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ARTICLE

Morphological and molecular characterization of *Geraldius galapagoensis* (Nematoda: Chambersiellidae) associated with lichens in Argentina

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Abstract. Lichens are symbiotic organisms, usually composed of a fungal partner, the mycobiont, and one or more photosynthetic partners, the photobiont, which is most often either a green alga or a cyanobacterium, that harbor a diverse community of invertebrates such as rotifers, tardigrades, mites, springtails, crustaceans, and nematodes. In this work, we isolated the nematode *Geraldius galapagoensis* (Chambersiellidae) associated with the lichen *Hyperphyscia syncolla* (Physciaceae) in a region of Buenos Aires Province, Argentina. This species was discovered in a tropical forest of Ecuador and is characterized mainly by a head offset by a constriction from the rest of the body, a esophagus with a cylindrical pharyngeal corpus without a median bulb, a narrow isthmus and an oval basal pharyngeal bulb with strong transverse/butterfly valve apparatus, a tail curved ventrally, ending in dorsally hooked end; the male with seven pairs of latero-ventral pre-anal papillae and three pairs of post-anal in the following positions: one pair latero-ventral and two pairs dorso-lateral and two slightly curved spicules with asymmetric manubrium with an anterior extension. The comparison of the morphometrics of our *G. galapagoensis* male with that of the original description shows that the body length is shorter, as are the distance of the excretory pore to the anterior end and the tail. On the other hand, the distance from the anterior end to the nerve ring and the esophagus length are greater. The head width, body diameter and spicule length are quite similar. We provide a morphological and morphometrical characterization of a *G. galapagoensis* second isolate and the first world report of molecular sequences belonging to this species.

Keywords. Nematodes; *Hyperphyscia syncolla;* Pycnidia; Molecular analysis; Eco Area.

INTRODUCTION

Lichens are symbiotic organisms, usually composed of a fungal partner, the mycobiont, and one or more photosynthetic partners, the photobiont, which is most often either a green alga or a cyanobacterium (Nash, 2008). They can tolerate the most extreme environments on Earth, such as hot deserts and arctic regions, and are characterized by low growth rates and nutrient requirements that enable them to play the role of pioneer vegetation in the colonization of fresh rocks (Chapin, 1980; De Vera, 2012; Raggio *et al.,* 2011). In turn, lichens harbor a diverse community of invertebrates, such as rotifers, tardigrades, mites, springtails, crustaceans and nematodes, and are considered micro-ecosystems that include complex food webs (Asplund & Wardle, 2017).

There are some 25,000 described species of nematodes, most of which are free-living, and others are parasites (Ruppert *et al.,* 2004). Many free-living nematodes are detritivores or

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decomposers and play an important role in recycling soil nutrients, whilst parasites are found in plants and animals. These worms inhabit moist interstitial environments in all habitats and are abundant in marine and freshwater benthic habitats and in the soil (Leduc & Zhao, 2023)

Geraldius galapagoensis Cid del Prado, 2012 is a nematode species belonging to the family Chambersiellidae and was discovered in a tropical forest of Ecuador (Cid del Prado, 2012). This species is recognized by the head being separated from the body by a conspicuous constriction, the short isthmus and an oval basal pharyngeal bulb with strong transverse/butterfly valve apparatus, the vulva on a prominent vulvar cone in the gravid female, the male with two slightly curved spicules and conspicuous gubernaculum, seven pairs of latero-ventral pre-anal papillae and three pairs of post-anal in the following positions: one pair latero-ventral and two pairs dorso-lateral, tail curved ventrally, terminuses dorsally hooked and slightly bifurcated (Cid del Prado, 2012). There are two other species in the genus, *Geraldius bakeri* (Sanwal, 1971), collected and described in Canada by Sanwal (1971) and later reported by Holovachov *et al.* (2003) from lichens hanging from a tree, and *Geraldius inserrai* (Cid del Prado-Vera *et al.,* 2021), described by Cid del Prado-Vera *et al.* (2021), collected from lichens and epiphytic plants in Mexico. The former species is characterized by having the head continuous with the rest of the body (lacks a constriction), in the female the vagina opens through a circular vulva placed on top of an elevated vulvar cone, the male with two spicules present not joined, gubernaculum present, and tail curved and ending in dorsally hooked terminus, whilst the latter is characterized by a < 200 µm long on average pharynx, an excretory pore < 150 µm from the anterior end on average, the female with the vulva positioned in an elevated vulvar cone and the male with two spicules, symmetrical, curved ventrally and gubernaculum present (Sanwal, 1971; Cid del Prado-Vera *et al.,* 2021) .

