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DNA barcoding supports identification of *Malacobdella* species (Nemertea: Hoplonemertea)

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Abstract

Background: Nemerteans of the genus *Malacobdella* live inside of the mantle cavity of marine bivalves. The genus currently contains only six species, five of which are host-specific and usually found in a single host species, while the sixth species, *M. grossa*, has a wide host range and has been found in 27 different bivalve species to date. The main challenge of *Malacobdella* species identification resides in the similarity of the external morphology between species (terminal sucker, gut undulations number, anus position and gonad colouration), and thus, the illustrations provided in the original descriptions do not allow reliable identification. In this article, we analyse the relationships among three species of *Malacobdella*: *M. arrokeana*, *M. japonica* and *M. grossa*, adding new data for the *M. grossa* and reporting the first for *M. japonica*, analysing 658 base pairs of the mitochondrial cytochrome *c* oxidase subunit I gene (*COI*). Based on these analyses, we present and discuss the potential of DNA barcoding for *Malacobdella* species identification.

Results: Sixty-four DNA barcoding fragments of the mitochondrial *COI* gene from three different *Malacobdella* species (*M. arrokeana*, *M. japonica and M. grossa*) are analysed (24 of them newly sequenced for this study, along with four outgroup specimens) and used to delineate species. Divergences, measured as uncorrected differences, between the three species were *M. arrokeana-M. grossa* 11.73%, *M. arrokeana-M. japonica* 10.62% and *M. grossa-M. japonica* 10.97%. The mean intraspecific divergence within the ingroup species showed a patent gap with respect to the interspecific ones: 0.18% for *M. arrokeana*, 0.13% for *M. grossa* and 0.02% for *M. japonica* (ranges from 0 to 0.91%).

Conclusions: We conclude that there is a clear correspondence between the molecular data and distinguishing morphological characters. Our results thus indicate that some morphological characters are useful for species identification and support the potential of DNA barcoding for species identification in a taxonomic group with subtle morphological external differences.

Keywords: DNA barcoding; *COI* gene; Bivalvia; Entocommensal nemertean; *Malacobdella*

Background

The phylum Nemertea is a group of organisms whose identification and taxonomy requires specialized methods, mainly histology. Recently, molecular methods have been a useful tool for ascertaining the actual biodiversity of these worms and increasing our knowledge of several of the problematic species (Chen et al. 2010; Fernández-Álvarez and Machordom 2013; Kvist et al. 2013). The nemertean genus *Malacobdella* de Blainville 1827 originally contained 13 nominal species, of which six are currently regarded as valid (Gibson 1995; Ivanov et al. 2002). The species of the genus are entocommensal in the mantle cavity of marine bivalves, mainly from the subclass Heterodonta (Jensen and Sadeghian 2005). The phylogenetic position of the genus *Malacobdella* is controversial within the phylum, mainly because this genus is always represented by sequences belonging only to the species *M. grossa* (Thollesson and Norenburg 2003; Andrade et al. 2012), the most studied and cosmopolitan species. The *Malacobdella* species are distributed in distant locations around the world: *M. japonica*, *M. macomae* and *M. siliquae* were described in the eastern (Japan) and western (west coast of the USA) Pacific Ocean; *M. grossa* was described in the Pacific (west coast of the USA) and Atlantic Ocean (northern Europe); and *M. arrokeana* was the only southern species described in the South Atlantic Ocean (Ivanov et al. 2002

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and references herein). The geographic distribution of the genus requires a huge sampling effort to work with all species. Identifying *Malacobdella* species is difficult because important diagnostic features were not initially recognized and thus not mentioned in earlier descriptions (Ivanov et al. 2002). In addition, the similarity of the external morphology between species (terminal sucker, number of intestinal loops (undulations), anus position and immature gonad colouration) and the illustrations provided in the original descriptions of the species do not allow reliable identification. There is little knowledge about the biology of *Malacobdella* species. The genus *Malacobdella* belongs to the hoplonemertean non-pilidiophora species (Thollesson and Norenburg 2003); this group presents direct development and non-feeding planuliform larvae (Maslakova and von Döhren 2009). However, there are no studies on its larval development, dispersion and settlement.

