

## Research Article

## Biological introductions or native ranges: two curious cases of new distributional records in the Panama Canal

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### Abstract

We report two new, curious records of freshwater worms collected in the Panama Canal, Panama: the ribbon worm, *Apatronemertes albimaculosa* Wilfert and Gibson, 1974, and the leech, *Placobdella parasitica* (Say, 1824). Identification of these specimens relied on morphological examinations and, more heavily, on molecular barcoding using the mitochondrial COI locus. Both phylogenetic and genetic distance analyses were employed to robustly establish the identities of the specimens. Whereas the native range of *A. albimaculosa* is currently unknown, *P. parasitica* was first described from Minnesota, USA and is possibly native to southern Canada and most of the USA. Neither species has previously been recorded from Central America. We briefly discuss possible scenarios for the dispersal of both species, and conclude that the introduction of *P. parasitica* into Panamanian waters was likely human-mediated in association with the introduction of its turtle host. Insofar as ours is the first record of wild-caught *A. albimaculosa* worldwide, we do not exclude the possibility of Panama being within the native range of the species. However, it is also possible that the species has been transported with ballast water into the Panama Canal; a relatively common phenomenon.

**Key words:** introduced species, Nemertea, ribbon worms, Hirudinea, leech, phylogeny, DNA barcoding

### Introduction

Linking the Atlantic Ocean to the Pacific Ocean, the Panama Canal is a major center for shipping activity, and one of the world's most heavily trafficked aquatic thoroughways (Mason and Rowlands 1938; Carse 2012). Every year, over 14,000 vessels traverse the narrow channel which stretches from Rio Chagres on the Caribbean slope in the north to Rio Grande on the Pacific slope in the south, effectively crossing the Continental Divide (Ruiz et al. 2006). This tremendous movement across the canal, coupled with the evacuation and loading of ballast water, has led to several cases of biological introductions to the Panama Canal, as well as the dispersal of organisms between the Atlantic and Pacific Oceans (Hanna 1966; Aron and Smith 1971; Jones 1972; Wasson et

al. 2001; Wonham et al. 2001; Smith et al. 2004; Cohen 2006; Ruiz et al. 2009; Schlöder et al. 2013). For example, the Harris mud crab *Rhithropanopeus harrisi* (Gould, 1841) native to the eastern coastal ranges of North America, currently seems to be well-established throughout several parts of the Panama Canal (Roche and Torchin 2007). Parasitic taxa, such as the Asian fish tapeworm *Schyzocotyle acheilognathi* (Yamaguti, 1934), have also been inadvertently introduced along with their hosts (Choudhury et al. 2013).

Nemertea (ribbon worms) is a predominantly marine taxon with only 22 out of the 1,280 species inhabiting freshwater (Gibson and Moore 1976; Kajihara et al. 2008; Sundberg and Gibson 2008; Strand and Sundberg 2015). The freshwater species *Apatronemertes albimaculosa* Wilfert and Gibson, 1974

(Nemertea: Heteronemertea: Lineidae) was originally described from aquarium plants (*Vallisneria spiralis* Linnaeus, 1758) in Düsseldorf, Germany (Wilfert and Gibson 1974), and was, by all accounts, artificially introduced from an unknown, but likely tropical location, together with plant material (Kajihara et al. 2016). Since then, the species has been reported from Austria, Spain, USA, and Japan (Senz 1993; Kajihara et al. 2016). In all cases, specimens were found in freshwater aquaria, suggesting that this species hitchhikes with plant material sold in the aquarium trade. Indeed, *A. albimaculosa* has been recorded in association with a wide variety of plants; a detailed list of potential carriers can be found in Kajihara et al. (2016). As a corollary, a different species of freshwater nemertean, *Prostoma graecense* (Böhming, 1892), has also been recorded from disjointed locations in Europe, Oceania and North America. Its odd distribution is a result of recent, accidental introduction by human activities (Lagunas-Calvo et al. 2018).

