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Taxonomy and systematics

Rediscovery of the nemertean *Prostoma graecense* from Xochimilco, 75 years after its first and only record

Redescubrimiento del nemertino Prostoma graecense *de Xochimilco, 75 años después de su primer y único registro*

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Abstract

The identity of the freshwater nemertean *Prostoma graecense* (Böhming, 1892) from Lake Xochimilco is solved 75 years after its first recording. Identification of the specimens was accomplished via both morphological and molecular means, the latter employing the mitochondrial cytochrome *c* oxidase subunit I. Recent literature and our results suggest that this species has a cosmopolitan distribution and has, presumably, been recently introduced to areas well removed from its original distribution, which is unknown. The current study provides a background for continuing the work with freshwater and marine nemerteans and underscores the importance of including molecular data in future studies of the group.

Keywords: Freshwater invertebrates; Nemertea; Tetrastemmatidae; Cox1; Taxonomy

Resumen

Se resuelve la identidad taxonómica del nemertino dulceacuícola *Prostoma graecense* (Böhming, 1892) del lago de Xochimilco 75 años después del primer registro. La identificación de los ejemplares se realizó con base en caracteres morfológicos y moleculares, esta última empleando el citocromo c oxidasa subunidad I mitocondrial. La literatura reciente y nuestros resultados sugieren que esta especie tiene una distribución cosmopolita y que ha sido introducida, presumiblemente, en áreas lejanas de su distribución original, la cual es desconocida. El presente estudio proporciona un contexto para continuar los estudios sobre nemertinos de agua marina y dulceacuícola y enfatiza la importancia de incluir datos moleculares en futuros estudios del grupo.

Palabras clave: Invertebrados dulceacuícolas; Nemertea; Tetrastemmatidae; Cox1, Taxonomía

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Introduction

Members of the phylum Nemertea (ribbon worms) are characterized by the presence of an eversible muscular proboscis housed within a cavity called a rhynchocoel; this structure is found in no other animal group. Nemerteans are unsegmented, bilaterian, vermiform animals with a closed circulatory system and protonephridia as excretory organs. The body size of these worms range in length from a few millimeters up to 30 meters (Sundberg & Strand, 2010; Strand & Sundberg, 2015), representing the longest metazoan on Earth. Members of Nemertea are mostly marine, with only 22 out of the 1,280 described species inhabiting freshwater (Kajihara et al., 2008; Sundberg & Gibson, 2008; Strand & Sundberg, 2015). Most of the species diversity within the group occurs in the Nearctic and Palearctic, but this may only reflect the intensity of sampling efforts and taxonomic work rather than a real biogeographical pattern (Strand & Sundberg, 2015). Because of the difficulty of identifying preserved specimens, several authors have suggested that morphological identification should always be accompanied by DNA sequence analyses (Chen et al., 2010; Sundberg & Strand, 2010; Sundberg et al., 2010, 2016).

Only a single freshwater nemertean is known from Mexico, originally referred to as *Stichostemma rubrum* (Leydi, 1850) from Lake Xochimilco, México City (Rioja, 1941). Gibson and Moore (1976) listed *S. rubrum* under the genus *Prostoma* and concluded that no typically North American species of *Prostoma* can be recognized, thus the Mexican records of *P. rubrum* should be considered as *P. graecense*. In the most recent taxonomic account of freshwater nemerteans from North America, only 2 species were recognized: *Prostoma canadiensis* Gibson & Moore, 1978, which possesses a frontal organ (including previous records of *P. graecense*), and *Prostoma asensoriatum* (Montgomery, 1896), which lacks such an organ (Strand & Sundberg, 2015). To robustly infer the presence of a frontal organ, histological preparations are required, a procedure that has yet to be used to characterize nemerteans from Mexico.