In this work, we report for the first time a population of *G. galapagoensis* associated with the lichen *Hyperphyscia syncolla* (Physciaceae) in Buenos Aires Province, Argentina. We provide a morphological and morphometrical characterization of *G. galapagoensis* and the first molecular sequence of this species.

MATERIAL AND METHODS

Sampling and study area

Lichens were collected in the Eco Area de Avellaneda reserve, Buenos Aires, Argentina (34°39′51.7″S, 58°18′56.3″W). The survey was carried out by ocular inspection on tree logs, a foliose lichen (*Hyperphyscia syncolla*) was selected and removed without stripping the bark with a metal blade, and the material was taken to the laboratory in paper bags. Lichens were processed using a scalpel to separate and open the pycnidia under a NIKON SMZ800N stereomicroscope.

Morphological study

Nematodes were transferred into a test tube with Ringer's solution, incubated in a water bath at 60°C for 2 minutes to instantaneously heat-kill the nematodes, and then fixed in T.A.F. (2% triethanolamine, 7.5% formaldehyde in distilled water) (Hazir *et al.,* 2022). The specimens were mounted on permanent glass slides for observation under light microscopy (LM). The nematodes were measured using an ocular micrometer in a Leica DM 500 microscope according to Cid del Prado-Vera *et al.* (2021). All measurements are provided in micrometers unless otherwise specified and followed by the range in parentheses.

Molecular study and phylogenetics

Identification of the nematodes was confirmed through a molecular approach. Genomic DNA was extracted using 50 µl of a 0.5% suspension of Chelex in deionized water and 2 µl of proteinase K (20 mg/ml), followed by overnight incubation at 56°C, boiling at 100°C for 10 min, and centrifugation at 12,000 g for 1 min. Forty µl of the supernatant were transferred to a new 1.5 ml tube and stored at −20°C until assayed for polymerase chain reaction (PCR). The 5′ end of the lsrDNA gene comprising the D1-D3 variable domains was amplified using forward primer LSU (5′-TAG GTC GAC CCG CTG AAY TTA AGC A-3′) with the reverse primer 1500R (5′-GCT ATC CTG AGG GAA ACT TCG-3′) (Tkach *et al.,* 2003). PCRs were performed with a Mastercycler thermocycler (Eppendorf) in a 50-µl reaction mixture containing 25 µl of PB-L master mix (PB-L Productos Bio-Lógicos, Quilmes, Argentina), 0.4 µM of each forward and reverse primer, and 4 µL of the template DNA, under the following conditions: 94°C for 3 min; 45 cycles of 94°C denaturation for 30 s, annealing at 48°C for 30 s, and extension at 72°C for 1.45 min, a single final extension period at 72°C for 2 min. PCR products were analyzed by electrophoresis on 1% agarose gels and visualized by staining with GelRed® (Biotium). The amplicon was sequenced in Macrogen Inc. (Korea) and edited with the Chromas software (http://technelysium.com.au/wp/chromas). The consensus sequences obtained were compared with sequences in the BLAST tool available in the NCBI database (https://www.ncbi.nlm.nih.gov) and submitted to the National Center for Biotechnology Information (NCBI) GenBank database (https://www.ncbi.nlm.nih.gov) under accession number OQ133527.