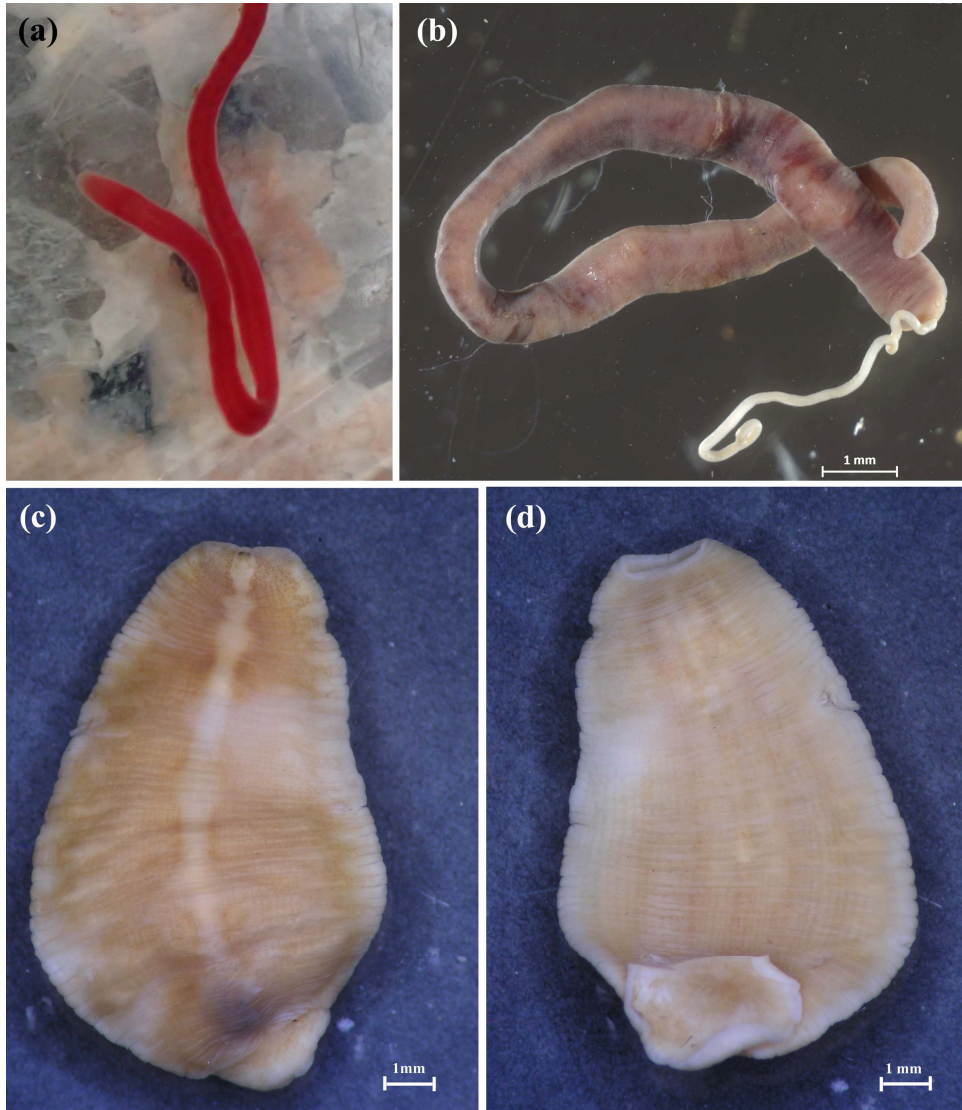
Equally prone to hitchhiking, the freshwater leech *Placobdella parasitica* (Say, 1824) (Annelida: Rhynchobdellida: Glossiphoniidae) is ectoparasitic on a wide range of freshwater turtles (Davy et al. 2009). The species feeds exclusively on blood from its hosts, and its known geographic range stretches from southern Canada in the north, to Texas, USA in the south (Klemm 1985; Sawyer 1986; Dodd Jr. 1988; Davy et al. 2009). According to historical and contemporary accounts (Sawyer 1986; Dodd Jr. 1988; Brooks et al. 1990; Ryan and Lambert 2005; Davy et al. 2009), *P. parasitica* is chiefly encountered attached to its hosts, suggesting that the host may limit its dispersal capabilities. *Placobdella parasitica* has been documented from a rather wide host range, including Blanding's turtle *Emydoidea blandingii* (Holbrook, 1838); northern map turtle *Graptemys geographica* (Lesueur, 1817); snapping turtle *Chelydra serpentina* (Linnaeus, 1758); wood turtle *Glyptemis insculpta* (Le Conte, 1830); painted turtle *Chrysemys picta* (Schneider, 1783); spotted turtle *Clemmys guttata* (Schneider, 1792); stinkpot *Sternotherus odoratus* (Latreille in Sonnini and Latreille, 1801); bog turtle *Glyptemis muhlenbergii* (Schoepff, 1801); eastern mud turtle *Kinosternon subrubrum* (Lacépède, 1788); pond slider *Trachemys scripta* (Thunberg in Schoepff, 1792); and spiny softshell turtle *Apalone spinifera* (Lesueur, 1827) (Davy et al. 2009 and references therein). Furthermore, despite robust collection efforts, *Placobdella parasitica* has never been recorded from Mexico or Central America (Cornejo et al. 2015; Ocegüera-Figueroa and Pacheco-Chaves 2012; Tessler et al. 2018).