More than 75 years have passed since the first and only record of the freshwater nemertean from Xochimilco, an area well known for its expansive biodiversity and number of endemics; arguably the most charismatic of these is Axolotl [*Ambystoma mexicanum* (Shaw & Nodder, 1798)] and the Montezuma leopard frog [*Lithobates montezumae* (Baird, 1854)]. During that time, Xochimilco has changed dramatically, due to a steep increase in human population numbers and the intentional and unintentional introduction of several exotic species that have reshaped the ecosystem, including the common carp *Cyprinus carpio* Linnaeus, 1758, tilapia *Oreochromis niloticus* (Linnaeus, 1758), and the water hyacinth *Eichhornia crassipes* (Mart.) Solms. (Cervantes-Sánchez & Rojas-Rabiela, 2000; Zambrano et al., 2010). The aim of the present study is to document the presence of the freshwater nemertean in Xochimilco, an area that has been severely impacted by human activities. In addition, this study aims to solidify the taxonomic and nomenclatural status of the nemertean and to generate the mitochondrial cytochrome *c* oxidase subunit I (cox1) DNA sequence, the preferred zoological barcoding region, which may serve as a framework for future studies of this charismatic taxon.

Materials and methods

In February, April, and October of 2016, 17 nemerteans were collected from "Embarcadero de Cuemanco", Xochimilco, México City, Mexico (19º17'20.934" N, 99º06'06.585" W). Specimens were found within the roots of water lily *Eichhornia crassipes* that were collected from the edges of canals. The aquatic plants were placed in trays and, after about 10 minutes, ribbon worms were recovered from the bottom of the tray with the use of delicate paintbrushes. For subsequent morphological procedures, worms were relaxed in 7% $MgCl_2$ and fixed in 4% formaldehyde. Two specimens were stained with Gomori trichrome and mounted on permanent slides with Canada balsam. For histological procedures, worms were fixed in 10% formaldehyde in phosphate-buffered saline (PBS) at pH 7.2, dehydrated with graded ethanol series and embedded in Epon epoxy resin. Semi-thin serial sections, obtained with a Leica Ultracut UTC Ultramicrotome, were stained with toluidine blue. In addition, 2 worms were dehydrated and critical point dried with CO_2, mounted and coated with a mixture of gold-palladium and subsequently observed in a Hitachi model SUI510 scanning electron microscope (SEM) at the Laboratorio Nacional de Biodiversidad, Instituto de Biología, Universidad Nacional Autónoma de México (IB-UNAM). Voucher specimens were deposited in the National Invertebrates Collection at the IB-UNAM (accession number CNINV-002, 003). For molecular analyses, cox1 sequences from 3 specimens were obtained via procedures described elsewhere (Kvist et al., 2014; Oceguera-Figueroa et al., 2005). The newly generated cox1 sequences were aligned and compared with 15 sequences of members of the suborder Monostilifiera (Class Enopla) available from GenBank (Table 1) and selected based on BLASTn (Altschul et al., 1990) matches. Nucleotide sequences were aligned with ClustalW (Thompson et al., 1997), implemented in the web-version of the software at http://www.genome.jp/tools/clustalw/. The edges of the alignment were trimmed to include sequences

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Table 1

Taxa used for the maximum likelihood analysis of nemerteans, including *Prostoma graecense* from Lake Xochimilco, Mexico with other specimens of the genus with localities and GenBank accession codes.

Taxon	Locality	GenBankaccession number
Gurjanovella littoralis	Kandalaksha Bay, White sea, Russia	AJ436904
Prosorhochmus nelsoni	Coquimbo, Chile	EF157586
Prosorhochmus belizeanus	Carrie Bow Cay, Belize	EF157591
Prosorhochmus cf. *claparedii*	Armintza, Bizkaia, Spain	EF157590
Prosorhochmus americanus	Turbeville, Pawley's Island, SC, USA	EF157588
Prosorhochmus chafarinensis	Savudrija, Adriatic Sea, Croatia	EF157587
Prostoma sp.	Los Angeles, USA	HQ938796
Prostoma sp.	Los Angeles, USA	HQ939311
Prostoma graecense	Ohrid Lake, Macedonia	JX017298
Prostoma graecense	West coast, Sweden	EF208981
Prostoma graecense	West coast, Sweden	EU489490
Prostoma graecense 1	Xochimilco, Mexico	KY523851
Prostoma graecense 2	Xochimilco, Mexico	MF314116
Prostoma graecense 3	Xochimilco, Mexico	MF314117
Nemertea sp.	Italy	KU840284
Prostoma cf. *eilhardi*	Eastbrook Woods, USA	HQ848594
Nemertopsis bivittata	South Carolina, USA	HQ848608
Psammamphiporus elongatus	Coruña, Galicia, Spain	HQ848609