For tree inference, the same sequences from Cid del Prado *et al.* (2023) were used. Global multiple alignment was made by using the ClustalO approach (https://www.ebi.ac.uk/Tools/msa/clustalo). The 28S gene sequences of species belonging to the Rhabditida order were selected and a Strongylida *Necator americanus* sequence (KU180694.1) chosen as the outgroup for rooting the tree. The evolutive model (TN+F+G4) was chosen with the IQTree server (http://iqtree.cibiv.univie.ac.at), following the Bayesian Information Criterion (BIC). The same server was used to construct the final ML-tree with an

ultrafast-bootstrap analysis = 10000. The tree was visualized in FigTree software v 1.4.4 and edited with Inkscape 1.2. The robustness of the branches was assessed using ultrafast bootstrap value ≥ 95 criterion.

RESULTS

Nematodes belonging to the species *Geraldius galapagoensis* (Cephalobida: Chambersiellidae) were isolated from asexual reproductive structures (pycnidia) of the lichen *Hyperphyscia syncolla* (Physciaceae) (Fig. 1A, B, C).

Family Chambersiellidae Thorne, 1937
Genus *Geraldius* Sanwal, 1971
Type species: *Geraldius bakeri* (Sanwal, 1957)
Geraldius galapagoensis Cid del Prado, 2012
(Fig. 1D)

Material examined: 2 males, 1 juvenile. Collected by Renato García and deposited in the Colección Helmintológica de Ciencias Naturales de La Plata with the accession number MLP-He 8063.

Morphology and morphometry

Male (n = 2): Body habitus an open C-shape, posterior end curved ventrally, upon fixation. Cuticle finely annulated. Head offset by a constriction from the rest of the body. Six outer setae, 4.6 µm long. Oval amphid aperture 9 µm from the anterior end. Head width 6-8 µm. Stoma 9.2 µm long by 5.8 µm wide. Esophagus with a cylindrical pharyngeal corpus without a median bulb, a narrow isthmus and an oval basal pharyngeal bulb with strong transverse/butterfly valve apparatus. Single gonad extends anteriorly, 454.7 µm long. Rectal glands present. Tail curved ventrally, ending in dorsally hooked end. Seven pairs of latero-ventral pre-anal papillae and three pairs of post-anal in the following positions: one pair latero-ventral and two pairs dorso-lateral. Phasmid at the same level as the second pair of latero-dorsal papillae. Two slightly curved spicules with asymmetric manubrium with an anterior extension, conspicuous gubernaculum present.

Juvenile (n = 1): total length: 414 µm, cephalic diameter: 6.9 µm, stoma length: 4.6 µm, stoma width: 2.3 µm, esophagus length: 158.7 µm, anterior distance to the basal bulb: 135.7 µm, greatest width: 23 µm, width at the level of the anus: 16.1 µm, tail length: 78.2 µm.

Male (n = 2) (Table 1): total length: 715.5 µm (711-720), cephalic diameter: 7.5 µm (6.9-8.1), stoma length: 9.2 µm, stoma width: 5.8 µm, distance from anterior end to the nerve ring: 142.6 µm (132.2-153), width at the level of the nerve ring: 35.9 µm (27.8-44.1), esophagus length: 193.7 µm (180.9-206.5), anterior distance to the basal bulb: 179.2 µm (160-198.3), distance from anterior end to the excretory pore: 127.6 µm, greatest width: 47.5 µm (46.4-48.7), width at the level of the anus: 34.8 µm (30.2-39.4), spicule length: 46.4 µm (44-48.7), spicule width: 8.1 µm, tail length: 79.3 µm (76.5-82.1).

Female: not found.

