

## Research Paper

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# Characterization of *Nothacrobeles lanceolatus* Abolafia & Peña-Santiago, 2003 (Rhabditida, Cephalobidae), a typical species of coastal dunes from Spain

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## Abstract

The nematode *Nothacrobeles lanceolatus* was described in arid soils from Cabo de Gata-Níjar Natural Park, province of Almería (Spain). Since then this species has not been found again. In this study, new data on the distribution and morphological, morphometrical and molecular characteristics (based on 18S and 28S ribosomal DNA) of *N. lanceolatus* is provided. This species is characterized by having a tessellated cuticle, with its inner layer bearing two rows of cuticular punctations per annulus, lips quadrangular with eight pinnae, labial probolae bifurcate with divergent prongs surrounded by pinnae, pharynx with corpus 2.5–4.0 times isthmus length, post-vulval uterine sac 1.4–2.2 times the corresponding body diameter, and female and male tails conical, bearing a conical mucro with acute terminus. The phylogenetic position of this and related species of the genus *Nothacrobeles* is discussed. Additionally, bacteria (*Pasteuria*) and fungi (*Catenaria*, *Nematocida*) feeding on *N. lanceolatus* are showed including light microscopy and scanning electron microscopy illustrations.

## Introduction

The genus *Nothacrobeles* was described by Allen & Noffsinger (1971) who found four new species from Israel, India and Australia, being the type species *Nothacrobeles sheri* Allen & Noffsinger, 1971. This genus is characterized by having lip region with labial probolae bifurcate with prongs having variable length, rarely absent, connecting basally by an expansion directed towards the secondary axil, and margins smooth or bordered by small membranous projections (pinnae). Later, Shahina & De Ley (1997) synonymized the genus *Namibinema* Rashid & Heyns, 1990 with it. Actually, the genus *Nothacrobeles* includes 21 valid species (Abolafia & Peña-Santiago, 2003; Ruiz-Cuenca & Abolafia, 2020).

One of these species, *Nothacrobeles lanceolatus*, was described by Abolafia & Peña-Santiago (2003) in xeric areas from Cabo de Gata-Níjar Natural Park (Spain). This species was considered as a possible endemism for this geographical area. In this study, new data about the distribution of this species, which can be considered now as Iberian littoral endemism, is provided. This new material is now characterized from the morphological, morphometric and molecular point of view.

## Material and methods

### Sampling and nematode extraction

The specimens of *N. lanceolatus* were extracted from the rhizosphere of xerophile plants from sand coastal dunes in the provinces of Huelva, Cádiz, Málaga, Almería, Murcia, Alicante, Valencia, Castellón, Tarragona and Barcelona (Spain) (fig. 1). The nematodes were extracted from soil samples using a modified Baermann's (1917) funnel technique, killed by heat and fixed in a 4% formalin solution. The nematodes were processed to anhydrous glycerine according to Siddiqi's (1964) method using lactophenol–glycerine solutions and were permanently mounted on glass microscope slides with the glycerine–paraffin method (de Maeseneer & d'Herde, 1963) somewhat modified using hot liquid paraffin.

### Light microscopy (LM)

Observations were made and measurements were taken using a Nikon Eclipse 80i (Nikon, Tokyo, Japan) microscope with differential interference contrast optics. Photomicrographs were taken with a Nikon Digital Sight DS-U1 camera and processed with Adobe® Photoshop® CS (Adobe Inc., San José, California, USA) and figures mounted using

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**Fig. 1.** Spanish localities with presence of *Nothacrobeles lanceolatus* Abolafia & Peña-Santiago, 2003.

Microsoft® PowerPoint® (Microsoft Corporation, Redmond, Washington, USA). Demanian indices (de Man, 1881) and other ratios were calculated. The terminology used for the morphology of probolae, stoma and spicules follows Ruiz-Cuenca & Abolafia (2021), De Ley *et al.* (1995) and Abolafia & Peña-Santiago (2017), respectively.

### Scanning electron microscopy (SEM)

The specimens preserved in glycerine were selected for observation under SEM according to Abolafia (2015). The nematodes were hydrated in distilled water, dehydrated in a graded ethanol-acetone series, critical-point dried, coated with gold and observed with a Zeiss Merlin microscope (5 kV) (Zeiss, Oberkochen, Germany).

### DNA extraction, polymerase chain reaction (PCR) and sequencing

Nematode DNA was extracted from single fresh individuals using the proteinase K protocol and PCR assays, as described by Castillo *et al.* (2003), somewhat modified (Archidona-Yuste *et al.*, 2016). The specimens were cut into small pieces using a sterilized dental needle on a clean slide with 18 ml of Tris-EDTA (ethylene-diamine-tetraacetic acid) buffer (10 mM Tris-Cl (tris hydrochloride) + 0.5 mM EDTA; pH 9.0), transferred to a microtube, adding 2 µl proteinase K (700 µg/ml<sup>-1</sup>) (Roche, Basel, Switzerland) and stored to -80°C within 15 min (for several days). The microtubes were incubated at 65°C (1 h), then at 95°C (15 min). For DNA amplification, 3 µl of the extracted DNA was transferred to a microtube containing: 0.6 µl of each primer (10 mM), 3 µl Master Mix Taq DNA Polymerase (5× Hot FirePol Blend Master Mix, Solis BioDyne, Tartu, Estonia) and double distilled water (ddH<sub>2</sub>O) to a final volume

of 20 µl. The primers used for amplification of the region of 18S ribosomal RNA (rRNA) gene were the forward primer 988 F (5'-CTCAAAGATTAAGCCATGC-3') and the reverse primer 1912R (5'-TTTACGGTCAGAACTAGGG-3') (Holterman *et al.*, 2006). The primers used for amplification of the D2-D3 region of 28S rRNA gene were the D2A (5'-ACAAGTACCGTGAGGG AAAGTTG-3') and the D3B (5'-TCGGAAGGAACCAGCTACTA-3') primers (Nunn, 1992; De Ley *et al.*, 1999). PCR cycle conditions were as follows: one cycle of 94°C for 15 min, followed by 35 cycles of 94°C for 45 s + annealing temperature of 55°C for 45 s + 72°C for 45 s, and finally one cycle of 72°C for 5 min. After DNA amplification, 5 µl of product was loaded on a 1% agarose gel in 0.5% Tris-acetate-EDTA (40 mM Tris, 20 mM glacial acetic acid and 2 mM EDTA; pH = 8) to verify the amplification using an electrophoresis system (Labnet Gel XL Ultra V-2, Progen Scientific, London, UK). The bands were stained with RedSafe (20,000×) previously added to the agarose gel solution. The sequencing reactions of the PCR products were performed at Sistemas Genómicos (Paterna, Valencia, Spain) according the Sanger *et al.* (1977) method. The sequences obtained were submitted to the GenBank database.

### Phylogenetic analyses

For phylogenetic relationships, analyses were based on 18S and 28S ribosomal DNA (rDNA) fragments. The newly obtained sequences were manually edited using BioEdit 7.2.6 (Hall, 1999) and aligned with another 18S or 28S rDNA sequence available in GenBank using the ClustalW (Thompson *et al.*, 1994) alignment tool implemented in MEGA7 (Kumar *et al.*, 2016). Alignment ends were trimmed using MEGA7. The best-fit model of nucleotide substitution used for the phylogenetic analysis was statistically selected using jModelTest 2.1.10 (Darriba *et al.*, 2012). The phylogenetic trees were generated with the Bayesian inference method

using MrBayes 3.2.6 (Ronquist *et al.*, 2012). *Drilocephalobus* sp. (AY284680) for 18S rDNA and *Teratolobus* sp. (KJ652552) for 28S rDNA were chosen as outgroup. The analysis under the general time-reversible plus invariant sites plus gamma distribution (GTR + I + G) model (Tavaré, 1986) was initiated with a random starting tree and run with the Markov Chain Monte Carlo (MCMC) (Larget & Simon, 1999) for  $1 \times 10^6$  generations. The trees were visualized and saved with FigTree 1.4.4 (Rambaut, 2018).

