First record of the terrestrial nemertean Geonemertes pelaensis Semper, 1863 (Hoplonemertea: Prosorhochmidae) for Cuba

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First record of the terrestrial nemertean *Geonemertes pelaensis* Semper, 1863 (Hoplonemertea: Prosorhochmidae) for Cuba

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Abstract

The terrestrial nemertean *Geonemertes pelaensis* Semper, 1863 (Hoplonemertea: Prosorhochmidae) is recorded for first time from Cuba based on specimens from Artemisa and La Habana provinces, western Cuba. Both external morphology and histology are consistent with *G. pelaensis* features. Maximum likelihood and Neighbor-joining analysis of mitochondrial cytochrome *c* oxidase I (COI) obtained from Cuban individuals form a well-supported clade with other specimens of *G. pelaensis* from Bermuda and Japan, as well as with *Geonemertes* sp. from Panama. The current record expands the distribution of *G. pelaensis* in the West Indies.

Key words: ribbon worm, DNA barcoding, cytochrome oxidase I, introduced species, range expansion, West Indies

Introduction

The terrestrial nemerteans constitute a small group of ribbon worms that originate from two separate families of marine hoplonemerteans, namely Plectonemertidae and Prosorhochmidae (Moore et al. 2001). For a long time all the species remained placed in the single genus *Geonemertes,* but further studies showed the existence of an assemblage of species that evolved independently (Moore and Gibson 1981; Kvist et al. 2014, 2015). Currently, about 13 species grouped in seven genera are known inhabiting fully terrestrial or intermediate habitats (*i.e.* swamp mangroves and upper littoral) (Moore and Gibson 1981; Moore et al. 2001).

Most of the species of terrestrial nemerteans are recorded from small oceanic islands, mainly from the Pacific and Indian oceans and show a restricted distribution. Only a few species, namely *Argonemertes dendyi* (Dakin, 1915), *Geonemertes pelaensis* Semper, 1863 and *Leptonemertes chalicophora* (Graff, 1879) present a wider distribution. *G. pelaensis* is the most widespread of the aforementioned species and is recorded from islands in the Pacific and Indian oceans and The Caribbean, as well as Florida, U.S.A. and Japan (Gibson and Moore 1998). The more probable *G. pelaensis*

mode of dispersal (as with other terrestrial nemerteans) appears to be by human activities such as the transport of exotic plants used in gardening and nurseries (Moore et al. 2001; Jones and Sterrer 2005). This success of *G. pelaensis* dispersal appears to be increased by being a hermaphroditic species (Moore et al. 2001).

Studies on Cuban nemerteans are quite scarce. Palacios-Lemagne et al. (2008) found a specimen of a terrestrial nemertean in western Cuba, identified as *Geonemertes* sp. Diez (2013) recorded two marine species from eastern Cuba, namely *Baseodiscus delineatus* (Delle Chiaje, 1825) and *Paranemertes* sp. In both reports the terrestrial and the marine nemerteans were identified based only on external morphology. The present paper constitutes the first record of a terrestrial nemertean from Cuba, including both external morphology and histology, as well as molecular techniques.

Materials and methods

Specimens collecting and fixation

Specimens were collected by hand under stones from Sierra de Anafe, Artemisa province, Cuba (n = 5) and the Instituto de Ecología y Sistemática, Havana province, Cuba (n = 5). They were measured alive with a ruler (± 1 mm) and photographed with a Nikon D5300 camera with an AF-S DX Micro NIKKOR 85 mm macro lens. The individuals were narcotized with 10% ethanol and a small fragment of the posterior end was cut from each specimen and stored in 96% ethanol for DNA studies. Worms were fixed in phosphate-buffered 4% formaline and stored in 70% ethanol.

Histological studies

Seven specimens were selected for histological studies. Tissue blocks of the anterior region and the stomach region were embedded in parafin wax. Sagittal and transversal 5–7 µm sections were stained with Cason's one step Mallory-Heidenhain trichrome (Winsor and Sluys 2018) and mounted in Canada balsam or Permount. Micrographs were taken with an AxioCam digital camera attached to a Carl Zeiss Axioskop 2 Plus compound microscope. Scale bars of all plates are given in micrometers. The studied material is deposited in the Colección Helmintológica de las Colecciones Zoológicas (CZACC), Instituto de Ecología y Sistemática, La Habana, Cuba.

