RESEARCH/ INVESTIGACIÓN

CHARACTERIZATION OF A POPULATION OF *HELICOTYLENCHUS DIHYSTERA* (COBB, 1893) SHER, 1961, PARASITIZING MAIZE ROOTS, IN SOUTHERN CÓRDOBA, ARGENTINA

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ABSTRACT

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Helicotylenchus are root parasites of cultivated and noncultivated plants. Species identification can be difficult because the nematode shares several diagnostic characters and exhibits an important interspecific variability. Species recognition is essential for planning appropriate control strategies or preventing the spread of these nematodes to other areas. In this work, morphological and morphometric characters from a single population were evaluated. Molecular analysis of D2-D3 of the 28s-rDNA and the 5.8s-ITS2 regions by PCR generated two new sequences that were deposited in Genbank. In addition, identification of the species was supported by phylogenetic analysis using Bayesian inference. This work is the first morphological, morphometric, and molecular characterization of a *H. dihystera* population from Argentina.

Key words: 28s-LSU, 5.8s-ITS2, Argentina, *Helicotylenchus dihystera*, maize, morphology, morphometric

RESUMEN

Brücher, E., E. E. Vuletic, F. A. Guerra, R. L. De Rossi, M. C. Plazas, G. D. Guerra, M. G. Molina, M. M. Gabriela, and M. E. Doucet. 2019. Caracterización de una población de *Helicotylenchus dihystera* (Cobb, 1893) Sher, 1961, parásitas de raíces de maíz en el sur de la provincia de Córdoba, Argentina. Nematropica 49:49-58.

Los nematodos en espiral del género *Helicotylenchus,* son parásitos de raíz de numerosas plantas cultivadas y no cultivadas. La identificación específica puede resultar compleja porque comparten diferentes caracteres diagnósticos similares y muestran una considerable variabilidad interespecífica. Su reconocimiento resulta esencial para poder establecer estrategias apropiadas de control o para prevenir su propagación a otras áreas. En este trabajo se evaluaron caracteres morfológicos y caracteres morfométricos de una población relacionada con el cultivo de maíz. Al mismo tiempo, análisis moleculares por PCR de una región del D2-D3 del gen 28s y la región 5.8s-ITS2 permitieron generar dos nuevas secuencias que fueron depositadas en Genbank. Además, por análisis filogenéticos utilizando inferencia Bayesiana, se

complementó la identificación de la especie. Este trabajo constituye la primera caracterización morfológica, morfométrica y molecular de una población de *Helicotylenchus dihystera* para Argentina.

Palabras claves: 28s-LSU, 5.8s-ITS2, Argentina, *Helicotylenchus dihystera*, maíz, morfología, morfometría

INTRODUCTION

Helicotylenchus Steiner, 1945, is a genus of plant-parasitic nematodes that comprises more than 200 recognized species (Marais, 2001; Uzma *et al.*, 2015) occurring in a variety of temperate and tropical environments (Anderson, 1974; Siddiqi, 2000; Marais, 2001). In general, species of *Helicotylenchus* are considered migratory ectoparasites that complete their cycle in the soil, where they feed on cortical parenchyma of the colonized roots. However, some species have been described as semi-endoparasites because they penetrate plant tissues with the anterior body region (Wouts and Yeates, 1994; Guzmán-Piedrahita, 2011) or endoparasites, with the entire body penetrating the host tissue (Siddiqi, 1973; Orbin, 1973). The true impact of *Helicotylenchus* on agriculture in Argentina remains unknown (Coronel and Devani, 2013; Doucet, *et al.*, 2015). Over the years, an increasing number of cases have been detected documenting the wide distribution of the genus in Argentina and its wide host range (Doucet, 1999; Doucet *et al.*, 2015). The genus has been reported to attack a large number of crops including several fruit trees, vegetable crops, maize and soybean. The species *H. dihystera* (Cobb, 1893) Sher, 1961; *H. digonicus* Perry, in Perri, Darling and Thorne, 1959; *H. multicinctus* (Cobb, 1893), Golden, 1956; and *H. nannus* Steiner, 1945 have been detected in the provinces of Salta, Jujuy, Tucumán, Entre Ríos, and Buenos Aires (Doucet, 1999).