of the same length. The final dataset included 18 terminals and 677 aligned characters. Phylogenetic analysis was run under the maximum likelihood (ML) criterion, employing a GTR+GAMMA+I model as suggested by jModelTest ver. 2 (Darriba et al., 2012). Likelihood inference (20 replicates), model parameters, and bootstrap (BS) support (500 pseudoreplicates) were implemented with RAxML v. 7.0.4 (Stamatakis, 2006). The resulting phylogenetic tree was visualized with the software FigTree ver. 1.4.2 (Rambaut, 2012). Based on the phylogenetic hypotheses of Andrade et al. (2012), *Nemertopsis bivittata* (Delle Chiaje, 1841) and *Psammamphiporus elongatus* (Stephenson, 1911) were selected as the outgroup. Genetic distances between selected terminals were calculated as uncorrected *p*-distances using MEGA ver. 5 (Tamura et al., 2011).

Results

Nemerteans small (8.45 ± 0.21 mm length by 0.67 ± 0.02 width), whitish-reddish, some specimens greenish when alive, number of eye spots variable, from 2-6 on the anterior end (Fig. 1A); proboscis large, with integument projections (Fig. 1B), with stylets at the mid-portion (Fig. 1C); rhynchocoel dorsal to the digestive tract, as large as proboscis (Fig. 1D). Histological analysis revealed a distinctive ciliated esophagus and no improvised ducts were observed (Fig. 1E). Frontal organ recovered with cephalic glands openings inside (Fig. 1F).

Maximum likelihood phylogenetic analysis (Fig. 2) recovered the 3 newly generated cox1 sequences from the Xochimilco specimens in a single group and nested within species of the genus *Prostoma* with 100% BS. *Prostoma graecense* from Sweden and Macedonia exhibit marginal genetic distances (> 0.2%) when compared to the Mexican samples. This group is sister to a moderately supported group (BS = 64%) formed by *Prostoma* cf. *eilhardi* from USA and a sample labeled as Nemertea sp. from Italy. The genetic distances between the members of these 2 latter groups are lower than 0.8%. Sister to this group are 2 identical sequences of unidentified *Prostoma* specimens from Los Angeles, California, USA with an average cox1 divergence of 3.2% with respect to the *P. graecense* group + the sample from Xochimilco.

Discussion

The placement of the 3 samples of nemerteans from Xochimilco, Mexico within a group of samples from