DNA extraction, amplification and sequencing

Genomic DNA was extracted with the NucleoSpin® Tissue kit (Macherey-Nagel, Düren, Germany) following manufacturer's protocol. Partial sequences of the DNA barcode mitochondrial COI gene (cytochrome *c* oxidase subunit I) were amplified with the universal primer set LCO1490 (5′-GGT CAA CAA ATC ATA AAG ATA TTG G-3′) and HCO2198 (5′-TAA ACT TCA GGG TGA CCA AAA AAT CA-3′) (Folmer et al. 1994).

PCR reactions were performed in a total volume of 11 µL with the Multiplex PCR Master Mix (Qiagen, USA). PCR cycling parameters consisted of an initial denaturation at 95 °C for 15 min followed by 40 cycles of 95 °C by 30 s, 48 °C by 30 s and 72 °C for 1 min and a final extension step of 72 °C for 10 min. The results of the PCR reactions were checked by agarose gel electrophoresis. PCR products were purified with Exonuclease I and FastAP™ (Thermo Scientific, USA) and the sequences were obtained from both strands using Big Dye Terminator v3.1 chemistry (Applied Biosystems, USA) and the same primers as the PCR reactions.

Raw sequences were manually edited with Sequencher 4.1.4 (http://genecodes.com). Sequences were deposited in GenBank NCBI (http://www.ncbi.nlm.nih.gov/genbank/). The accession numbers are shown in the phylogram.

Phylogenetic analysis

Several sequences of hoplonemerteans (Prosorhochmidae, Malacobdellidae) were selected from GenBank for the phylogenetic analyses (accession numbers in the phylogram). Three species of *Amphiporus* (Amphiporidae) were used as the outgroup taxa.

COI sequences were translated to amino acids, examined for stop codons or gaps, then manually aligned with MEGA6 (Tamura et al. 2013) and retranslated to nucleotides. MEGA6 was also used to determine the optimal model of sequence evolution (GTR+G+I) following the Akaike Information Criterion (AIC), and perform the Maximum likelihood (ML) analysis. Branch support for the ML tree was inferred by bootstrap using 1,000 iterations. Also, MEGA6 was used to perform a Neighbor-Joining (NJ) analysis based on Kimura two-parameter distances (K2P) with 1,000 bootstrap replicates.

Results and discussion

Material examined

Voucher CZACC 18.007; Cuba, Artemisa province, Caimito, Sierra de Anafe (eastern slope); 22°57′27.75″N; 82°36′37.34″W; 30/X/2017; L.F. de Armas coll.; serial sagittal sections (5–7 µm) of the anterior region in 2 slides; a small portion of the posterior end removed for molecular studies; rest of specimen in 70% ethanol.

Voucher CZACC 18.008; Cuba, Artemisa province, Caimito, Sierra de Anafe (eastern slope); 22°57′27.75″N; 82°36′37.34″W; 30/X/2017; L.F. de Armas coll.; a small portion of the posterior end removed for molecular studies; rest of specimen in 70% ethanol.

Voucher CZACC 18.009; Cuba, Artemisa province, Caimito, Sierra de Anafe (eastern slope); 22°57′27.75″N; 82°36′37.34″W; 30/X/2017; L.F. de Armas coll.; a small portion of the posterior end removed for molecular studies; rest of specimen in 70% ethanol.

Voucher CZACC 18.010; Cuba, Artemisa province, Caimito, Sierra de Anafe (eastern slope); 22°57′27.75″N; 82°36′37.34″W; 30/X/2017; L.F. de Armas coll.; serial sagittal sections (5–7 μm) of the anterior region in 4 slides; a small portion of the posterior end removed for molecular studies; rest of specimen in 70% ethanol.

Voucher CZACC 18.011; Cuba, Artemisa province, Caimito, Sierra de Anafe (eastern slope); 22°57′27.75″N; 82°36′37.34″W; 30/X/2017; L.F. de Armas coll.; serial transverse sections (5–7 μm) of the stomach region in 6 slides; a small portion of the posterior end removed for molecular studies; rest of specimen in 70% ethanol.