The host specificity of the *Malacobdella* species is generally high; five of the six known species have been reported from only one or two bivalve species: *M. arrokeana* Ivanov et al. 2002 from *Panopea abbreviata* (Heterodonta: Hiatelloidea) (Ivanov et al. 2002; Alfaya et al. 2013); *M. japonica* Takakura 1897 from *Spisula sachalinensis* (Heterodonta: Mactroidea) (Takakura 1897); *M. macomae* Kozloff 1991 from *Macoma nasuta* and *Macoma secta* (Heterodonta: Tellinoidea) (Kozloff 1991); *M. minuta* (Coe 1945) from *Yoldia cooperii* (Protobranchia: Nuculanoidea) (Coe 1945); and *M. siliquae* Kozloff 1991 from *Siliqua patula* (Heterodonta: Solenoidea) (Kozloff 1991). The sixth species, *M. grossa*, is the type specimen of the genus and was originally obtained from *Dosinia exoleta* (Linnaeus 1758) (Heterodonta: Veneroidea) (Müller 1776). However, the most complete morphological description of *M. grossa*, reported by Riepen (1933), was based on material from *Arctica islandica* (Linnaeus 1767) (Heterodonta: Arcticoidea). Nemerteans are traditionally identified and classified using morphological criteria, but the relatively low number of qualitative morphological characters, the lack of adequate fixation procedures for histological studies, vague descriptions in the original papers and the paucity of species-specific characters make species delimitation problematic, especially when comparing closely related species (Chen et al. 2010; Sundberg et al. 2010; Fernández-Álvarez and Machordom 2013; Kvist et al. 2013). The difficulty of morphological recognition of a great part of the more than 1,280 species included in the phylum Nemertea has been previously discussed (Andrade et al. 2012; Sundberg and Strand 2010; Kvist et al. 2013, among others), and different authors have advocated new tools and comprehensive studies for correctly identifying species and thus providing accurate biodiversity knowledge. The combination of molecular and morphological methods has been useful in elucidating nemertean taxonomy in other genera (Sundberg et al. 2009; Junoy et al. 2010; Puerta et al. 2010; Kajihara et al. 2011; Taboada et al. 2013). DNA barcoding accelerated the discovery ratio of new species (Wiens 2007) and identified some inconsistencies between species assignment and previously sequenced specimens (Kvist et al. 2013). Nevertheless, only a small number of nemerteans have been analysed through DNA barcoding (Sundberg et al. 2009; Chen et al. 2010; Fernández-Álvarez and Machordom, 2013; Kvist et al. 2013; Strand et al. 2014). The potential use of DNA barcoding needs to be substantiated in well-established taxonomic groups before it can be fully exploited in all nemertean genera. Currently, only 6% of the phylum Nemertea has an associated barcode sequence (Bucklin et al. 2011; Fernández-Álvarez and Machordom 2013).

Our objectives are to provide the most comprehensive molecular data set for the *Malacobdella* genus and test the usefulness of this data in the delimitation of the three most widely distributed species of the *Malacobdella* genus, and to provide for the first time *COI* sequences for *M. japonica*. We also estimate the phylogenetic relationship between 64 individuals from the 3 species based on partial sequences of *COI*.

Methods

Specimen collection and sequences used

Thirty-eight sequences of *M. arrokeana* were obtained from specimens collected at three northern Patagonian gulfs in Argentina (previously studied by Alfaya et al. 2013): San Matías Gulf ($n = 23$), San José Gulf ($n = 7$) and Nuevo Gulf ($n = 8$) (Table 1). Each of the *M. arrokeana* specimens was collected from a different host specimen of *P. abbreviata*. Specimens of *M. japonica* were collected from 15 different *S. sachalinensis* bivalves at Shinryu Beach in Akkeshi, Hokkaido, Japan. Individuals of *M. grossa* ($n = 11$) were obtained from different *A. islandica* clams in the waters of Tjärnö, Skagerak, Sweden. Additionally, two *M. grossa COI* sequences from GenBank (HQ848591 and AJ436905, from Tjärnö, Sweden, and the White Sea, Russia, respectively) were included in the analysis (Table 1). Fresh specimens were stored in absolute ethanol, and DNA was extracted from preserved tissues using a DNeasy extraction kit (Qiagen, Inc., Hilden, Germany), following the manufacturer's protocol.

To test for the monophyly of the genus, we sequenced *Ramphogordius sanguineus* specimens collected along the Argentinean coast to use as the outgroup. We also used *COI* sequences available from GenBank for nemerteans that are closely related to *Malacobdella* and have been previously used in other phylogenetic studies (Andrade et al. 2012; Mateos and Giribet 2008; Thollesson and Norenburg 2003), including two *R. sanguineus* outgroup sequences (Table 1).