In the present study, we report the first records of the nemertean *Apatronemertes albimaculosa* and the leech *Placobdella parasitica* from the vicinity of the Panama Canal, and discuss potential circumstances leading to their disjointed distributions.

## Material and methods

### *Specimen collection and DNA sequencing*

Eleven leech specimens were collected in December 2015 from the exposed skin of the pond slider (*Trachemys scripta*) in Rio Chagres, Gamboa (09°06.96017'N; 79°41.60917'W). Four specimens of ribbon worms were collected in June 2017 from submerged logs and rocks in a small, unnamed pond directly adjacent to Lago Miraflores (Panama Canal) (09°00.680'N; 79°35.730'W). The fauna associated with the nemerteans included various crustaceans (mainly crabs and isopods), freshwater polychaetes, insect larvae, and smaller fish. Nemertean specimens were photographed prior to preservation and all specimens were preserved in 95% EtOH. A small portion of the posterior end (= caudal sucker of *P. parasitica* to avoid contamination by the blood meal) was cut and used for DNA sequencing. The present study targeted a 658 bp region of the mitochondrial locus cytochrome *c* oxidase subunit I (COI), commonly used for zoological DNA barcoding (see Hebert et al. 2003) and that has been successfully applied to the identifications of both leeches and nemerteans in previous studies (Lagunas-Calvo et al. 2018; Kvist et al. 2010). Extraction of DNA was carried out using the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA, USA) following the manufacturer's protocol. Sequences for COI were amplified for each sample using 25 µl PCR reactions, consisting of 16.39 µl ddH<sub>2</sub>O; 2.5 µl buffer; 2.5 µl MgCl<sub>2</sub>; 1 µl of each primer [HCO2198 (5'-TAAACTTCAGGGT GACCAAAAATCA-3') and LCO1490 (5'-GGTC AACAAATCATAAAGATATTGG-3'); Folmer et al. 1994] at 10 µM concentration; 0.56 µl dNTPs at 10 mM concentration; 0.05 µl Platinum *Taq* polymerase (Invitrogen, Carlsbad, CA); and 1 µl total genomic DNA. PCR products were subsequently purified with ExoSAP-IT (Affymetrix, Santa Clara, CA) according to the manufacturer's protocol. Cycle sequencing reactions (10 µl total volume) were carried out using 0.5 µl each of ABI Big Dye Terminator V 3.1 and Big Dye 5x Sequencing Buffer (Applied Biosystems, Carlsbad, CA); 2 µl primer at 10 µM concentration; and 2.5 µl purified PCR product. Reaction mixtures were heated to 96 °C for 1 min. followed by 30 cycles of 96 °C (10 s), 50 °C (5s) and 60 °C (4 min). The samples were sequenced on