Identification of a nematode species based only on morphological and morphometric characters is often a difficult task. Several reports have indicated a considerable interspecific variability within the genus *Helicotylenchus* and the difficulty involved in making reliable species recognition (Fortuner, 1979; Fortuner *et al.*, 1981; Fortuner, 1984; Marais, 2001; Subbotin *et al.,* 2011). Molecular diagnostic provides a quick and much efficient identification of a species, however, several reports indicated the existence of genetic variability within a nematode species (Arias *et al.*, 2009; Leach *et al.,* 2012; Subbotin *et al.* 2015; Khanal *et al.*, 2016; Palomares-Ruis *et al.*, 2017). In the last 8 years, the population densities of *Helicotylenchus sp.* in diverse areas of Argentina has increased from 10-30 to 1300-2000 specimens per 100 grams of soil (Doucet *et al.*, 2015). The evaluations of morphological and morphometric characters of a population detected in soil and roots of a maize crop suggested the presence of *H. dihystera*, species which had already been identified in Argentina only in soils cultivated with sugarcane, cotton and citrus (Costilla *et al.*, 1976). However, populations of this genus were never characterized in Argentina. The aim of this research was to characterize a population of *Helicotylenchus*.

MATERIALS AND METHODS

Soil and root samples were taken from a maize crop that exhibited patches with plants of reduced size cultivated in Adelia María, Córdoba province, Argentina (33°44'17.4"S 63°44'32.3"W). Healthy maize plants and maize plants showing symptoms of poor growth and chlorotic leaves were selected for the study (Fig. 1A). Plants were uprooted, and the soil surrounding the root at the depth of 30-40 cm and the root system were placed in different polyethylene bags (Fig. 1B and 1C). Nematodes were separated from the soil using density centrifugation (Jenkins, 1964), after being subjected to sedimentation (Dalmasso, 1966).

The roots were gently washed with running water to remove adhering soil particles and cut into 0.5-1 cm long segments, and nematodes present in the tissues were released using the method of Whitehead and Hemming (1965). Twenty-five female nematodes were fixed in hot 4:1 FA solution, dehydrated, cleared, and subsequently mounted on pure anhydrous glycerin on glass slides (Seinhorst, 1962). Both visual recognition and measurement of morphometric characters were performed with a microscope supported with the software AxioVision LE (Carl Zeiss). The frequency of appearance of the different

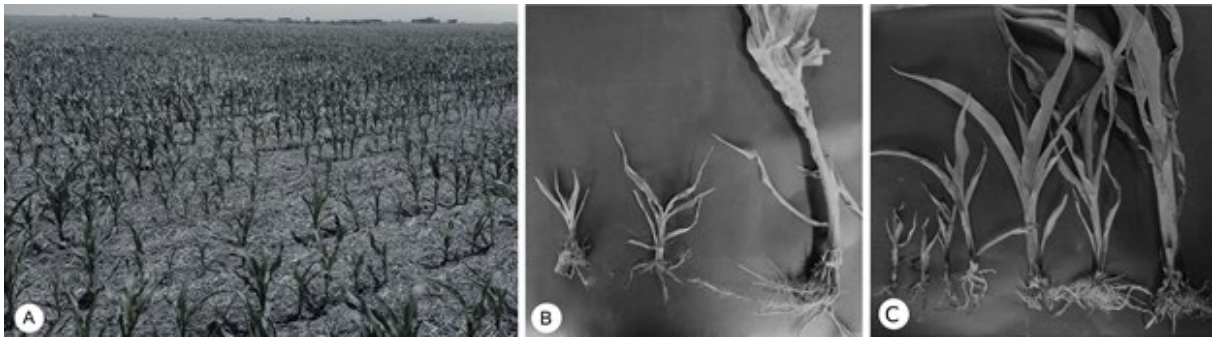

Figure 1. A: Maize seedlings at the time of sampling. B: Left: Maize in affected patches. Right: Plant showing normal development. C: Plants at different development stages. Right: normal plant; left: plants collected from affected patches.

morphological characters in the population was quantified. The following morphometric characters values were considered: minimum, maximum, range, mean, standard deviation, and coefficient of variation.