Voucher CZACC 18.012; Cuba, La Habana, Boyeros, Instituto de Ecología y Sistemática; 23°02′02.41″N; 82°22′44.60″W; J. Morffe, L. Véliz coll.; serial sagittal sections (5–7 μm) of the anterior region in 1 slide; serial transverse sections (5–7 μm) of the stomach region in 1 slide; a small portion of the posterior end removed for molecular studies; rest of specimen in 70% ethanol.

Voucher CZACC 18.013; Cuba, La Habana, Boyeros, Instituto de Ecología y Sistemática; 23°02′02.41″N; 82°22′44.60″W; J. Morffe, L. Véliz coll.; serial transverse sections (5–7 μm) of the stomach region in 3 slides; a small portion of the posterior end removed for molecular studies; rest of specimen in 70% ethanol.

Voucher CZACC 18.014; Cuba, La Habana, Boyeros, Instituto de Ecología y Sistemática; 23°02′02.41″N; 82°22′44.60″W; J. Morffe, L. Véliz coll.; serial sagittal sections (5–7 μm) of the anterior region in 2 slides; serial transverse sections (5–7 μm) of the stomach region in 3 slides; a small portion of the posterior end removed for molecular studies; rest of specimen in 70% ethanol.

Voucher CZACC 18.015; Cuba, La Habana, Boyeros, Instituto de Ecología y Sistemática; 23°02′02.41″N; 82°22′44.60″W; J. Morffe, L. Véliz coll.; a small portion of the posterior end removed for molecular studies; rest of specimen in 70% ethanol.

Voucher CZACC 18.016; Cuba, La Habana, Boyeros, Instituto de Ecología y Sistemática; 23°02′02.41″N; 82°22′44.60″W; J. Morffe, L. Véliz coll.; serial sagittal sections (5–7 μm) of the anterior region in 2 slides; a small portion of the posterior end removed for molecular studies; rest of specimen in 70% ethanol.

The color pattern of the Cuban specimens corresponds with what has been described for most of the localities where the species have been recorded, including the West Indies and Florida, U.S.A. (Gibson and Moore 1998). The background color of the dorsum is cream coloured, with a mid-dorsal dark brown stripe (Figure 1). The single lateral stripe is present on both sides, very faint and incomplete. The ventral side is pale buff in color (Figure 1D). One specimen from the Instituto de Ecología y Sistemática (CZACC 18.014) presented the posterior end of the body dark