**Figure 1.** Select photos of collected specimens. (a) live habitus of a specimen of *Apatronemertes albimaculosa* from a pond adjacent to Lago Miraflores (Panama Canal), (b) preserved specimen of *Apatronemertes albimaculosa* from a pond adjacent to Lago Miraflores (Panama Canal), (c) dorsal view of preserved specimen of *Placobdella parasitica* from Rio Chagres, Gamboa, (d) ventral view of preserved specimen of *Placobdella parasitica* from Rio Chagres, Gamboa. Photographs by Alejandro Ocegüera-Figueroa.

an ABI PRISM 3730XL DNA Analyzer (Applied Biosystems, Carlsbad, CA). All resulting sequences were deposited in GenBank (accession numbers MH128987–MH128991). Leech specimens were deposited in the Colección Nacional de Helmintos (CNHE: 10678) at the Universidad Nacional Autónoma de México, Mexico and nemerteans were deposited in the Colección Nacional de Invertebrados (CNINV: 107) at the Universidad Nacional Autónoma de México, Mexico. Specimens were collected under permit SE/A-61-17 issued by MiAmbiente, Dirección de Áreas Protegidas y Vida Silvestre, Panama.

#### *Morphological and molecular identifications*

Morphological examinations were carried out using a Zeiss Discovery V8 (Toronto, Canada) dissecting microscope, and provisional identifications were affixed to the specimens. Nemerteans were identified to order-level, whereas the leeches were identified to species-level; see Figure 1 for select photographs of the specimens; for detailed morphological accounts of *A. albimaculosa*, see Wilfert and Gibson (1974) and Kajihara et al. (2016); and for accounts of *P. parasitica*, see Moser et al. (2013). Provisional

identifications were later corroborated and refined with the aid of DNA sequences, employing a standard BLASTn search approach against the non-redundant (nr) database on GenBank.

Additionally, phylogenetic trees were constructed for each taxon in order to provide robust identifications of the newly collected specimens under a tree-based framework. One analysis for each target taxon was conducted using a subset of the data from de Carle et al. 2017 (for the leech) and Kvist et al. 2015 (for the nemertean). The datasets were separately aligned using the online version of MAFFT ver. 7 (Katoh and Standley 2013) employing the “auto” option and default settings. PartitionFinder ver. 1.1.1 (Lanfear et al. 2012) was used to estimate the optimal partitioning scheme and model of nucleotide evolution under the Akaike Information Criterion, considering each codon position. Maximum likelihood analyses were then performed with RAxML ver. 8 (Stamatakis 2014) on the CIPRES platform (Miller et al. 2010). For the nemertean dataset, analyses used a GTR+ $\Gamma$  model of nucleotide evolution for three partitions (two of these also included a proportion of invariable sites parameter). For the leech dataset, two partitions (both following a GTR+ $\Gamma$  model of nucleotide evolution) were suggested by PartitionFinder and one of these included a proportion of invariable sites. The RAxML runs consisted of 1,000 iterations with 25 initial GAMMA rate categories that were allowed to fluctuate independently for each partition, and final optimization with four GAMMA shape categories. Support values for the clades were estimated via 100 iterations of the rapid bootstrap algorithm using the same settings as above.

Uncorrected *p*-distances were calculated in PAUP\* ver. 4.0b10 (Swofford 2002) applying the following settings: function of minimal evolution, ignoring gaps for affected pairwise comparisons (although no gaps were present in the resulting alignments) and equal rates for variable sites.

## Results

### *Morphological identifications*

Based on external morphology only (no internal morphological analysis was carried out, as these are most often not needed for accurate specimen identifications [see Sundberg et al. 2016]), our nemertean specimens agree with the description of *Apatronemertes albimaculosa*. Importantly, live specimens showed a bright red coloration, with small flecks of white; a bluntly rounded head that is not demarcated from the remainder of the body; cephalic grooves extending from tip of the head to close to the mouth;

and a lack of eye-spots, frontal organ and caudal cirrus (Figure 1a, b). See Sundberg et al. (2016) for an in-depth discussion on the utility of external morphology for the identification of nemertean species.