For the molecular study of this population, the genomic regions D2-D3 expansion segment of the 28s-rDNA (Subbotin *et al.,* 2007) and the 5.8s-ITS2 (Tanha Maafi *et al.,* 2003) were selected. The following primers were used for each region: for D2-D3 expansion segments of the 28s-rDNA, the D2A forward primer (5′ACA AGT ACC GTG AGG GAA AGT TG 3′) and the D3B reverse primer (5′TCG GAA GGA ACC AGC TAC TA 3′), and for the 5.8s-ITS2 region, the TW81 forward primer (5′GTT TCC GTA GGT GAA CCT GC 3′) and the AB28 reverse primer (5′ATA TGC TTA AGT TCA GCG GGT 3′) were used. DNA was extracted from a group of 20 females present both in roots and soil, using Proteinase K protocol (Tanha Maafi *et al.*, 2003). Each polymerase chain reaction (PCR) amplification was designed for a final volume of 25 µl and a MultiGene Labnet International Inc. thermocycler. The reaction mixture contained 5 µl 5X Green GoTaq® Reaction Buffer, 2.5 µl 200 µM dNTPs, 0.125 µl 1 unit of GoTaq® DNA Polymerase (Promega) and 2.5 µl 0.5 µM of each oligonucleotide synthesized by IDT (Integrated DNA Technologies, USA) and 4 ng template DNA. The optimum PCR conditions for the ribosomal DNA markers were used. For D2-D3 expansion segments of the 28s-rDNA, an initial denaturation was at 94°C for 4 min followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1.5 min, extension at 72°C for 2 min and final extension at 72°C for 10 min. For the 5.8s-ITS2 region, an initial denaturation was at 94°C for 2 min, followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 59°C for 1 min, extension at 72°C for 1 min, and final extension at 72°C for 10 min. Gel electrophoresis was conducted by preparing 1% p/v agarose gel stained with ethidium bromide. The gel was run at 50 volts for 1 hr. Five microliters of PCR products were visualized under UV light (FirstLight® UV Illuminator, UVP). PCR products that remained after running the gel were purified using a commercial kit (illustra GFX PCR DNA and Gel Band Purification) and submitted to Macrogen, South Korea, for sequencing, then aligned and compared by Blast method for species determination.

For phylogenetic analysis several sequences were downloaded from NCBI (Basic local alignment search tool; https://ncbi.nlm.nih.gov) (Altschul *et al.,* 1990) and incorporated to the database. The database obtained from NCBI and the new sequences obtained in this research were aligned with Muscle v.3.8.31 (Edgar, 2004), using default parameters, and the alignments were checked and processed using Mesquite (Maddison and Maddison, 2017). The data set of sequences was analyzed by Bayesian inference using the GTR+G+I model implemented in MrBayes 3.1.2 (Huelsenbeck and Ronquist, 2001). Intervals of 10,000,000 generations and a sampling of a tree every 1000 generations were selected. The phylogenetic tree was graphically represented using the software FigTree v1.4.3 (Rambaut, 2009).

RESULTS

Morphological characters

Females

After fixing, the body acquired particular spiral shape. In most cases (88%), the body appeared straight from the anterior end to approximately a little more than one third of its length, whereas the remaining two thirds was spiral-shaped (Fig. 2A). In 8% of the species studied, body of nematode was completely spiraled while the remaining 4% were slightly curved, C-shaped (Fig. 2B and 2C). Lip region rounded, separated from the body by a slight constriction, with two or three transverse striate that were