## Results

### *Nothacrobeles lanceolatus* Abolafia & Peña-Santiago, 2003

#### Material examined

In this study, 20 females and 20 males from several Iberian coastal dunes were examined.

#### Measurements

For measurements, see table 1.

#### Description

**Adult** (figs 2–5). Body cylindrical, with 0.6–0.8 mm long. Cuticle with tessellated annuli; annuli 3  $\mu\text{m}$  wide at midbody. Lateral fields with three longitudinal incisures (or two longitudinal alae) occupying 16–25% of the body diameter at midbody. Lip region with dentate rectangular lips, grouped in pairs. Primary axils deep with two elongate guard processes and secondary axils shallow lacking guard processes. Oral opening surrounded by three labial probolae with dentate margin bearing rounded to conoid pinnae, 10–14  $\mu\text{m}$  long, bifurcated, with a prominent basal ridge protruding toward the lips; each probolae with furca elongate. Amphid openings ovoid. Stoma cephaloboid, robust, with short cheilostom with large and rounded cheilorhabdia, very reduced gymnostom with minute gynnorhabdia, and well developed stegostom with minute stegorhabdia and large dorsal tooth. Pharynx also cephaloboid, with subcylindrical pharyngeal corpus having procorpus and metacarpus well differentiated, both with similar length; pharyngeal corpus–isthmus junction well demarcated and isthmus narrower than corpus; basal bulb spheroid with valvular apparatus (grinder) well developed. Nerve ring at 70–81% of neck length, surrounding the isthmus. Excretory pore at 66–78% of neck length, at isthmus level. Deirid at 77–93% of neck length, at isthmus or basal bulb level. Intestine without distinct specializations but having narrower walls at anterior part.

**Female.** Reproductive system monodephic–prodelphic, cephaloboid. Ovary long, without flexures with oocytes in only one row; oviduct very short; spermatheca well developed, swollen, 1.0–1.4 times the body diameter, with large spermatozoa; uterus with elongate tubular distal part having thicker walls and short proximal swollen part having thinner walls; vagina short, about one fourth of the body diameter; vulva a transversal slit; post-vulval uterine sac swollen, 1.4–2.2 times the corresponding body diameter long. Rectum shorter than the anal body diameter. Anus well developed, curved. Tail conical, spear-shaped, ending in an acute, conoid mucro, with cuticle slightly wider at posterior region. Phasmids located at 32–40% of tail length.

**Male.** Reproductive system monorchid, with testis ventrally reflexed anteriorly. Tail conical, ventrally curved with an acute mucro at the end. Phasmids located at 30–36% of tail length. Post-cloacal genital papillae five pairs, two at middle length of tail, one lateral and one ventrosublateral, and three near the tail tip, one subdorsal and two subventral. Spicules paired and symmetrical, ventrally curved having rounded manubrium, conoid

calamus and ventrad curved lamina with reduced ventral velum and acute terminus. Gubernaculum well developed, almost straight with ventrad curved at its anterior part.

#### Molecular characterization

Four 18S rDNA sequences of *N. lanceolatus* were obtained having 849 bp (OK042922), 801 bp (OK042923), 742 bp (OK042924) and 795 bp (OK042925) from sand dunes in Artola-Cabopino, Málaga (Spain), being 100% similar between them, in a segment in common with 678 bp. On the other hand, two 28S rDNA sequences with 797 bp (OK042914, OK042915) are obtained from the same specimens being 100% similar.

#### Localities and habitats

The new examined populations of *N. lanceolatus* have been found in coastal sand dunes from several Spanish localities and beaches (fig. 1): El Altet Beach, Guardamar del Segura (province of Alicante); Salinas de Cabo de Gata, San José (province of Almería); Gavá (province of Barcelona); Barbate, Bolonia Beach, El Chato Beach, La Barrosa Beach, Rota, Valdelagrana (province of Cádiz); Prat de Cabanes-Torreblanca (province of Castellón); El Rompido, Isla Cristina, Islantilla, Punta Umbria (province of Huelva); Artola-Cabopino Beach, Torrox (province of Málaga); Calblanque Beach, San Pedro del Pinatar (province of Murcia); Deltebre (province of Tarragona); El Saler (province of Valencia). All of these populations are associated with a community of xerophilic plants, including *Alyssum loiseleurii* P. Fourn., *Ammophila arenaria* (L.) Link, *Anthyllis cytisoides* L., *Carpobrotus edulis* (L.) N.E. Br., *Otanthus maritimus* Hoffmanns. & Link and *Pancratium maritimum* L.

#### Remarks

Eight specimens (two males, three females and three juveniles) from two localities were found with bacteria or fungi feeding on them. Thus, one specimen collected from Islantilla (province of Huelva) was found with the bacterium of the genus *Pasteuria* Metchnikoff, 1888 (Firmicutes, Pasteuriaceae) adhered at the cuticle of the nematode (fig. 3e, g).

Also, two species of fungi have been found in the surface and inside the body of nematode specimens from Artola-Cabopino (province of Málaga). The fungus of the genus *Catenaria* Sorokin, 1876 (Blastocladiomycota, Blastocladiales) has been found in seven specimens of *N. lanceolatus* (figs 4a–g and 5a, b, g–k) appearing attached in groups of flagellate zoospores surrounding the oral, vulval and anal openings of the nematodes, generating long hyphae which penetrate inside the body along the digestive tract. Other fungus, found in two specimens, belonging to the genus *Nematocida* Troemel, Félix, Whiteman, Barrière & Ausubel, 2008 (Microsporidia, Minisporida), an obligate intracellular parasite, appears inside some intestinal cells (fig. 4h–j). Unfortunately, it is unknown if these nematode specimens were parasitized in their natural habitat or during the extraction process, which maintains the soil samples in water for several days.