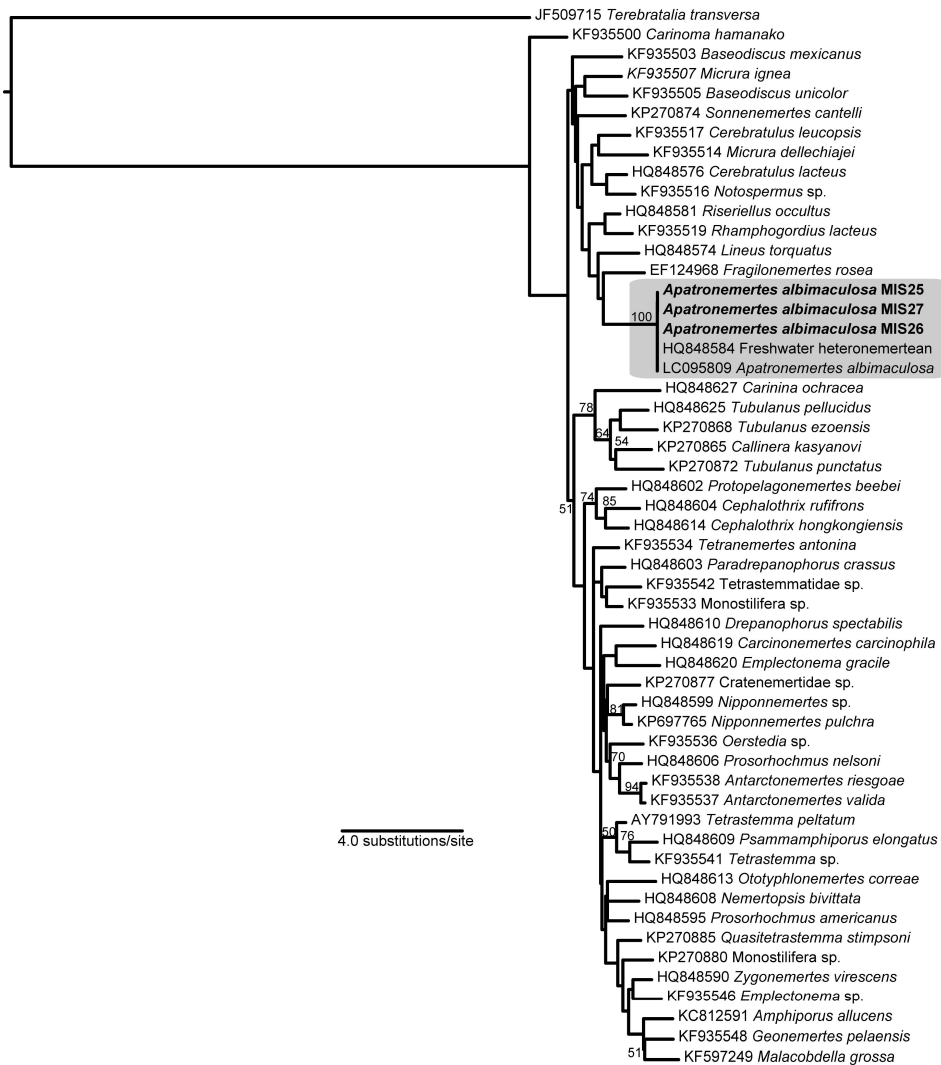
The newly collected specimens of *Placobdella parasitica* are compatible with the original description (Say 1824), as well as the redescription (Moser et al. 2013) of the species. In particular, our specimens display a smooth (unpapillated) dorsum with a yellow medial line, and 8–12 darker, sometimes blue, anteroposterior lines on its venter (Figure 1 c, d).