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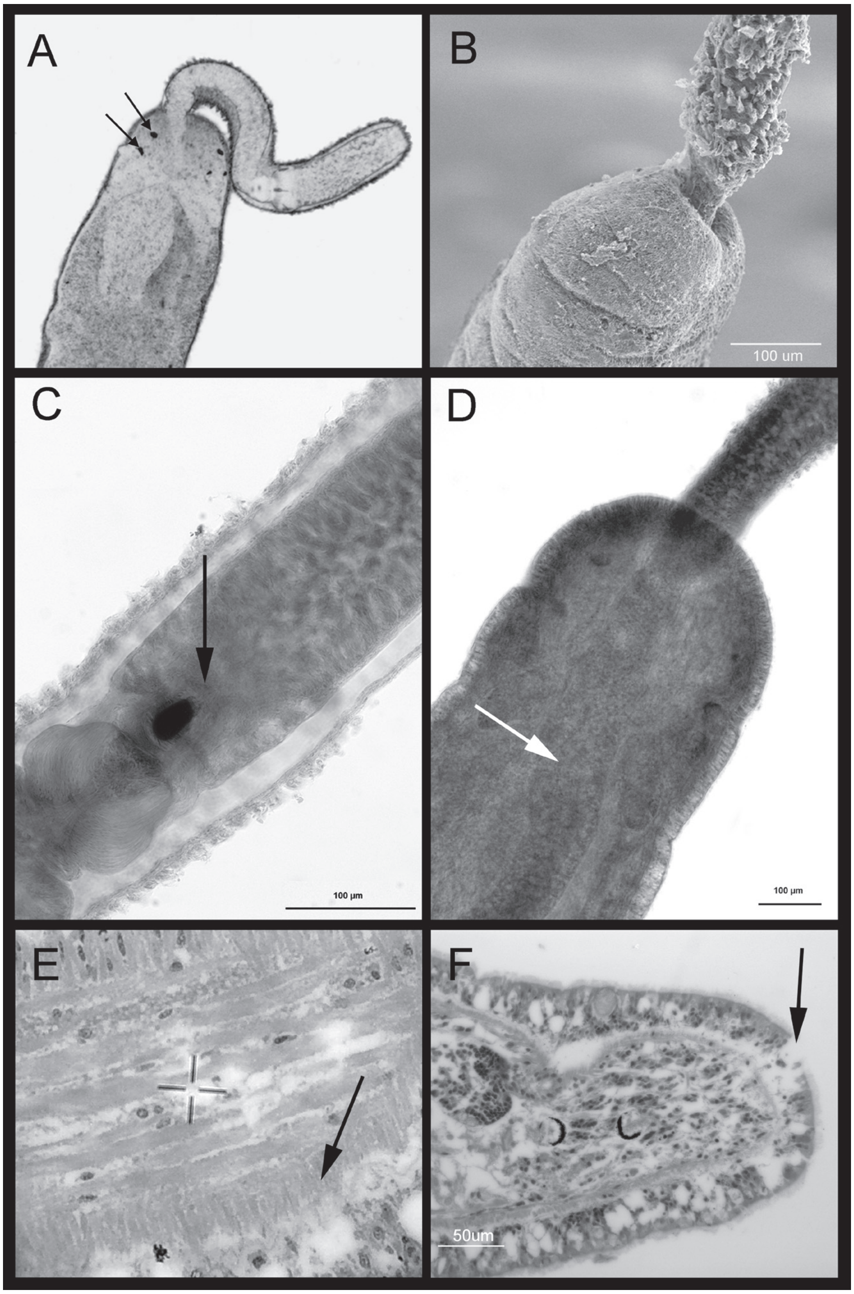

Figure 1. *Prostoma graecense* from Xochimilco, Mexico. Living specimen: A) eyes spots (black arrow). Microphotography with SEM: B) details of integument projections of the proboscis. Specimens stained with Gomori trichrome: C) stylet in the middle portion of proboscis (black arrow); D) rhynchocoel (white arrow). Sagittal section through the body: E) ciliated epithelium of the esophagus (black arrow) at middle body region; F) cup-shaped frontal organ (black arrow) at the cephalic region.

disparate areas including Macedonia, Italy, Sweden, and USA, and with genetic distances lower than 1%, strongly suggests that all of these specimens belong to the same species. This result contrast with the most recent taxonomic treatment of the group by Sundberg and Gibson (2008) and Strand and Sundberg (2015) who applied the name *Prostoma canadiensis* for the North American freshwater nemerteans in possession of a frontal organ and maintained the name *Prostoma graecense* for specimens from other major biogeographic areas in possession of the