Molecular analysis

The large subunit 28S ribosomal RNA gene sequence of *G. galapagoensis* was 916 bp. The phylogenetic analysis placed *G. galapagoensis* (OQ133527) as a member of a clade related to 3 populations of *Geraldius.* The clade of *Geraldius galapagoensis* (OQ133527) and *Geraldius* sp. (GU062821.1) grouped with a support of PP = 100%. The percentage of divergence observed between both sequences was 0.55. Divergences of 7.85 and 9.71% were observed between *G. galapagoensis* (OQ133527) and *Geraldius inserrai* (OK012568.1 and OK12567.1, support

Table 1. Morphometrics (in µm) of males of *Geraldius galapagoensis* and *Geraldius* spp. with the range in parentheses.

Character	*Geroldius galapagoensis* (This study, 2023)	*G. bakeri* (Sanwal, 1971)	*G. galapagoensis* (Cid del Prado, 2012)	*G. inserrai* (Cid del Prado-Vera *et al.*, 2021)
Total length	715.5 (711-720)	1.100-1.300	1000 (800-1100)	1000 (910-1100)
Cephalic diameter	7.5 (6.9-8.1)			
Stoma length	9.2			
Stoma width	5.8			
Distance from anterior end to the nerve ring	142.6 (132.2-153)	170	133.6 (107-157)	153.8 (138-168)
Width at the level of the nerve ring	35.9 (27.8-44.1)			
Esophagus length	193.7 (180.9-206.5)	240-242	183 (122.5-226)	223.4 (198-224)
Anterior distance to the basal bulb	179.2 (160-198.3)			
Distance from anterior end to the excretory pore	127.6	194-195	145 (108-169)	160.3 (138-183)
Greatest width	47.5 (46.4-48.7)	40-42.5	43 (32-55)	36.6 (34-40)
Width at the level of the anus	34.8 (30.2-39.4)			
Spicule length	46.4 (44-48.7)	50-52	49 (44-59)	42.7 (39-49)
Spicule width	8.1			
Gubernaculum length	21.2 (20.8-21.6)	24	22 (20-25)	19.6 (18-21)
Tail length	79.3 (76.5-82.1)	115-121	88.7 (78-98)	89.4 (77-101)