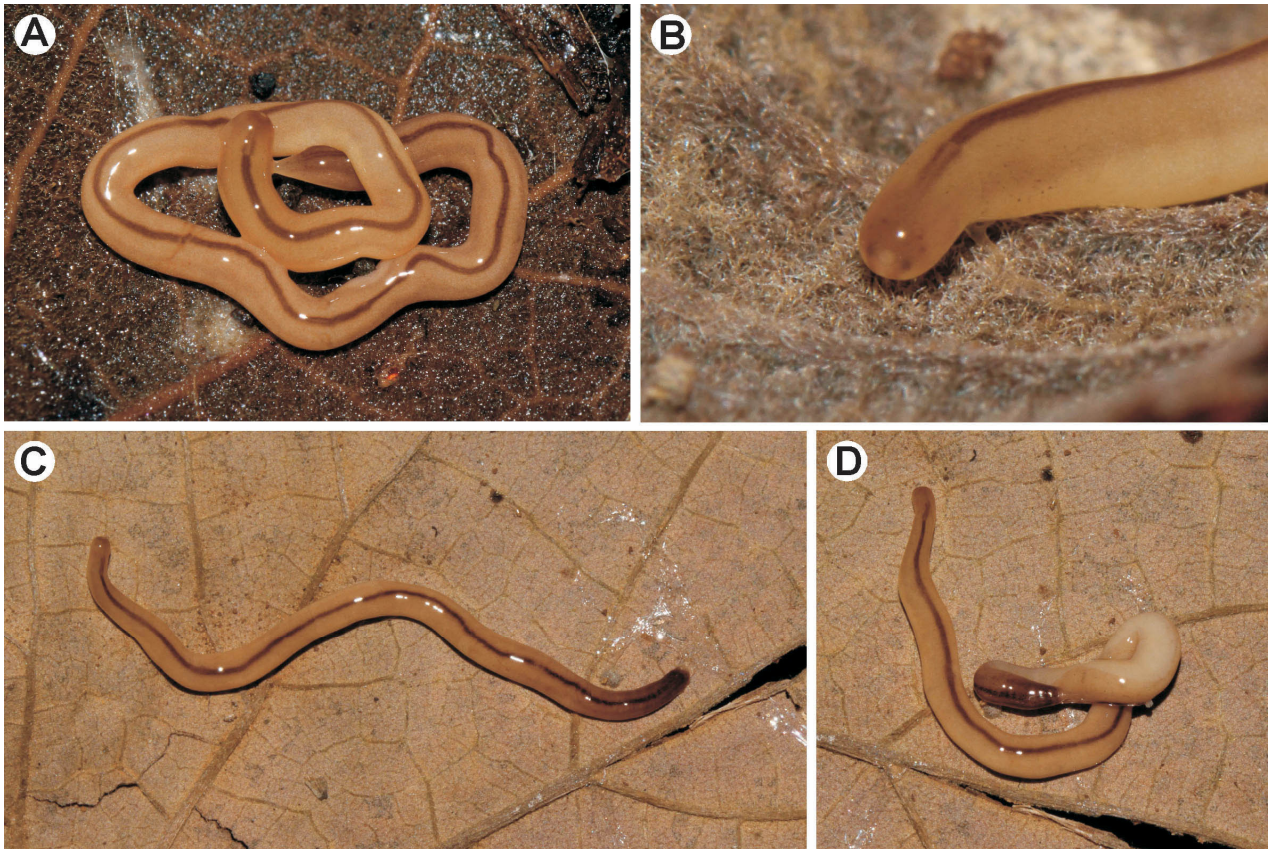

Figure 1. *Geonemertes pelaensis* Semper, 1863 (Nemertea: Hoplonemertea: Prosorhochmidae) from Cuba. Individual from Sierra de Anafe, Caimito, Artemisa province, Cuba deposited in the Helminthological Collection of the Zoological Collections, Instituto de Ecología y Sistemática, Havana, Cuba under the collection number CZACC 18.011. A. Entire specimen. B. Detail of the head. Individual from the Instituto de Ecología y Sistemática, La Habana province, Cuba deposited in the Helminthological Collection of the Zoological Collections, Instituto de Ecología y Sistemática, Havana, Cuba under the collection number CZACC 18.014. C. Entire specimen. D. Entire specimen showing the ventral side. Scale bars not available. Photographs by Jans Morffe.

brown and the mid-dorsal stripe turned blackish towards that region (Figure 1C, D). By the aforementioned color pattern *G. pelaensis* can be readily differentiated from the other two nominal species of the genus. *G. rodericana* (Gulliver, 1879) presents the dorsal background dark green and the mid-dorsal stripe is white. In the case of *G. philippinensis* Gibson & Moore, 1998 the mid-dorsal stripe is cream, on a dark brown background (Gibson and Moore 1998).

The specimens included in our study present six eyes arranged as an anterior, larger pair of eyes and two pairs of smaller and less distinct eyes (Figure 1B). This falls within the most common range of the eye number recorded for the species: 4–6 (Gibson and Moore 1998). In life, the length of the specimens from Sierra de Anafe range 24 mm to 48 mm and in the case of the individuals from the Instituto de Ecología y Sistemática range 40 mm to 55 mm. These measures are within the values of 10–60 mm observed by Gibson and Moore (1998). Jones and Sterrer (2005) observed some specimens with a body length of 70 mm.

The flame cells observed in the Cuban specimens are binucleate and reinforced by longitudinal and transverse support bars (Figure 2A). This coincides with the morphology of the flame cells typical of the genus