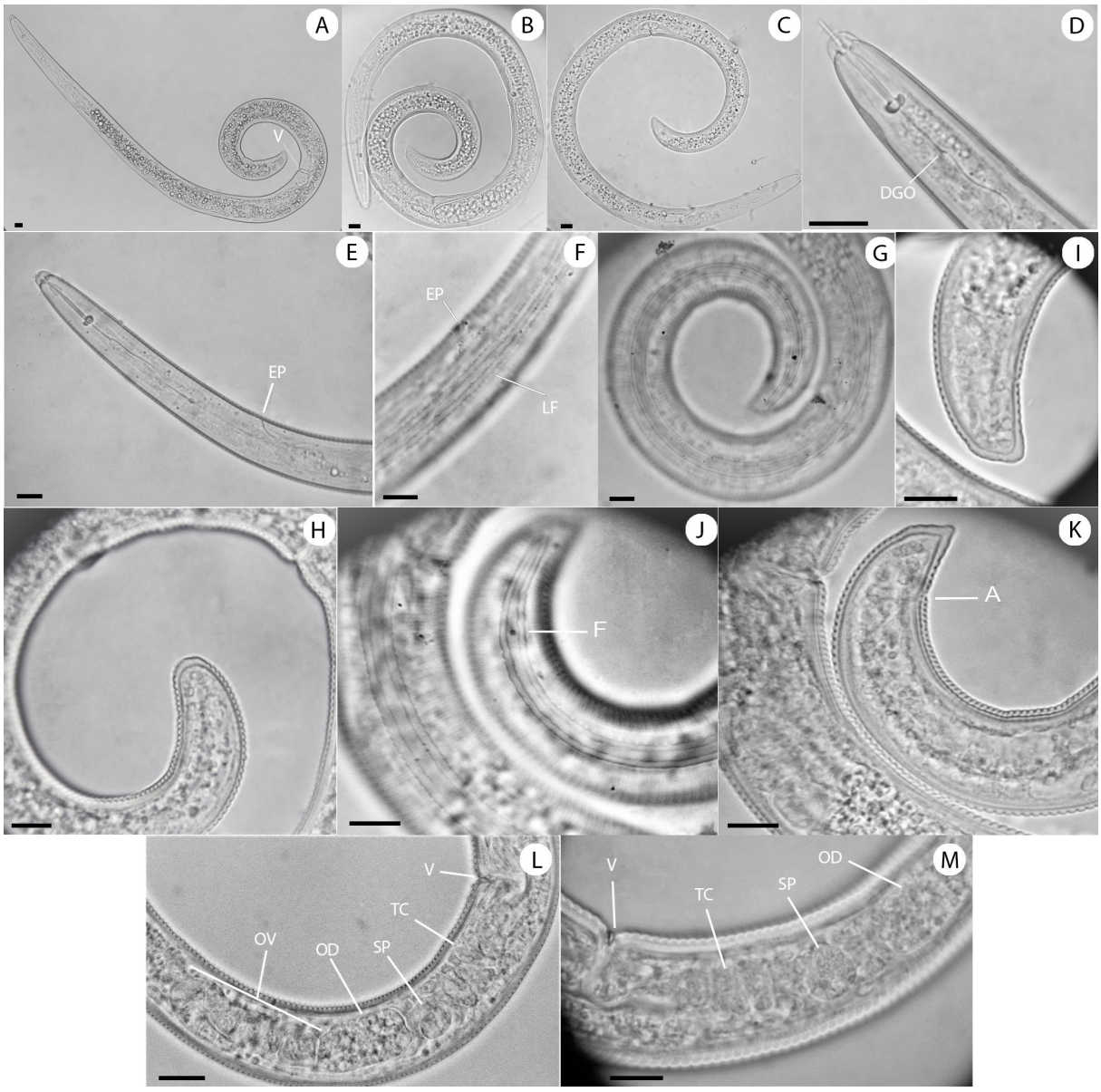

Figure 2. Light microscope images of *Helicotylenchus dihystera*. A-M Females. A-C Posture of body after treatment with lethal temperature. D Anterior region, dorsal gland opening (DGO). E Excretory pore (EP). F, G and J Lateral fields (LF). H, I and K Caudal region. J and K Phasmid and anus position. L and M. Reproductive system of female, ovary (OV), oviduct (OD), spermatheca (SP), tricolumella (TC) and vulva (V). Reference, scale: 10 µm.

difficult to observed in most cases. Stylet well developed, with the external margin of the anterior side of the knobs slightly projected towards the anterior region (Fig. 2D and 2E). Esophageal glands overlapping the intestine ventrally. Reproductive system composed of two symmetric branches. Ovary with a single row of oocytes; oviduct followed by the ovary by a constriction (Fig. 2L and 2M); spermatheca round, with clear borders, empty and slightly displaced from the axial line of the reproductive branch (Fig. 2L). Uterus composed of two parts (Hirschmann and Triantaphyllou, 1968); the distal part, tricolumnela, represented by 12 cells arranged in 3 rows, followed by the proximal part, the uterine lumen and the vagina; the latter was composed of thick cuticle walls that extend to mid body (Fig. 2L). Lateral fields smooth, delimited by four lines along the body, ending variably shaped (V- or Y-shaped) in the caudal region (Fig. 2F, 2G, and 2J). Phasmid 6 to 8 annules (Fig. 2J) anterior to anus (Fig. 2K). Tail dorsally curved in 68% of specimens, with a slight subterminal projection on its ventral side (Fig. 2K), 16% were round and the remaining 16% were slightly truncated (Fig. 2H and 2I). The number of tail annules ranged between 6 and 8.

Males

No males were present.

The set of characters evaluated, the interpretation of taxonomic keys, and the comparison with records in the GenBank confirm that the studied population belongs to *H. dihystera* (Cobb, 1893) Sher, 1961.

Morphometric characters

Table 1 shows 22 morphometric characters obtained from the evaluations of 25 adult females. The mean, standard deviation (±), the maximum and minimum values, and the coefficient of variation were considered.