### *Molecular identifications*

DNA from three ribbon worm specimens and two leech specimens was successfully sequenced. The final alignments each consisted of 658 aligned sites for 54 (Nemertea) and 37 (Hirudinea) terminals. Initial BLASTn searches suggested that the ribbon worms belong to the species *Apatronemertes albimaculosa* (e-values = 0; identity scores 98%–99%), and that the leeches belong to *Placobdella parasitica* (e-values = 0; identity scores 99%). The resulting phylogenetic hypotheses corroborate these identifications (Figures 2 and 3), as both taxa nest within highly supported clusters of conspecific individuals with short branch lengths. Average uncorrected *p*-distances between our specimens of *Apatronemertes albimaculosa* and that of the only other nominal specimen deposited in GenBank, which was collected in Japan (accession number LC095809; Kajihara et al. [2016]), is 0.06%  $\pm$  0.009. The same distance measure for the specimens of *Placobdella parasitica*, when compared to specimens from a variety of North American localities is 1.62%  $\pm$  0.27; when compared only to specimens collected at the type locality of the species (see Moser et al. 2013), the same value is 1.53%  $\pm$  0.08. It is worth noting that relatively high levels of genetic diversity seem to be common in *P. parasitica*: sequences for this species deposited in GenBank show a maximum pairwise COI distance of 2.04%. Notwithstanding this genetic disparity, specimens of *Placobdella parasitica* are easily identified using unequivocal morphological characters.

## Discussion

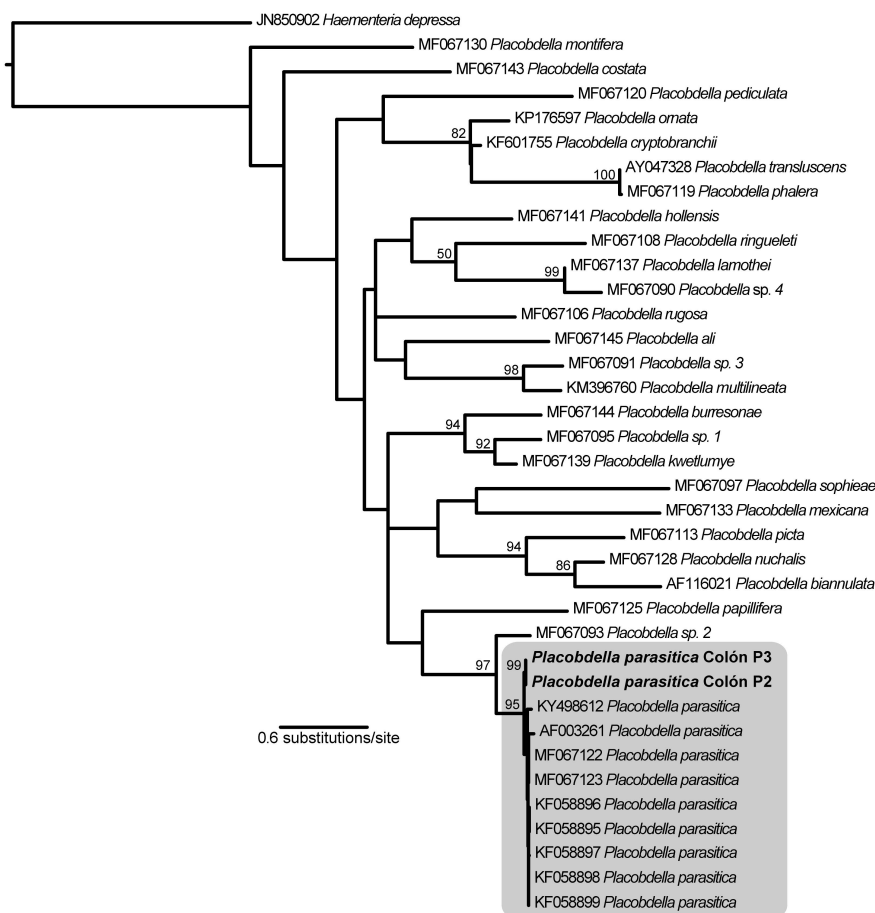
One nemertean species, *Apatronemertes albimaculosa*, and one hirudinean species, *Placobdella parasitica*, are recorded for the first time from the Panama Canal, Panama. The nemertean species has hitherto only been recorded in conjunction with plant material related to the aquarium trade in Europe, Japan, and USA. Our new records represent the first



**Figure 2.** Maximum likelihood tree ( $lnL = -11845.231952$ ) resulting from the analysis of the nemertean COI dataset. Maximum likelihood bootstrap values above 50% are shown above each node, newly sequenced taxa are shown in bold font and the target taxa are highlighted by a grey background. GenBank accession numbers are noted before each taxon name.