## Discussion

### Morphological and morphometrical analyses of *N. lanceolatus*

In general, the morphology of *N. lanceolatus*, in the present study, is similar to the type population described by Abolafia & Peña-Santiago (2003) from Cabo de Gata-Níjar Natural Park. However, the morphometrical analysis shows some important differences, namely, the measurements of the type population are slightly

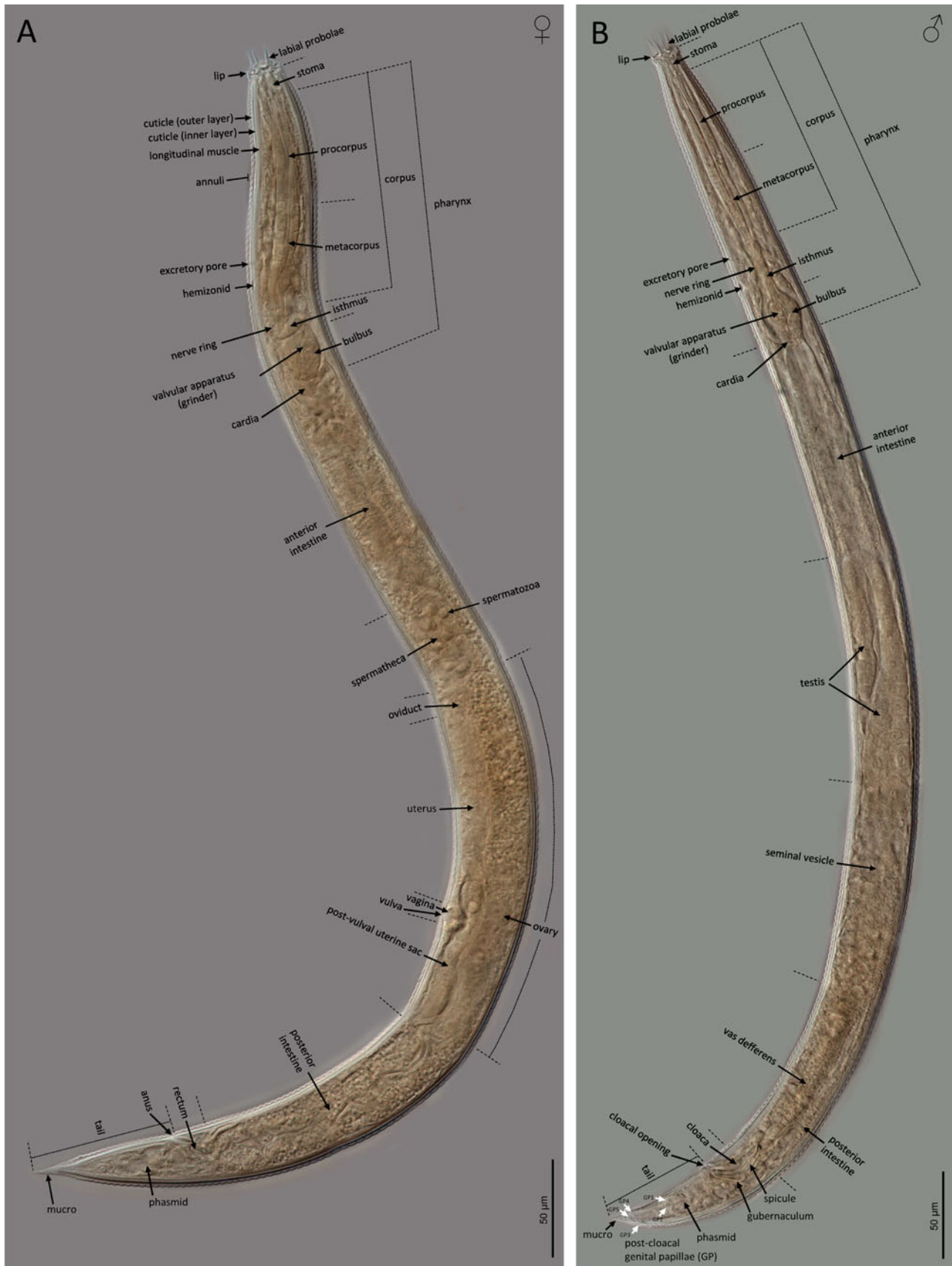
**Table 1.** Morphometrics of *Nothacrobeles lanceolatus* Abolafia & Peña-Santiago, 2003. All measurements in  $\mu\text{m}$ .