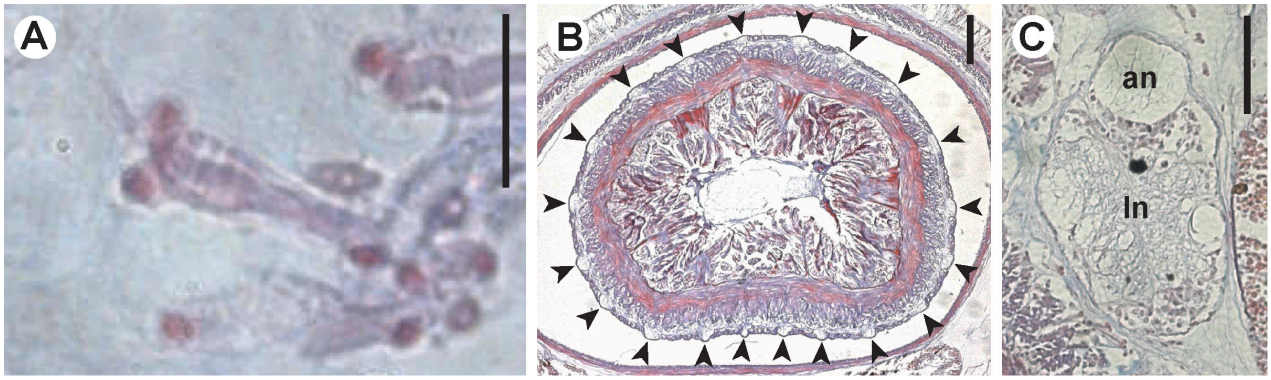

Figure 2. *Geonemertes pelaensis* Semper, 1863 (Nemertea: Hoplonemertea: Prosorhochmidae) from Cuba. Transversal sections at level of the stomach region. A. Binucleate flame cell. B. Proboscis, arrowheads point the proboscis nerves. C. Main lateral nerve cord (ln) and accessory lateral nerve (an). Scale bars: A. 10 µm. B. 100 µm. C. 50 µm. Micrographs by Jans Morffe.

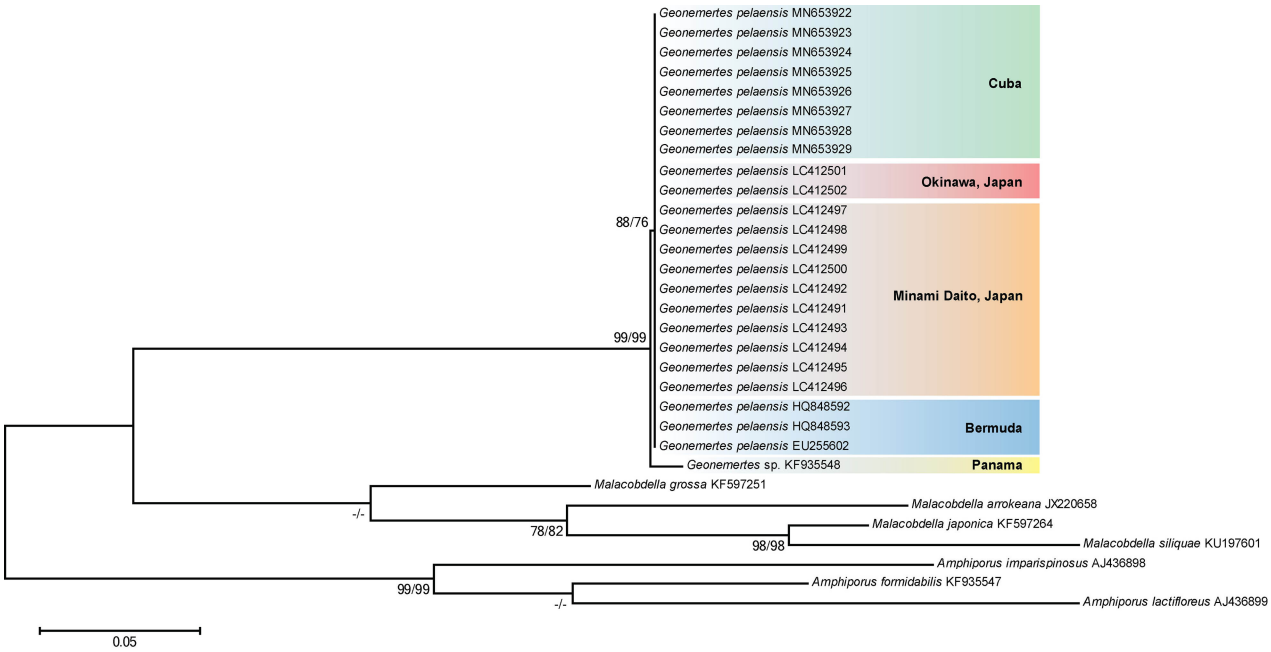

Figure 3. Maximum likelihood (ML) tree inferred from the cytochrome *c* oxidase subunit I (COI) gene for several species of hoplonemerteans (Nemertea: Hoplonemertea). Three species of *Amphiporus* (Amphiporidae) were used as outgroup taxa. Values at the nodes correspond to ML bootstrap resampling (≥ 70)/NJ bootstrap resampling (≥ 70).