Molecular characters and phylogenetic analysis

Amplification of the D2-D3 expansion segment of the 28s-rDNA region and 5.8s-ITS2 region yielded products of approximately 800 pb and 1100 pb, respectively. Two new sequences were obtained from this study and were included in GenBank (MG733983.1; MG733982.1). The result of alignments in GenBank (https://www.ncbi.nlm.nih.gov/) allowed to determine that the specimen studied corresponds to *H. dihystera* with 99% accuracy and 100% alignment coverage (Accession number: KF443217.1 and KF486503.1) of the D2-D3 expansion segment of the 28s-rDNA region, and 97% accuracy and 100% alignment coverage (Accession number: DQ309585.1, KM506883.1 and KM506884.1) for the 5.8s-ITS2 region.

Species of the genus *Helicotylenchus* are separated by a few morphological and morphometric characters, making their differentiation difficult. For this reason, a phylogenetic analysis including closely related species was performed. Alignment of the D2-D3 expansion segment of the 28s-rDNA and 5.8s-ITS2 regions consisted of 14 590-bp sequences and of 10 471-bp sequences, respectively, after removal of ambiguous regions from alignment. Figures 3 and 4 show the phylogenetic relationships derived from a Bayesian inference using the GTR + I + G model. The tree topology is congruent, which supports the identification of the isolate as *H. dihystera*, with posterior probabilities higher than 70% providing appropriate clades.

DISCUSSION

The morphological and morphometric description of the studied population coincides with records reported for *H. dihystera.* *T*he population has the characteristics typical of the genus: specimens exhibited a spiral position, esophageal glands covered the intestine ventrally, the orifice of the dorsal esophageal gland was one fourth or more of stylet length and the reproductive system had two symmetric branches (Sher, 1966; Fortuner, 1984; Uzma *et al.,* 2015). At the species level, the characteristics that identified this population are mean body length (686 µm), vulva position (average 63.85%), phasmid located 6-7 annules anterior to anus, inner incisures of lateral fields fused in the tail, the tail tip dorsally curved and with a slight projection in the angle of the ventral side, empty spermatheca, and the absence of males. The morphometric characters with lowest coefficient of variation were vulva (V) (3.17%), excretory pore (4.26%), m (5.67%), and stylet length (6.3%). These values are consistent with

Table 1. Morphometric characters of females of a *Helicotylenchus dihystera* population from Argentina. The values appear as follows: mean, standard deviation (±); maximum and minimum values (in parentheses), and coefficient of variation.

Characters[z]	*Helicotylenchus dihystera* (n= 25)
L	686 ± 62.5 (527-796) 9.11
a	26 ± 3.1 (22.5-32.6) 11.41
b	6.75 ± 0.8 (5.6-8.2) 11.41
b´	4.9 ± 0.5 (4.0-5.9) 9.37
c	41.2 ± 5.2 (34.15-49.2) 12.76
c´	1.2 ± 0.1 (0.8-1.4) 11.37
DGO	11.3 ± 0.9 (10-12.25) 7.65
o	44 ± 4.4 (37.8-54)10.04
m	49 ± 2.8 (45-60) 5.67
V	63.85 ± 2 (61.2-69.2) 3.17
Stylet length	25.6 ± 1.6 (23.7-28) 6.3
Conus length	12.4 ± 1.15 (9.33-15.7) 9.29
Knobs width	4.79 ± 0.39 (3.87-5.56) 8
Knobs height	1.91 ± 0.2 (1.53-2.2) 9.8
Esophagus length	39.4 ± 7.3 (24.12-53.72) 18.54
Distance between the anterior end and the esophagus	107.4 ± 9.97 (85.11-128.2) 9.28
Distance between the anterior end and the esophagus-intestinal junction	146.8 ± 11.4 (122.5-168.3) 7.77
Body width	25.6 ± 3.21 (19.63-31.6) 12.5
Body width at anus level	14.18 ± 1.32 (10.76-16.18) 9.32
Tail length	16.95 ± 2.16 (11.38-21.25) 12.76
Distance between the anterior end and the vulvar opening	436.36 ± 38.51 (341.48-501.15) 8.82
Distance between the anterior end and excretory pore	110 ± 4.69 (105.4-118.6) 4.26

[z]All measurements are in µm, except for o and V that are in %.

those described by Sher (1966), Fortuner *et al.* (1981), and Marais (2001) for *H. dihystera*.