Province Habitat	Present paper								Abolafia & Peña-Santiago (2003)	
	Cádiz Sand dunes		Huelva Sand dunes		Málaga Sand dunes		Valencia Sand dunes		Almería Salty sand dunes	
<i>n</i>	5 ♀♀	5 ♂♂	5 ♀♀	5 ♂♂	5 ♀♀	5 ♂♂	5 ♀♀	5 ♂♂	10 ♀♀	3 ♂♂
Body length	813.2 ± 46 (749–862)	825.8 ± 23.6 (800–850)	775.6 ± 76.8 (651–857)	792.2 ± 12.5 (784–814)	793.8 ± 33.5 (760–843)	728.3 ± 25.5 (708–757)	744.2 ± 79.4 (650–863)	768 ± 59.5 (703–852)	560.0 ± 51.5 (447–603)	552.3 ± 30.4 (530–587)
<i>a</i>	19.7 ± 2 (17.0–22.2)	21.9 ± 0.3 (21.6–22.3)	19.1 ± 1.3 (17.5–20.9)	21.1 ± 2.7 (17.9–24.6)	19.9 ± 1.3 (18.7–22)	19.5 ± 0.5 (18.9–20)	19.6 ± 2.7 (17.1–22.7)	20.2 ± 2.6 (17.5–23.5)	18.1 ± 2.0 (14.4–21.3)	19.0 ± 0.2 (18.9–19.3)
<i>b</i>	4.4 ± 0.3 (3.8–4.7)	4.7 ± 0.1 (4.6–4.8)	4.4 ± 0.1 (4.3–4.7)	4.5 ± 0.3 (4.2–5)	4.2 ± 0.2 (3.9–4.5)	4.2 ± 0.1 (4.1–4.3)	4.0 ± 0.2 (3.9–4.4)	4.2 ± 0.3 (3.8–4.7)	4.0 ± 0.3 (3.2–4.4)	4.1 ± 0.1 (4.0–4.3)
<i>c</i>	12.2 ± 0.8 (10.8–12.8)	12.1 ± 0.3 (11.6–12.6)	11.1 ± 0.6 (10.1–11.7)	12.2 ± 0.5 (11.7–13.1)	11.9 ± 0.7 (10.7–12.6)	12.5 ± 0.3 (12.2–12.8)	10.9 ± 1.6 (9.2–12.8)	11.8 ± 0.7 (10.9–12.9)	10.9 ± 0.7 (9.5–11.8)	11.8 ± 0.5 (11.3–12.3)
<i>c'</i>	2.3 ± 0.1 (2.2–2.3)	2.5 ± 0.1 (2.4–2.5)	2.5 ± 0.1 (2.4–2.7)	2.3 ± 0.1 (2.2–2.4)	2.5 ± 0.3 (2.3–3.1)	2.3 ± 0.1 (2.2–2.4)	2.4 ± 0.1 (2.3–2.5)	2.2 ± 0.1 (2.1–2.5)	2.3 ± 0.1 (2.2–2.5)	2.2 ± 0.1 (2.0–2.2)
<i>V</i>	64.5 ± 2.6 (63–69)	–	64.9 ± 2.9 (63–70)	–	63.2 ± 1.3 (62–65)	–	64.2 ± 4.7 (59–72)	–	61.5 ± 2.6 (56–65)	–
Labial probolae length	12.2 ± 0.4 (12–13)	11.4 ± 0.8 (11–13)	12.0 ± 1.4 (11–14)	13.2 ± 0.8 (12–14)	13.0 ± 1.0 (12–14)	12.3 ± 0.5 (12–13)	11 ± 1.7 (10–14)	11.8 ± 1.0 (10–13)	8.4 ± 0.8 (7–10)	8.0 ± 0.0 (8)
Lip region width	18.6 ± 0.5 (18–19)	17.4 ± 0.8 (17–19)	18.2 ± 0.8 (17–19)	17.4 ± 0.8 (16–18)	17.8 ± 0.4 (17–18)	17.3 ± 1.1 (16–18)	17.6 ± 1.1 (16–19)	17.2 ± 1.3 (15–18)	14.9 ± 0.8 (14–16)	13.0 ± 1.7 (11–14)
Stoma length	12.6 ± 1.1 (11–14)	12.8 ± 0.8 (12–14)	12.0 ± 0.0 (12)	11.4 ± 0.5 (11–12)	11.8 ± 1.3 (10–13)	11.6 ± 0.5 (11–12)	14.0 ± 1.2 (13–16)	12.8 ± 1.9 (10–15)	12.4 ± 1.4 (10–14)	12.0 ± 1.0 (11–13)
Pharyngeal corpus length	104.8 ± 1.6 (103–107)	101.2 ± 1.3 (100–103)	105.2 ± 12.3 (84–114)	100.6 ± 2.7 (97–104)	110.0 ± 5.4 (103–116)	107.3 ± 2 (105–109)	108.4 ± 7.5 (100–119)	104.4 ± 2.8 (102–109)	76.6 ± 4.4 (68–81)	72.3 ± 0.6 (72–73)
Isthmus length	35 ± 5.4 (29–42)	32.4 ± 4.6 (26–39)	28.4 ± 2.7 (25–32)	33.0 ± 6.2 (25–39)	34.6 ± 4.3 (27–38)	29.6 ± 3.7 (27–34)	32.0 ± 3.3 (28–36)	33.4 ± 4.2 (28–38)	26.9 ± 3.2 (22–32)	27.0 ± 2.6 (25–30)
Bulbus length	30.8 ± 3.7 (27–37)	27.0 ± 3.0 (24–32)	27.6 ± 3.3 (24–33)	28.2 ± 2.3 (25–31)	29.4 ± 3.6 (25–34)	23.3 ± 2.5 (21–26)	28.6 ± 3.7 (25–33)	28.2 ± 1.7 (26–30)	23.7 ± 0.8 (22–25)	23.0 ± 1.0 (22–24)
Pharynx length	170.6 ± 6.1 (163–180)	160.6 ± 4.0 (157–167)	161.2 ± 16 (133–172)	161.8 ± 8.7 (151–173)	174.0 ± 11.7 (158–186)	160.3 ± 3.7 (156–163)	169 ± 12.8 (153–183)	166.0 ± 3.6 (160–170)	139.8 ± 5.2 (132–147)	134.3 ± 3.2 (132–138)
Nerve ring – anterior end	136.2 ± 3.9 (131–140)	135.4 ± 6.4 (127–145)	142.0 ± 3.6 (138–146)	135.6 ± 9.2 (128–146)	136.8 ± 3.6 (134–142)	127.3 ± 5.8 (123–134)	150.0 ± 11.3 (142–158)	135.0 ± 6.6 (130–144)	76.1 ± 12.9 (61–98)	88.3 ± 6.8 (83–96)
Excretory pore – anterior end	139.5 ± 3.5 (137–142)	133.3 ± 3.0 (130–136)	133.5 ± 3.0 (130–136)	139.5 ± 2.3 (137–142)	122.5 ± 14.8 (112–133)	116.3 ± 1.5 (115–118)	137.0 ± 9.8 (130–144)	128.5 ± 3.5 (126–131)	78.1 ± 14.6 (61–101)	87.5 ± 14.8 (77–98)
Deirid – anterior end	165 ± 1.4 (164–166)	155.5 ± 0.7 (155–156)	143.7 ± 10.8 (130–155)	152.0 ± 2.9 (149–156)	146.7 ± 11.8 (133–154)	137.5 ± 3.5 (135–140)	151.3 ± 17.8 (146–160)	158.6 ± 11.8 (145–166)	96.3 ± 16.5 (77–122)	98.3 ± 11.4 (89–111)
Neck length	183.2 ± 6.6 (176–194)	173.4 ± 4.1 (169–180)	173.2 ± 16 (145–184)	173.2 ± 9.2 (162–185)	185.8 ± 11.4 (170–199)	172.0 ± 3.4 (168–174)	183.0 ± 13.1 (166–196)	178.8 ± 3.3 (173–181)	?	?
Annuli width	3.0 ± 0.0 (3)	3.0 ± 0.0 (3)	3.0 ± 0.0 (3)	3.0 ± 0.0 (3)	3.0 ± 0.0 (3)	3.0 ± 0.0 (3)	3.0 ± 0.0 (3)	3.0 ± 0.0 (3)	3.2 ± 0.4 (3–4)	3.0 ± 0.0 (3)