Geonemertes (Moore and Gibson 1985; Gibson and Moore 1998). The number of proboscis nerves is variable intraspecifically and *G. pelaensis* presents a number of 16–23. Our results (19–20) fall within this range (Figure 2B). Accessory lateral nerves are present, as is characteristic of the genus *Geonemertes* (Figure 2C). In our individuals, such structures constitute *ca.* 40% of the length of the main lateral nerves. This is consistent with the observations of Gibson and Moore (1998), which recorded that the length of the accessory lateral nerves range from "a quarter or less" to "a third or slightly more" of the diameter of the main lateral nerve.

The topology of both the ML and NJ phylograms was identical and only the former is shown (Figure 3). The Cuban specimens belong to the same haplotype of the available sequences of *G. pelaensis* from Bermuda and two islands of the Japanese archipelago, Minami-Daito and Okinawa, forming

a well-supported monophyletic group. Thus, the phylogenetic analyses support the morphological and histological data and confirm the present specimens as *G. pelaensis*.

The native range of *G. pelaensis* appears to be the Indopacific region where the other congeneric species, namely *G. philippinensis* and *G. rodericana* occur (Moore and Gibson 1981; Gibson and Moore 1998). These taxa share characters that contributes to differentiate them from the other genera of terrestrial nemerteans native from Australia and New Zealand (Moore and Gibson 1981). The current study extends the distribution of *G. pelaensis* in the West Indies. Previously, the species was recorded from Jamaica (Moore and Gibson 1986) and Dominica (Moore and Moore 1982). In the Americas *G. pelaensis* is also present in Florida (Gibson and Moore 1998) and Bermuda (Jones and Sterrer 2005). In addition, Kvist et al. (2014) included a specimen identified as *Geonemertes* sp. from Boca del Toro, Panama in their phylogeny of Nemertea. According to the photograph shown in the paper, the specimen presented the color pattern typical of *G. pelaensis*, with the background of the dorsum cream coloured and the dorso-median dark brown stripe. In the present study, the COI sequences of this individual differ in five homologous positions (in an alignment of 561 bp) and the distance with the sequences of *G. pelaensis* is 0.9%. Still, in order to clarify if these *Geonemertes* belong to another haplogroup of *G. pelaensis* or constitute a new species, additional studies are required, including increased sampling of Panamanian individuals from the same locality and applying histological and molecular techniques.

Geonemertes pelaensis have been found in man-modified areas, such as nurseries and gardens, under plant pots or soil sacks and beneath moist cement blocks and bricks (Jones and Sterrer 2005). Coinciding with the results of these authors, our specimens were also found in areas disturbed by human activities. The individuals from Sierra de Anafe were collected under rocks in a highly altered semi-deciduous forest growing on karst (Figure 4A, B). This is the vegetation unit typical of the hills of western Cuba. The specimens from the Instituto de Ecología y Sistemática were collected under a rock in a garden area (Figure 4C, D). This institution is located in the outskirts of Havana city, in a former country estate that was also a school of horticulture. Due to that, exotic plants and soil were routinely brought to the area for gardening purposes.

