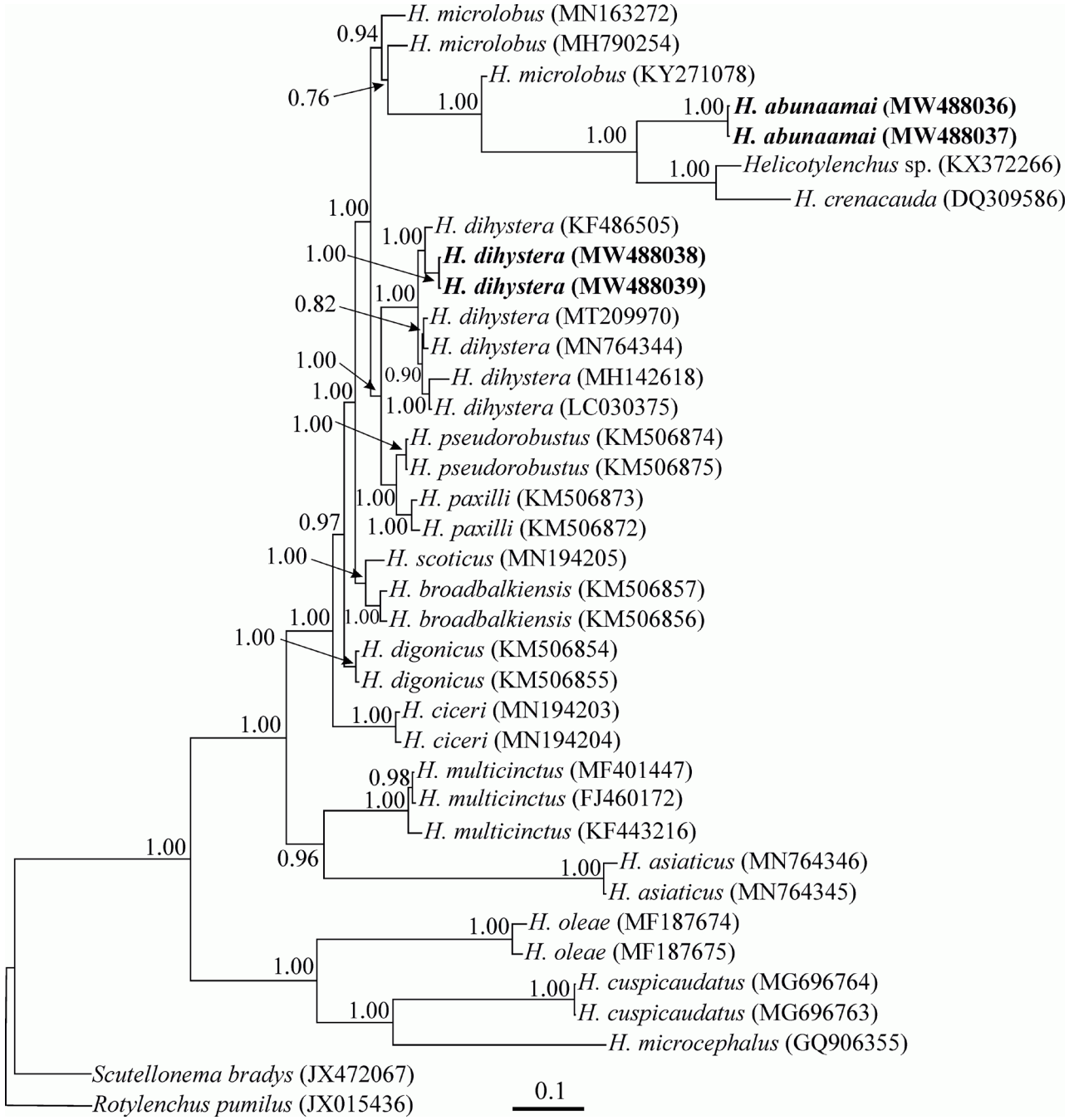

Fig. 6. Bayesian 50% majority rule consensus tree inferred using ITS rDNA sequences of *Helicotylenchus abunaamai* and *H. dihystera* under the GTR + G + I model. Bayesian posterior probability values more than 0.50 are given for appropriate clades. Newly generated sequences are indicated in bold.

Molecular characterisation and phylogenetic relationships. Two identically aligned sequences for the D2-D3 expansion segments of LSU (MW481640, MW481641) were obtained for *H. abunaamai*. The BLAST search using these sequences revealed they have 94.85% and 95.05% identity with an unidentified species of the genus (DQ077794). One LSU sequence (MW481642) was obtained for *H. dihystera*, showing a 99.55% identity with other sequence of this species (*e.g.*, MT193461). The included sequences of this species in LSU phylogeny had a 0.8-1.0% intraspecies variation.