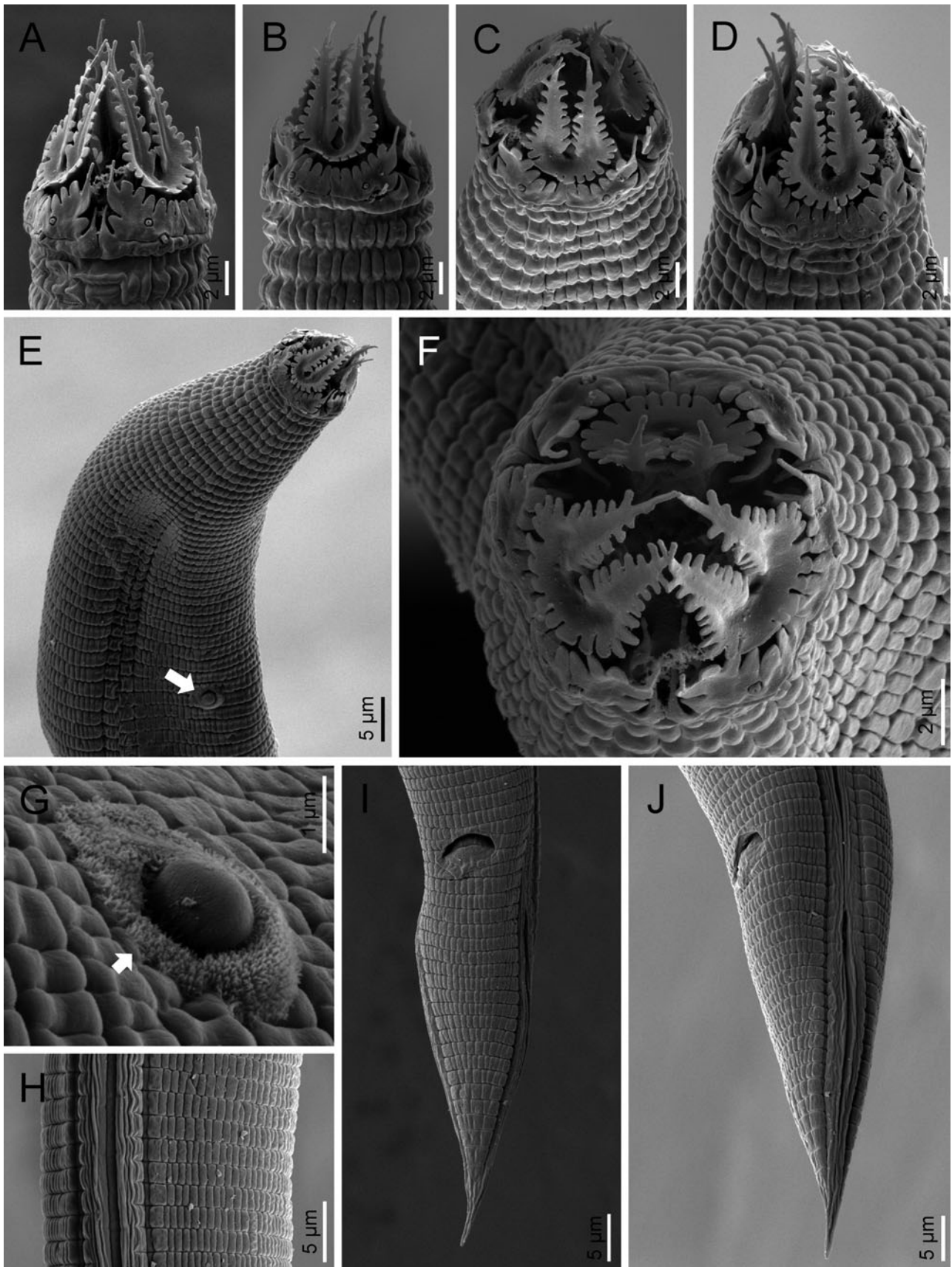


Cuticle at midbody	3.0 ± 0.0 (3)	3.0 ± 0.0 (3)	3.0 ± 0.0 (3)	3.0 ± 0.0 (3)	3.0 ± 0.0 (3)	3.0 ± 0.0 (3)	3.0 ± 0.0 (3)	3.0 ± 0.0 (3)	2.0 ± 0.2 (2)	2.0 ± 0.0 (2)
Body diameter at neck base	39.4 ± 3.7 (35–44)	35.0 ± 1.8 (33–37)	37.2 ± 2.4 (34–39)	36.4 ± 3.4 (33–42)	37.2 ± 2.4 (35–41)	36.0 ± 3.4 (34–40)	38.6 ± 0.8 (38–40)	37.4 ± 2.7 (33–40)	31.0 ± 3.9 (22–34)	28.7 ± 0.6 (28–29)
Body diameter at midbody	41.6 ± 5.2 (36–50)	37.6 ± 0.5 (37–38)	40.4 ± 2.4 (37–43)	38.0 ± 4.5 (33–44)	40 ± 3.7 (35–45)	37.3 ± 2.3 (36–40)	37.7 ± 2.0 (35–40)	38.2 ± 3.6 (32–41)	31.0 ± 1.8 (28–34)	29.0 ± 1.7 (28–31)
Lateral field width	8.2 ± 0.9 (7–9)	8.2 ± 0.5 (8–9)	7.2 ± 1.3 (6–9)	8.0 ± 0.0 (8)	7.3 ± 0.5 (7–8)	7.6 ± 1.1 (7–9)	8.0 ± 0.0 (8)	7.6 ± 1.1 (6–9)	6.0 ± 0.9 (4–7)	6.0 ± 0.0 (6)
Anterior ovary/testis	178.3 ± 18.9 (165–200)	191.2 ± 27.0 (170–230)	204.5 ± 9.1 (198–211)	215.4 ± 30 (180–262)	196.1 ± 26.8 (177–215)	211.3 ± 23.1 (196–238)	246.5 ± 58.6 (205–288)	169.6 ± 37.6 (134–231)	134.5 ± 26.8 (97–177)	–
Spermatheca length	56.0 ± 0.0 (56)	–	49.5 ± 4.9 (46–53)	–	43.7 ± 13.5 (31–61)	–	38.0 ± 0.0 (38)	–	36.3 ± 8.7 (25–46)	–
Anterior uterus length	91.3 ± 11 (80–102)	–	63.6 ± 25.4 (48–93)	–	104.5 ± 48.3 (32–132)	–	127.0 ± 67.8 (79–175)	–	69.7 ± 8.1 (57–81)	–
Post-vulval sac length	69.6 ± 5.5 (64–75)	–	69.4 ± 12.2 (50–84)	–	66.4 ± 5.5 (60–74)	–	68.5 ± 10.6 (61–76)	–	27.1 ± 3.2 (23–32)	–
Vagina length	13.0 ± 1.4 (12–14)	–	12.2 ± 0.5 (12–13)	–	10.6 ± 0.5 (10–11)	–	12.0 ± 0.0 (12)	–	9.3 ± 1.3 (8–11)	–
Vulva – anterior end	524.4 ± 19.4 (506–545)	–	503.8 ± 52.2 (419–548)	–	501.8 ± 24.8 (471–529)	–	477.0 ± 45.8 (416–545)	–	345.0 ± 40.1 (249–384)	–
Rectum length	23.8 ± 3.3 (18–26)	35.6 ± 2.9 (32–39)	21.7 ± 2.3 (20–25)	33.2 ± 2.1 (31–36)	23.0 ± 2.2 (20–26)	32.0 ± 2.0 (30–34)	23.2 ± 1.5 (22–25)	36.6 ± 1.8 (35–39)	19.9 ± 1.5 (18–22)	∞
Anal body diameter	28.2 ± 0.4 (28–29)	27.0 ± 0.0 (27)	27.2 ± 1.6 (26–30)	28.0 ± 1.2 (27–30)	26.2 ± 2 (23–28)	24.6 ± 0.5 (24–25)	27.7 ± 0.5 (27–28)	28.4 ± 2.1 (25–31)	22.0 ± 1.1 (21–24)	21.7 ± 2.1 (20–24)
Tail length	66.4 ± 2.1 (63–69)	68.2 ± 2.0 (66–70)	69.6 ± 4.9 (64–76)	64.6 ± 3.3 (60–69)	66.8 ± 5.6 (60–73)	58.0 ± 2.0 (56–60)	67.7 ± 2.8 (64–70)	64.6 ± 1.3 (63–66)	51.2 ± 3.2 (47–56)	46.7 ± 2.5 (44–49)
Phasmid–anus distance	25.5 ± 0.7 (25–26)	23.5 ± 2.1 (22–25)	23.0 ± 1.7 (20–24)	23.5 ± 2.1 (22–25)	23.0 ± 4.2 (20–26)	19.0 ± 1.0 (18–20)	24.3 ± 3.2 (22–28)	19.5 ± 0.7 (19–20)	13.6 ± 2.8 (8–17)	14.3 ± 1.5 (13–16)
Spicules length	–	34.4 ± 1.9 (31–36)	–	36.4 ± 1.1 (35–38)	–	34.3 ± 2.1 (32–36)	–	37.6 ± 2.7 (35–41)	–	28.3 ± 1.2 (27–29)
Gubernaculum length	–	18.8 ± 1.1 (18–20)	–	21.0 ± 1.0 (20–22)	–	20.0 ± 0.0 (20)	–	19.4 ± 1.1 (18–21)	–	16.3 ± 1.2 (15–17)

Measurements in  $\mu\text{m}$  and in the form: mean  $\pm$  standard deviation (range) where appropriate. Demanian indices (de Man, 1981): a, body length/body diameter; b, body length/pharynx length; c, body length/tail length; c', tail length/anal body diameter; V, distance from anterior region to vulva/body length  $\times 100$ .