case of wild-caught specimens, possibly suggesting that the species is native to the area surrounding the Panama Canal. The freshwater biota of the Panamanian Isthmus is known to be heavily influenced by marine ecosystems (Hildebrand 1939; Menzies 1968; San Martín et al. 1998; Ruiz et al. 2006; Galil et al. 2008), such that several species from predominantly marine taxonomic groups seem to be able to persist in freshwater habitats of Panama. The presence of *A. albimaculosa* in this freshwater canal may represent another example of this phenomenon. To further support this hypothesis, Wilfert and Gibson (1974) and Kajihara et al. (2016) suggest a tropical origin

for the plant material with which *A. albimaculosa* has historically been associated. The plant genus *Vallisneria*, for instance, is hypothesized to be very widespread (Good 1964; Les et al. 2003) and, whereas its native range has yet to be established (Les et al. 2008), it does occur in the neotropics of Central America (e.g. Les et al. 2008; Balciunas and Center 1981). Alternatively, because of the sheer number of vessels that move through the Panama Canal – and, by extension, the high volume of ballast water and elevated potential for hull fouling, it is possible that the species was introduced to Panamanian waters from a different locality. There are already numerous



**Figure 3.** Maximum likelihood tree ( $lnL = -5950.074003$ ) resulting from the analysis of the hirudinean COI dataset. Maximum likelihood bootstrap values above 50% are shown above each node, newly sequenced taxa are shown in bold font and the target taxa are highlighted by a grey background. GenBank accession numbers are noted before each taxon name (note that GenBank numbers beginning with KF0588 represent specimens collected at the type locality [see Moser et al. 2013]).

accounts of non-endemics in the Panama Canal (e.g. Cohen 2006), and this species could potentially be added to the ever-growing list. The reproductive cycle of *Apatronemertes albimaculosa* certainly facilitates dispersal: the species lays eggs in a mucous sheet that is then attached to plant material (Wilfert and Gibson 1974), which may be sold in the aquarium trade or be taken up as ballast. It is unclear how resilient the eggs are to changes in external conditions (pollution, temperature, pH) that would inevitably arise from the invasion of new areas, but the adult worms seem to be rather susceptible to changes in temperature: exposure to temperatures below 10 °C is fatal (Kajihara et al. 2016). Based on our current knowledge of the species, it is impossible to establish the direction of introduction, yet its presence in disparate areas is clearly a byproduct of human activity.

The presence of *Placobdella parasitica* in Panama is less ambiguous. Given the species' abundance in North American ponds and lakes, and its absence south of the USA, our new record likely represents an introductory event (see Cornejo et al. 2015 for an account of current knowledge of Panamanian leeches). The turtle host, *Trachemys scripta*, was introduced to Panama, likely through the pet trade (Moll and Legler 1971). Both species occur throughout large parts of North America: *P. parasitica* occurs from the southernmost areas of Canada – between New Brunswick and Saskatchewan – as far south as Texas, USA, and is abundant throughout its range (Dodd Jr. 1988; Donaldson and Echtenacht 2005; Davy et al. 2009); while *T. scripta* is distributed between southern Virginia and Florida, resulting in an expansive geographic overlap between host and parasite. In

both adult and juvenile stages, *Placobdella parasitica* is a more-or-less stationary ectoparasite, presumably only detaching from its hosts to reproduce. After mating, Glossiphoniid leeches show a high degree of parental care, brooding their eggs on their venters until the juveniles hatch, and are delivered to their first blood meal (Siddall et al. 2005). It is therefore difficult to envision a scenario in which the eggs of *P. parasitica* would be inadvertently taken up as ballast, and it seems even less likely that a parasitized turtle would persist in a ballast tank for extended periods, much less survive transportation through the filtering systems that mitigate ballast water intake. Equally improbable is the notion that the leeches would be transported attached to the hull of a vessel, as this species cannot tolerate long periods of exposure to salt water. We therefore hypothesize that *Placobdella parasitica* was inadvertently introduced via the pet trade along with its turtle host. It seems clear that the geographic distribution of this particular leech is underlain by intricacies and serendipity.

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