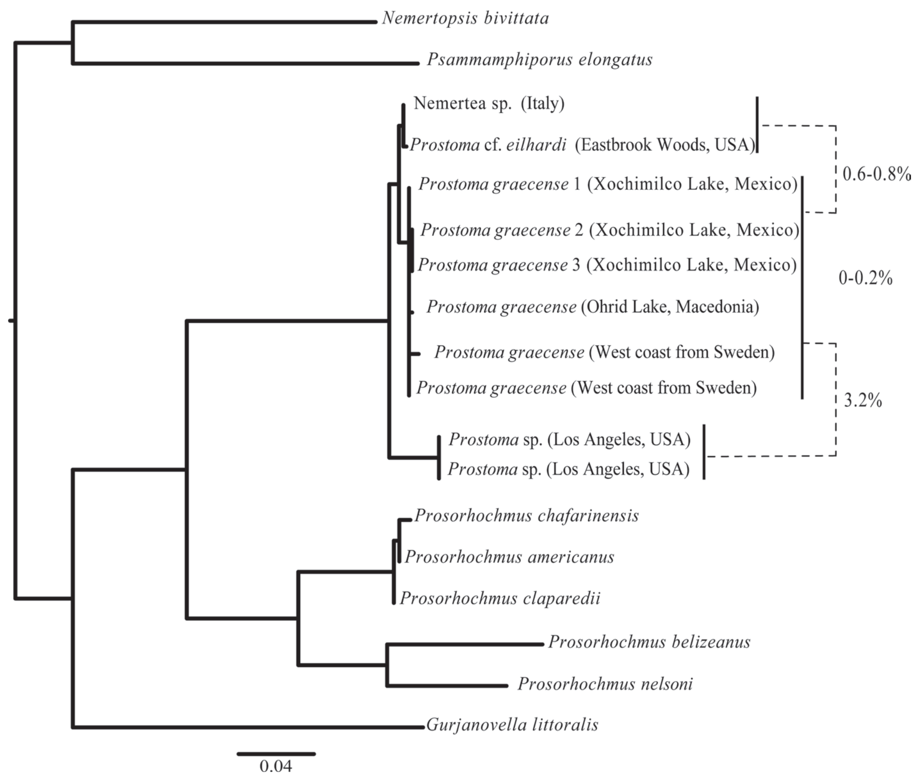

Figure 2. Phylogenetic tree obtained from the analysis of maximum likelihood including 3 samples of *Prostoma graecense* from Xochimico, Mexico in bold. Dotted lines show the genetic distances between the groups of *Prostoma*.

same organ. The presence of a frontal organ in the samples from Mexico and the negligible genetic divergence found in the 3 Mexican specimens and between the samples labeled as *P. graecense* from North America and Europe, allowed us to suggest that all of them should be considered the same species for which the name *P. graecense* should be used. The unusual disjunct biogeographic distribution accompanied with low genetic divergences found by Strand and Sundberg (2005) in their analysis of partial 18S rRNA sequences of 3 *P. graecense* samples from Sweden and New Zealand and a sample labeled as *P. eilhardi*, was interpreted as evidence of a recent introduction, and suggested that these taxa should potentially be synonymized. The cox1 locus has been suggested as a reliable marker for species delineation in nemerteans under the assumption that 4-5% divergence indicates a difference in species-level identity (Sundberg et al., 2016). In our results, the low genetic divergence in cox1 (less than 1%) when comparing the samples from Xochimilco and other localities is also interpreted as a relatively recent introduction. The fact that international efforts to introduce the South American water hyacinth, at least in Europe and North America were conducted intensively in the second half of the 19th Century, may explain the current distribution of *P. graecense*, a species that might be transported inadvertently when introducing this aquatic plant. This phenomenon also has been recorded in other freshwater invertebrates such as leeches (Garduño-Montes de Oca et al., 2016; Reyes-Prieto et al., 2013) and the red crayfish *Procambarus clarkii* (Hernández et al., 2008). *Prostoma graecense* has, based mainly on morphological data to be confirmed by molecular studies, a cosmopolitan distribution: in the Americas (from USA to Argentina), Europe, Asia, Africa, Australia, and Oceania (Cordero, 1943; Corrêa, 1986; Pennak, 1989; Tamburi & Cazzaniga, 2006; Weidenbach, 1995).

Phylogenetic analyses by Kvist et al. (2015) with 158 terminals and 4 molecular markers, recovered a monophyletic group with *Prostoma* cf. *eilhardi* and brackish and marine sacconemertids (Sacconemertidae), suggesting that at least in this part of the tree, the transition from marine to brackish/freshwater habitat may be the result of a single transitional event. Unfortunately, this hypothesis cannot be tested with our dataset given that our taxon selection, based on BLASTn matches, only recovered species of *Prosorhochmus* as the most similar sequences to those of *Prostoma*, leaving the transitional hypothesis to be tested in future studies after the inclusion of more species of *Prostoma* and additional markers.

Enrique Rioja Lo Bianco, who first reported the nemertean from Xochimilco, also contributed to the characterization of Mexican fauna of annelids and crustaceans (Caso, 1964; Dosil-Mancilla & Cremades-Ugarte, 2004). In addition, he studied freshwater sponges, hydrozoans, and bryozoans (Rioja, 1940a, b, c; 1953a, b). These taxa, like Nemertea, have not been studied in the last 75 years in Mexico. The omission of taxonomic studies in several phyla of invertebrates is a serious problem for the current studies of comparative biology and biodiversity.

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