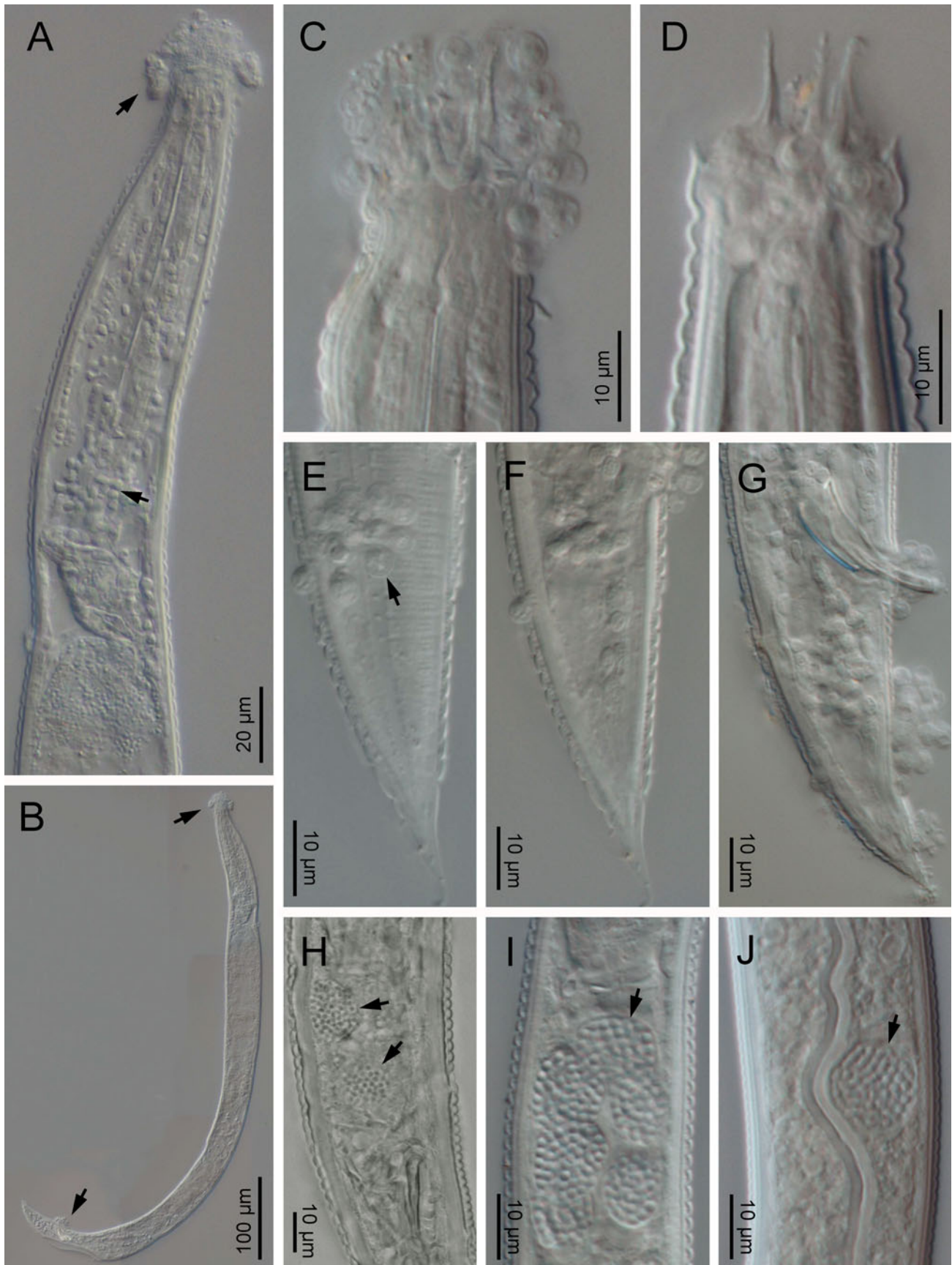


**Fig. 2.** LM of *Nothacrobeles lanceolatus* Abolafia & Peña-Santiago, 2003. (A) Female; (B) male.



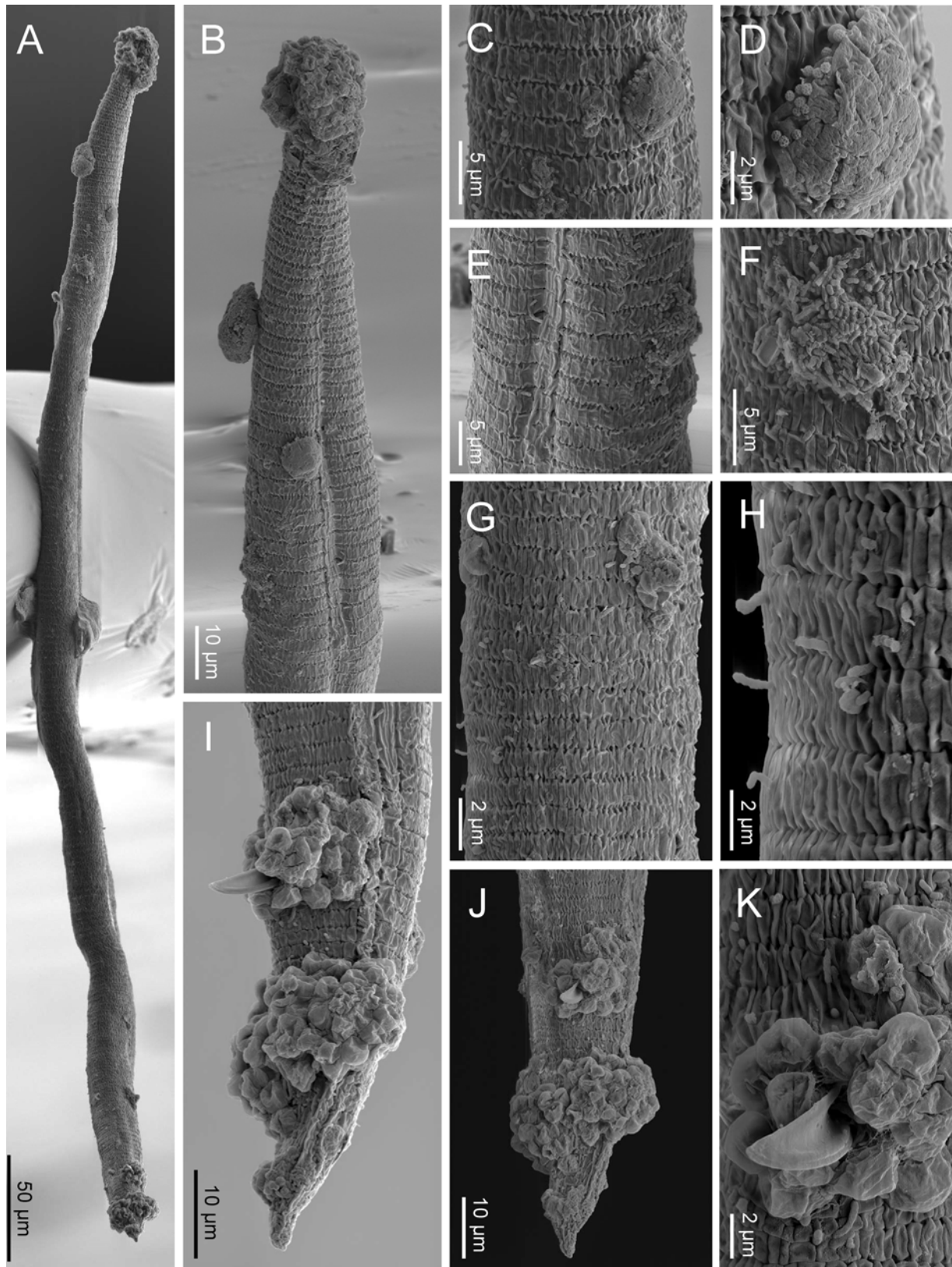
**Fig. 3.** SEM of *Nothacrobeles lanceolatus* Abolafia & Peña-Santiago, 2003. (A–D, F) Lip region in ventral, left lateral, subfrontal, right lateral and frontal view, respectively; (E) anterior region (arrow pointing the bacterium *Pasteuria*); (G) sporangium of *Pasteuria* (arrow) at cuticle; (H) lateral field; (I, J) tail in ventral and lateral view, respectively.