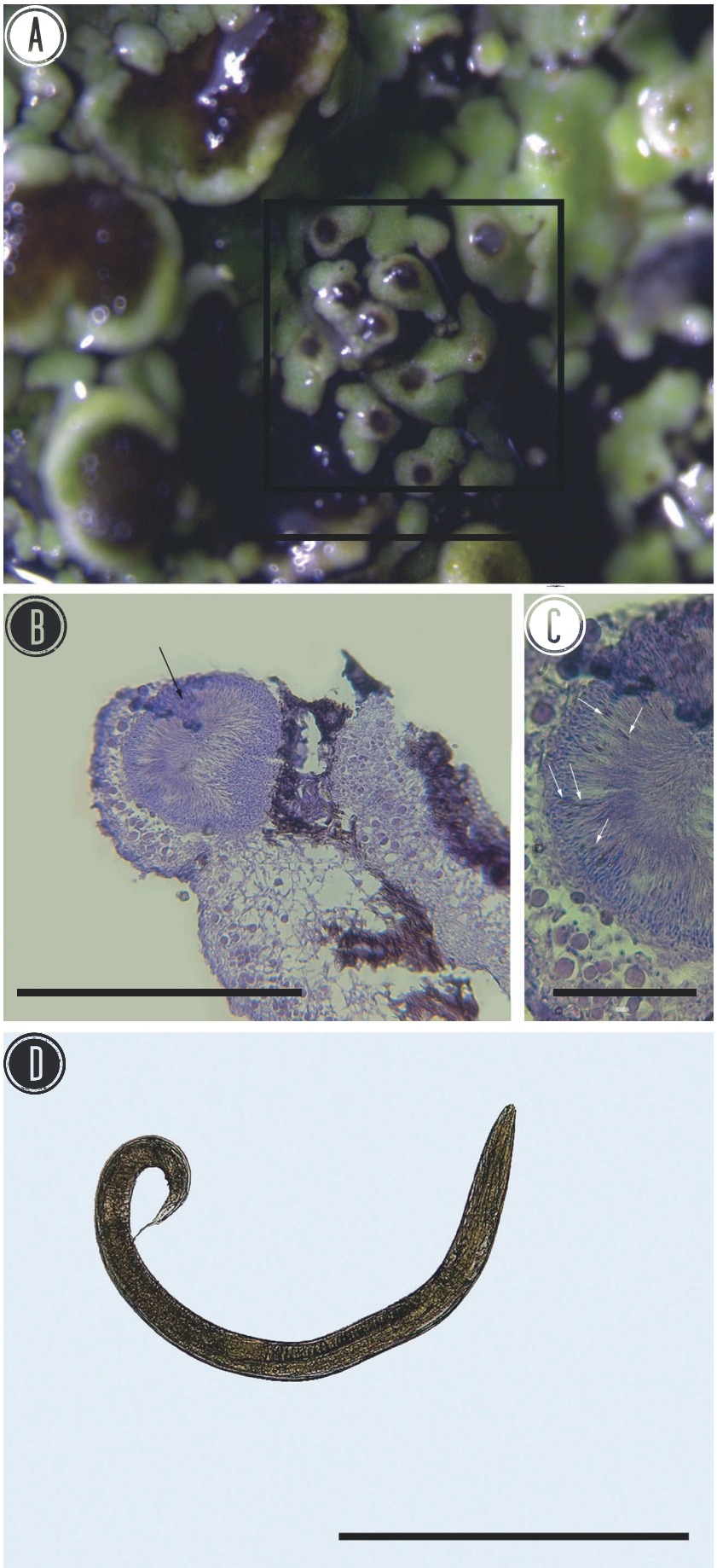

Figure 1. *Hyperphyscia syncolla.* (A) Pycnidia in the thallus (black square). (B) Histological section of one pycnidium (black arrow). (C) Nematodes inside the pycnidium (white arrows). (D) *Geraldius galapagoensis* male (scale bars: A = 2 cm, B = 120 µm, C = 30 µm, D = 500 µm).