Fortuner and Quénéhervé (1980) conducted an assay with *H. dihystera* in which they evaluated the changes that occurred in the morphometric characters when they parasitized different hosts. Compared with the population of this study, only some characters coincide with those described by the authors for maize. These characters are a, m, DGO and V, where V is one of the most constant characters for the species. Possibly this may be because the population in this work comes directly

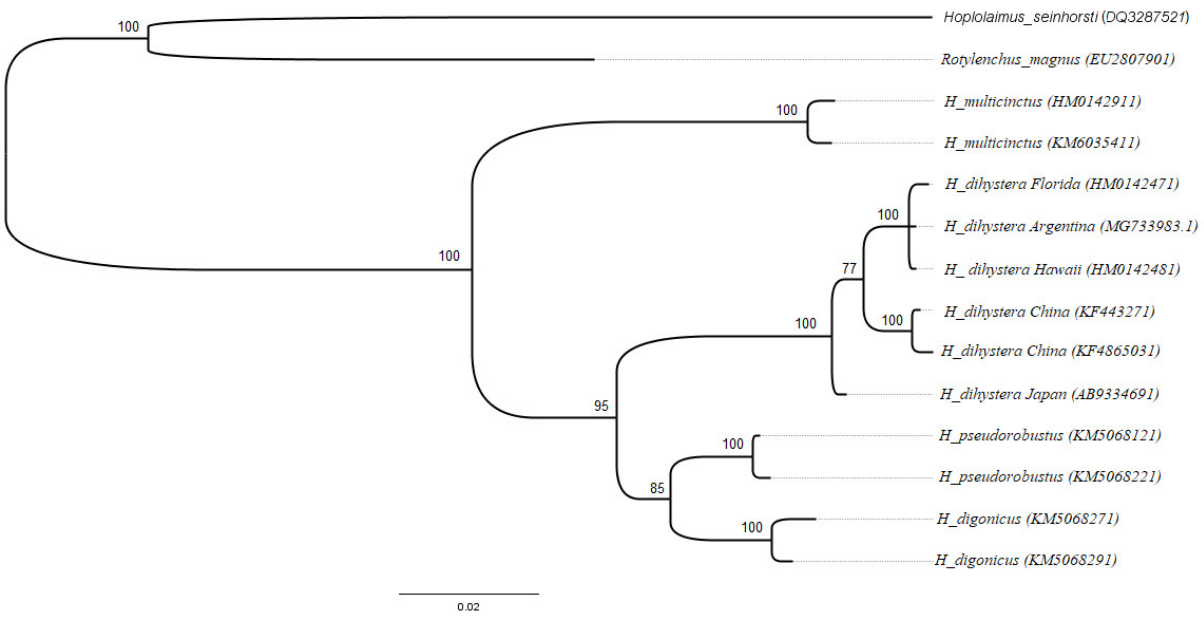

Figure. 3. Phylogenetic relationships of *Helicotylenchus* species. Bayesian 50% majority rule consensus tree from two runs as inferred from analysis of D2-D3 of 28 rDNA gene sequence alignment under GTR+I+G model. Posterior probabilities higher than 70% are given for appropriate clades. The new sequence is in bold.

Figure 4. Phylogenetic relationships of *Helicotylenchus* species. Bayesian 50% majority rule consensus tree from two runs as inferred from analysis of 5.8s-ITS2 rDNA gene sequence alignment under GTR+I+G model. Posterior probabilities higher than 50%. The new sequence is in bold.

from the field, unlike those used by Fortuner and Quénéhervé (1980), who used a population originated from a single female. In contrast, a great similarity of the morphometric characters of this population under study was observed with the populations determined by Marais (2001) in South Africa, Martinique, Guadeloupe, and French Guiana. Possibly these slight variations are due to what Subbotin *et al.* (2015) mentions as geographical variants.

Given the close relationship of *H. dihystera* with other species of the genus, the taxonomic results were complemented with the analysis of the two genomic regions. In addition, the study population was delimited using a phylogenetic method based on Bayesian inference, which demonstrated that it forms a well-defined group that is separated from closely related species. Although the thresholds of divergence were straightforward in the differentiation for the 5.8 ITS region, both phylogenetic analyzes delimit the species closely related to *H. dihystera* and tentatively show an intraspecific geographic delimitation.

This study is the first detailed and comprehensive description of the morphology, morphometry, and sequence and phylogenetic analyses conducted in an *H.dihystera* population from Argentina.

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