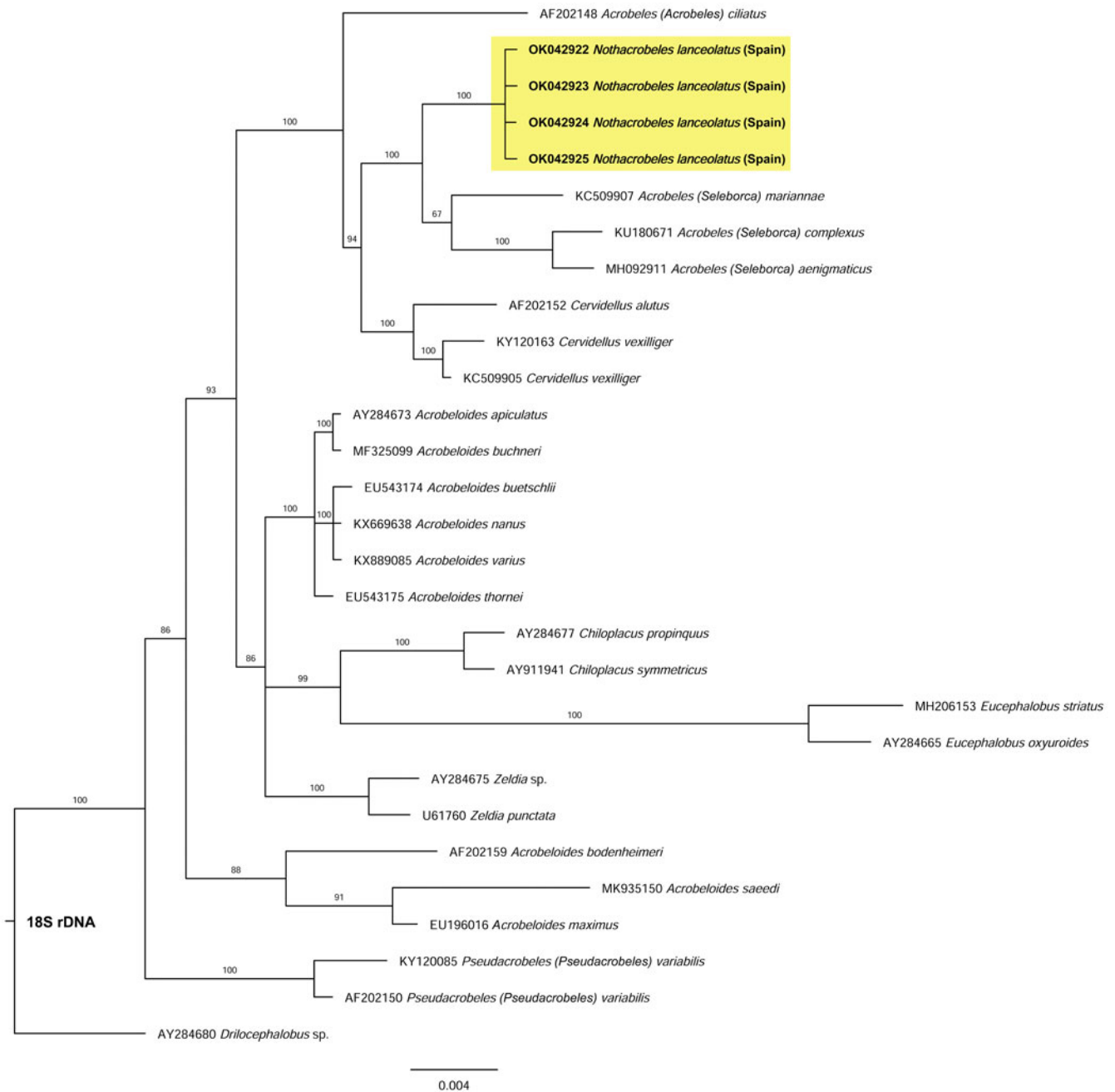


**Fig. 4.** LM of *Nothacrobeles lanceolatus* with fungi. (A–G) Zoospores of *Catenaria* (arrows) at anterior end and pharynx; (H–J) *Nematocida* (arrows) at intestine cells.





**Fig. 5.** SEM of *Nothacrobeles lanceolatus* with fungi and bacteria. (A) Entire body; (B) anterior end with zoospores of *Catenaria*; (C, D) cuticle with sticky mess with pollen grains; (E, F) excretory pore with bacteria; (G, H) sporangia of *Catenaria* emerging through the cuticle; (I, J) male tail in lateral and ventral views, respectively, with zoospores of *Catenaria* at cloaca and tail; (K) detail of cloaca showing the spicule and cloacal aperture surrounded by zoospores.



**Fig. 6.** Bayesian inference tree from known and the newly sequenced *Nothacrobeles lanceolatus* based on sequences of the 18S rDNA region. Bayesian posterior probabilities (%) are given for each clade. Scale bar shows the number of substitutions per site.

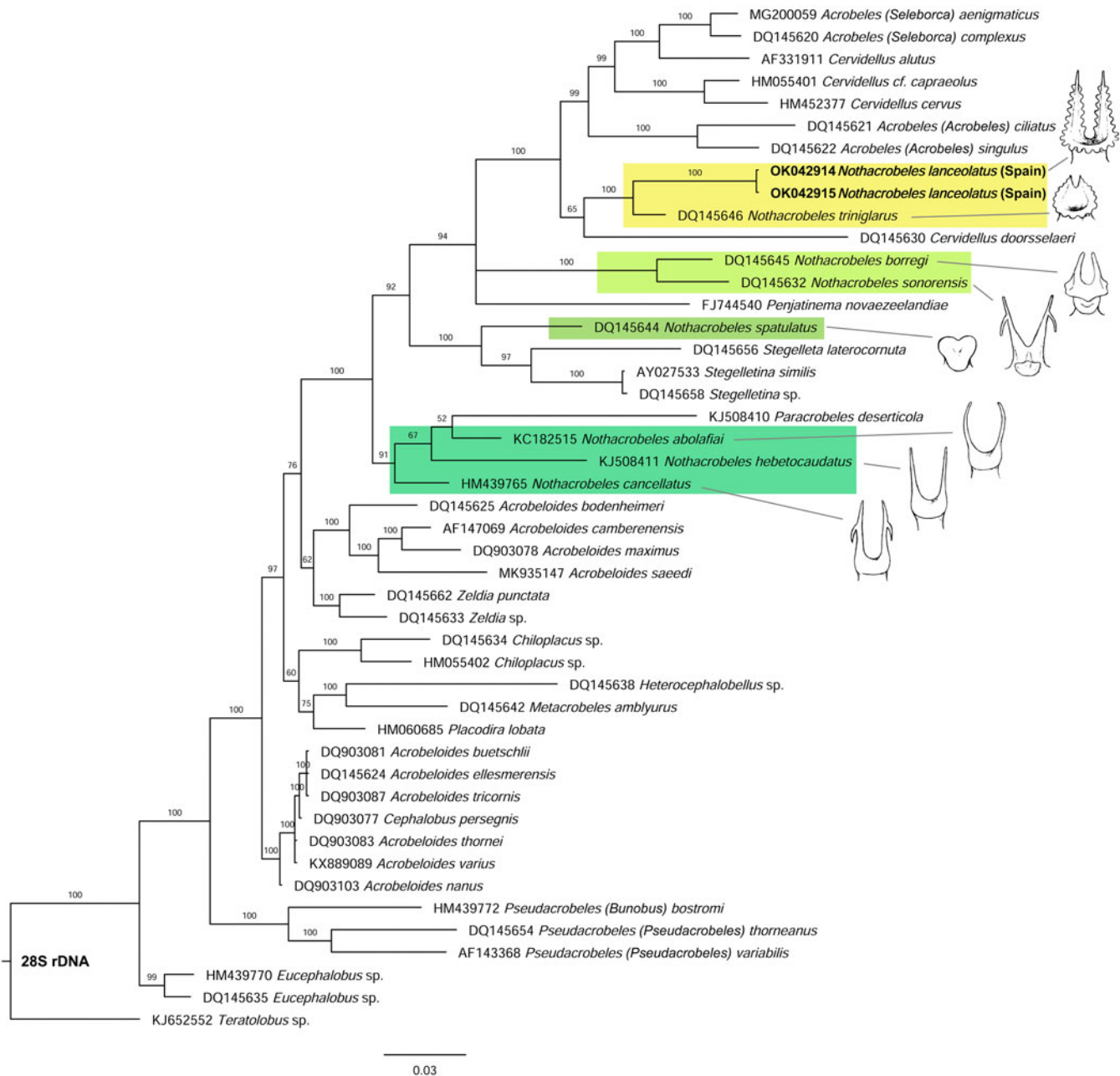
smaller than in the specimens examined in the present study: body length (0.4–0.6 mm vs. 0.6–0.8 mm), labial probolae length (4–10  $\mu\text{m}$  vs. 10–14  $\mu\text{m}$ ), female tail length (47–56  $\mu\text{m}$  vs. 60–76  $\mu\text{m}$ ), male tail length (44–49  $\mu\text{m}$  vs. 56–73  $\mu\text{m}$ ), post-vulval sac length (23–32  $\mu\text{m}$  vs. 50–84  $\mu\text{m}$ ) and spicules length (27–29  $\mu\text{m}$  vs. 31–41  $\mu\text{m}$ ). These differences are considered as geographical variability.