A total of 56 sequences of *Helicotylenchus* spp. and two sequences of *Hoplolaimus seinhorsti* Luc, 1958 and *H. galeatus* (Cobb, 1913) Thorne, 1935 as outgroup taxa (DQ328752 and EU626787,

respectively), were selected for the LSU phylogeny. This dataset comprised 755 total characters. The phylogenetic tree inferred using this dataset is presented in Figure 5. In this tree, the newly generated sequences of the Iranian population of *H. abunaamai* formed a maximally supported clade with *H. caudatus* Sultan, 1985 and *H. digonicus* Perry, in Perry, Darling & Thorne, 1959. The newly generated sequence of Iranian population of *H. dihystera* formed a maximally supported clade with other sequences of the species.

Amplification and sequencing of the ITS rDNA of the Iranian population of *H. abunaamai* yielded two identically aligned fragments. The BLAST search using these sequences (MW488036, MW488037), revealed they have 78.60% and 78.63% identity with an unidentified species of the genus (KX372266). Two almost identical ITS sequences (MW488038, MW488039) (five degenerated nucleotides were observed) were obtained for the studied population of *H. dihystera*, which showed 97.97% and 98.34% identity with another sequence of the species (KF486505). The included sequences of the species in ITS phylogeny had a 0.0-5.0% genetic distance.

A total of 37 sequences of *Helicotylenchus* spp. and two sequences of *Scutellonema bradys* (Steiner & LeHew, 1933) Andrássy, 1958 and *Rotylenchus pumilus* Perry in Perry, Darling & Thorne, 1959 as outgroup taxa (JX472067, JX015436), were selected for ITS phylogeny. This dataset comprised 1448 total characters. The phylogenetic tree inferred using this dataset is presented in Figure 6. The sequences of the Iranian population of *H. abunaamai* formed a maximally supported clade with *H. crenacauda* Sher, 1966 and an unidentified species of the genus (KX372266). The sequences of the Iranian population of *H. dihystera* formed a maximally supported clade with other sequences of the species.

DISCUSSION

The objectives of this study were the morphological and molecular characterisation of the Iranian populations of *H. abunaamai* and *H. dihystera*. The previous studies on these species occurring in Iran have been solely based on traditional methods (Ghaderi *et al.*, 2018). In the present study, Iranian populations of *H. abunaamai* and *H. dihystera* were molecularly characterised for the first time. Currently, the molecular data of the genus *Helicotylenchus* are poor. The molecular data of the type species, or other populations of the known species will further shed light on the genetic structure of its species. As already showed in this paper, intraspecies genetic diversity between several isolates of the studied species were observed for both the LSU and ITS marker.

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N. Pour Ehtesham, S. Azimi and M. Pedram. Первая молекулярная характеристика *Helicotylenchus abunaamai* Siddiqi, 1972 и *H. dihystera* (Cobb, 1893) Sher, 1961 (Tylenchomorpha: Hoplolaimidae) из Ирана.

Резюме. В ходе изучения биологического разнообразия фитопаразитических нематод полей бамии в провинции Хузестан (юго-западный Иран) были найдены два вида геликотиленхов: *Helicotylenchus abunaamai* и *H. dihystera*. Приводится описание их морфологии с морфометрическими данными и обсуждаются отличия от других популяций этих видов, в том числе, типовой. По мнению авторов, это первое сообщение об этих двух видах на полях бамии. С использованием Байесова анализа проведен филогенетический анализ последовательностей D2-D3 сегмента большой субъединицы (LSU rDNA) и внутренних транскрибируемых спейсеров (ITS rDNA) рибосомальных повторов. Иранская популяция *H. abunaamai* по результатам анализа D2-D3 сегмента объединялась с максимальной поддержкой с *H. caudatus* и *H. digonicus*. Иранская популяция *H. dihystera* по результатам анализа этой последовательности попадала в кладу, объединяющую несколько изолятов этого вида. В филогении, построенной по результатам анализа ITS-участка, иранская популяция *H. abunaamai* образовывала кладу с максимальной поддержкой, включающую *H. crenacauda* и еще один неопределенный вид рода, а иранская популяция *H. dihystera* образовывала кладу с другими изолятами данного вида. Обсуждается вариабельность последовательностей D2-D3 и ITS участков *Helicotylenchus dihystera* в сравнении с другими имеющимися последовательностями этого вида. Данные по нуклеотидным последовательностям *H. abunaamai* получены впервые, тогда как для *H. dihystera* получены первые нуклеотидные данные для особей из Ирана.