The previous record of a terrestrial nemertean from Cuba consisted of a single specimen from the Valley of Viñales, Pinar del Río province, western Cuba. The specimen, identified as *Geonemertes* sp., was fragmented during collection (Palacios-Lemagne et al. 2008). These authors did not provide a detailed description or photograph of the individual and it was not properly fixed and deposited in a collection. Thus, information is not enough to determine whether the record of Palacios-Lemagne et al. (2008)

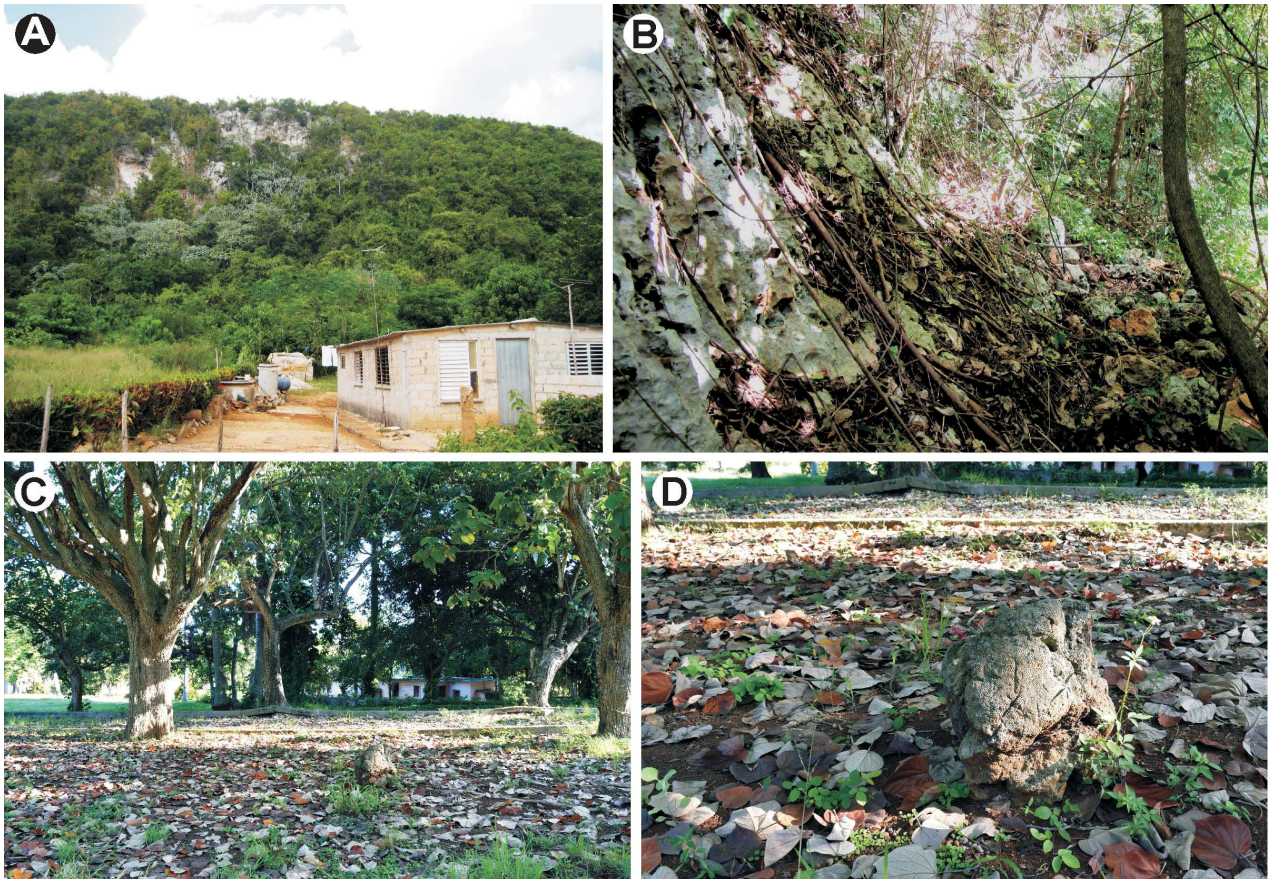

Figure 4. Collecting localities of *Geonemertes pelaensis* Semper, 1863 (Nemertea: Hoplonemertea: Prosorhochmidae) from western Cuba. A, B. Sierra de Anafe, Caimito municipality, Artemisa province. C, D. Instituto de Ecología y Sistemática, Boyeros municipality, La Habana province. Photographs by Luis F. de Armas (A, B) and Jans Morffe (C, D).

corresponds to a species of *Geonemertes* (*i.e.* the herein recorded *G. pelaensis*) or belongs to another taxon. Further surveys on the area are needed in order to collect terrestrial nemerteans and conduct proper morphological, histological and molecular studies.

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