#### ***New geographical distribution of N. lanceolatus***

After the original description provided by Abolafia & Peña-Santiago (2003), *N. lanceolatus* appears with a more

extended distribution along the sand dunes of Atlantic and Mediterranean Spanish coasts. All populations examined of this species appeared in xeric areas, which have soils with very low humidity and some salinity, the Cabo de Gata zone (Southern Iberian Peninsula) being the most arid area (Capel-Molina, 1982) where the species has been found.

Other additional localities have been sampled in coastal dunes from the north Iberian Peninsula, as well as numerous samples across the Iberian geography, in several habitats, and this species was not found. For this reason, it could be considered as a bioindicator of these xeric habitats.



**Fig. 7.** Bayesian inference tree from known and the newly sequenced *Nothacrobeles lanceolatus* based on sequences of the 28S rDNA region. Bayesian posterior probabilities (%) are given for each clade. Scale bar shows the number of substitutions per site. Detail of the labial probolae of the *Nothacrobeles* species is included.

### Molecular analysis of *N. lanceolatus*

In the phylogenetic tree based on 18S rDNA (fig. 6), the sequenced specimens of *N. lanceolatus* appear to be related with species of the genus *Acrobeles* von Linstow, 1877, having a similarity of 94.7% (36 bp differences among insertions, deletions and substitutions), specifically with members of the subgenus *Seleborca*, with 96.5% (24 bp differences) similarity, while those of the subgenus *Acrobeles* maintain 97.7% similarity (11 bp differences). Also, the genus *Cervidellus* Thorne, 1937 appears in the same clade, having 98.4% similarity (11 bp differences).

In the phylogenetic tree based on 28S rDNA (fig. 7), *N. lanceolatus* appears to be related with *Nothacrobeles trinigrarus*

Tandingan De Ley, De Ley, Baldwin, Mundo-Ocampo & Nadler, 1999 (DQ145646), which shares a similarity of 90.5% (64 bp differences). With *Nothacrobeles sonorensis* (Ragsdale, Mundo-Ocampo, Bumbarger & Baldwin, 2011) Ruiz-Cuenca & Abolafia, 2020 (DQ145632) and *Nothacrobeles borregi* Poiras, Baldwin, Mundo-Ocampo & Bumbarger, 2002 (DQ145645) who appear further, *N. lanceolatus* shares a similarity of 84.3% (105 bp differences) and 81.1% (133 bp differences), respectively. Also, according to previous studies (Mehdizadeh & Shokoohi, 2013; Abolafia, Divsalar, Panahi & Shokoohi, 2014; Ruiz-Cuenca & Abolafia, 2020), the distance with other species of the same genus is even longer: *Nothacrobeles abolafiai* Mehdizadeh & Shokoohi, 2013 (KC182515), *Nothacrobeles*



*cancellatus* (Thorne, 1925) Ruiz-Cuenca & Abolafia, 2020 (HM439765), *Nothacrobeles hebetocaudatus* Abolafia, Divsalar, Panahi & Shokoohi, 2014 (KJ08411) and *Nothacrobeles spatulatus* Tandingan De Ley, De Ley, Baldwin, Mundo-Ocampo & Nadler, 1999 (DQ145644), with which *N. lanceolatus* shares a similarity of 83.1% (113 bp differences), 83.4% (111 bp differences), 75.5% (161 bp differences) and 79.5% (137 bp differences), respectively. Also, in this phylogenetic tree, the sequences of the Spanish *Nothacrobeles* appear near to genera *Acrobeles* and *Cervidellus*, with similarities of 76.9% (154 bp differences) and 73.9% (175 pb differences), respectively.

### Phylogenetic relationships of the *Nothacrobeles* species

The phylogenetic tree based on 28S rDNA fragment (fig. 7) shows that the genus *Nothacrobeles* is clearly polyphyletic. Although only eight of the 21 species of the genus have been sequenced, the clades in the tree show an evolutionary development of the lip region. Thus, three species (*N. abolafiai*, *N. cancellatus* and *N. hebetocaudatus*), related with the genus *Paracrobeles* Heyns, 1968, have similar a lip region pattern appearing at the basal clades, all of which have with labial probolae smooth with long bifurcations and stipe swollen, probably a plesiomorphic condition. *Nothacrobeles spatulatus*, related with the genera *Stegelleta* Thorne, 1938 and *Stegellina* Andrassy, 1984, has low labial probolae lacking bifurcations and pinnae. Two species (*N. borregi* and *N. sonorensis*) have bifurcated labial probolae with smooth bifurcations and low stipe with an expansion on the outer side. Another two species (*N. lanceolatus* and *N. triniglarus*), related with the genera *Cervidellus* and *Acrobeles*, have labial probolae with long or short bifurcated prongs and basal concave expansion on the outer side, both species with pinnae. This last morphological pattern is a probable apomorphic condition, appearing also in species of the genus *Acrobeles*, while some species of the genus *Cervidellus* such as *Cervidellus doorsselaeri* (De Clerck & De Ley, 1990) Boström & De Ley, 1996, morphologically related with *Acrobeles*, present labial probolae with incipient lateral pinnae.

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**Conflicts of interest.** None

**Ethical standards.** All procedures contributing to this study comply with the ethical standards of the relevant national and institutional guides on the care and use of animals.

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