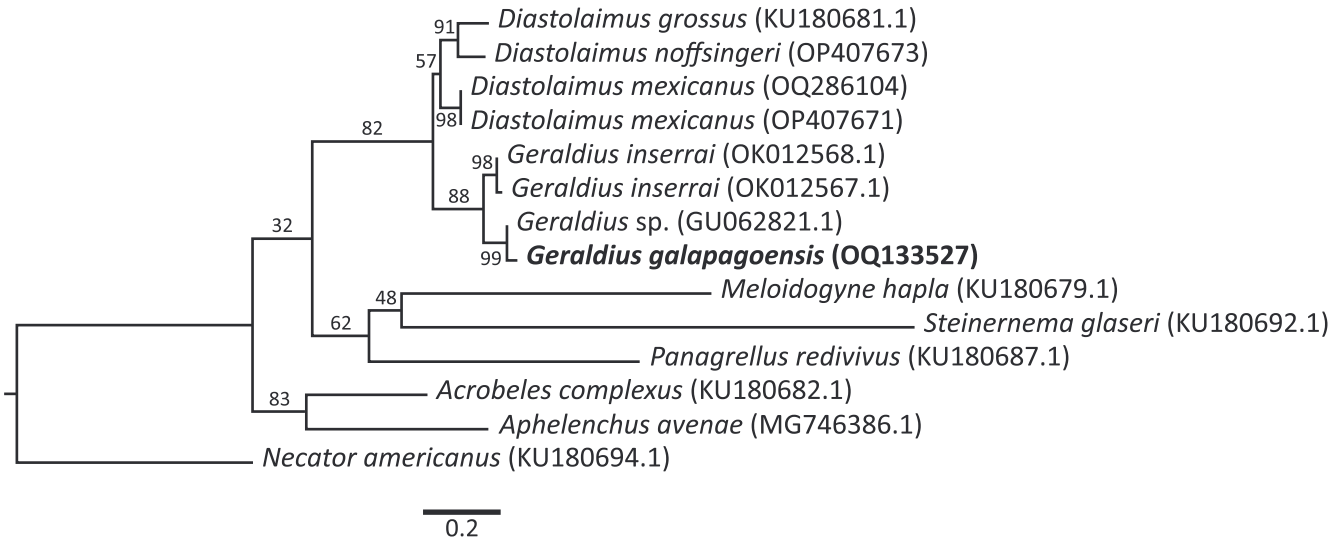

Figure 2. ML-tree based on 28S ribosomal RNA gene, including the *G. galapagoensis* identified in this report, highlighted in bold. The branch numbers represent the ultrafast bootstrap support.

73%), respectively. Additionally, *Diastolaimus grossus* (KU180681.1), *Diastolaimus noffsingeri* (OP407673) and *Diastolaimus mexicanus* (OQ286104 and OP407671) were grouped within a monophyletic clade, supported by PP = 57%. Lastly, the clade conformed by the species of *Diastolaimus* is supported by PP = 82% with the clade conformed by the species of *Geraldius* (Fig. 2).

DISCUSSION

The presence of *G. galapagoensis* in the pycnidia of *H. syncolla* is the first record of this species of nematode associated with the structure. The pycnidium is a structure formed only by the fungus, for asexual reproduction purposes, characterized by a rounded shape with an operculum. It is unusual to find other organisms inside these structures, but in some cases it has been observed that the interaction of other organisms with lichens can generate galls; fungi and mites have been observed to form these structures on the thallus (Hawksworth, 1982; De los Rios & Grube, 2001). In the case of nematodes, Siddiqi & Hawksworth (1982) recorded the presence of galls generated on *Cladonia glauca,* in which specialized nematode fauna lived, supporting unknown species from other habitats. In this study, the nematodes seem to penetrate these structures through the operculum without generating apparent damage to the lichen thallus.

The comparison of the morphometrics of our *G. galapagoensis* male with that of the original description shows that the body length is shorter (0.71 mm vs 1 mm), as are the distance of the excretory pore to the anterior end (127.6 µm vs 145 µm) and the tail (79.3 µm vs 88.7 µm). On the other hand, the distance from the anterior end to the nerve ring (142.6 µm vs 133.2 µm) and the esophagus length (193.7 µm vs 183 µm) are greater. The head width (7.5 µm vs 8.1 µm), body diameter (47.5 µm vs 43 µm) and spicule length (46.4 µm vs 49 µm) are quite similar.

Our study found a 99.45% similarity between the molecular sequences of *Geraldius galapagoensis* (OQ133527) and those of *Geraldius* sp. (GU062821.1), also from Argentina. Cid del Prado-Vera *et al.* (2021) determined that sequences of *Geraldius inserrai* (OK012567 and OK012568) obtained from two populations in Mexico differed from *Geraldius* sp. (GUO6281.1) by 5.7 and 7.0% (41 and 47 bp respectively), whilst the same sequences differed from *G. galapagoensis* (OQ133527) by 9.71 and 7.85%, respectively. Based on these results, with a divergence below 1%, *Geraldius* sp. (GUO6281.1) and *G. galapagoensis* (OQ133527) could be considered the same species. However, there are no reports with a morphological and morphometric description of *Geraldius* sp. (GUO628.1). Further studies are needed to corroborate this hypothesis.

CONCLUSION

Geraldius galapagoensis was collected by Cid del Prado in 2012, from mosses from the family Meteoriaceae, growing abundantly in endemic trees *Scalesia pedunculata* Hook. (Asteraceae) in a tropical forest on the twin volcanoes of Isla Santa Cruz, Galápagos Islands, Ecuador. This is the second report of this species, now associated with the lichen *Hyperphyscia syncolla* (Physciaceae) in Buenos Aires Province, Argentina. We provide a morphological and morphometrical characterization of *G. galapagoensis* and the first molecular sequence of this species.

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