Table 1 List of species used in the analysis, including the sample locality, number of specimens analysed (*N*) and GenBank accession numbers

Species	Specimen ID	Locations	Position	*N*	GenBank acc. number	References or voucher numbers[a]
Malacobdella arrokeana	Ma1	San Matías Gulf, Argentina	40°50′S/65°04′W	23	JX220596	CNP-INV 1879
	Ma2				JX220597	CNP-INV 1880
	Ma4				JX220599	CNP-INV 1881
	Ma5				JX220600	CNP-INV 1882
	Ma6				JX220601	CNP-INV 1883
	Ma7				JX220602	CNP-INV 1884
	Ma8				JX220603	CNP-INV 1885
	Ma9				JX220604	CNP-INV 1886
	Ma10				JX220605	CNP-INV 1887
	Ma11				JX220606	CNP-INV 1888
	Ma13				JX220607	CNP-INV 1889
	Ma14				JX220608	CNP-INV 1890
	Ma15				JX220609	CNP-INV 1891
	Ma16				JX220610	CNP-INV 1892
	Ma17				JX220611	CNP-INV 1893
	Ma18				JX220612	CNP-INV 1894
	Ma19				JX220613	CNP-INV 1895
	Ma20				JX220614	CNP-INV 1896
	Ma21				JX220615	CNP-INV 1897
	Ma22				JX220616	CNP-INV 1898
	Ma23				JX220617	CNP-INV 1899
	Ma24				JX220618	CNP-INV 1900
	Ma27				JX220620	CNP-INV 1901
	MaA1	Nuevo Gulf, Argentina	42°55′S/64°30′W	7	JX220621	CNP-INV 1902
	MaA2				JX220622	CNP-INV 1903
	MaA3				JX220623	CNP-INV 1904
	MaA4				JX220624	CNP-INV 1905
	MaA6				JX220625	CNP-INV 1906
	MaB1				JX220626	CNP-INV 1907
	MaB2				JX220627	CNP-INV 1908
	GSJ5A	San José Gulf, Argentina	42°20′S/64°10′W	8	JX220629	CNP-INV 1909
	GSJ5B				JX220630	CNP-INV 1910
	GSJJ5				JX220638	CNP-INV 1911
	GSJJ6				JX220639	CNP-INV 1912
	GSJJ7				JX220640	CNP-INV 1913
	GSJJ8				JX220641	CNP-INV 1914
	GSJJ9				JX220642	CNP-INV 1915
	GSJJ10				JX220643	CNP-INV 1916
M. japonica	Mja1	Hokkaido, Japan	43°3′N/144°51′E	13	KF597252	MNCN-5.02/3
	Mja2				KF597253	MNCN-5.02/4
	Mja4				KF597254	MNCN-5.02/5
	Mja5				KF597255	MNCN-5.02/6
	Mja6				KF597256	MNCN-5.02/7

Table 1 List of species used in the analysis, including the sample locality, number of specimens analysed (*N*) and GenBank accession numbers *(Continued)*

	Mja7				KF597257	MNCN-5.02/8
	Mja8				KF597258	MNCN-5.02/9
	Mja10				KF597259	MNCN-5.02/10
	Mja11				KF597260	MNCN-5.02/11
	Mja12				KF597261	MNCN-5.02/12
	Mja13				KF597262	MNCN-5.02/13
	Mja14				KF597263	MNCN-5.02/14
	Mja15				KF597264	MNCN-5.02/15
M. grossa	Mgrossa197	Tjärnö, Sweden	58°53′N/011°5′E	12	KF597241	MNCN-5.02/16
	Mgrossa198				KF597242	MNCN-5.02/17
	Mgrossa201				KF597243	MNCN-5.02/18
	Mgrossa202				KF597244	MNCN-5.02/19
	Mgrossa203				KF597245	MNCN-5.02/20
	Mgrossa204				KF597246	MNCN-5.02/21
	Mgrossa205				KF597247	MNCN-5.02/22
	Mgrossa206				KF597248	MNCN-5.02/23
	Mgrossa207				KF597249	MNCN-5.02/24
	Mgrossa208				KF597250	MNCN-5.02/25
	Mgrossa209				KF597251	MNCN-5.02/26
	Mgrossa GB 1				HQ848591	Andrade et al. (2012)
	Mgrossa GB 2	White Sea, Russia		1	AJ436905	Thollesson and Norenburg (2003)
Ramphogordius sanguineus	R.s1	Maine, USA		1	HQ848580	Andrade et al. (2012)
	R.s2	Anglesey, UK		1	AJ436938	Thollesson and Norenburg (2003)
	Li2	Nuevo Gulf, Argentina		4	KM387723	CNP-INV 1917
	Li3				KM387724	CNP-INV 1918
	Li4				KM387725	CNP-INV 1919
	Li5				KM387726	CNP-INV 1920
Amphiporus lactifloreus	A.l	Anglesey, UK	53°17′N/04°03′W	1	HQ848611	Andrade et al. (2012)
Paradrepanophorus crassus	P.c	Galicia, Spain		1	HQ848603	
Geonemertes pelaensis	G.p1	St. Davis, Bermuda		2	HQ848592	
	G.p2	Bermuda			EU255602	Mateos and Giribet (2008)

[a]Voucher numbers are given only for *Malacobdella arrokeana* specimens from the previous work of Alfaya et al. (2013), and for *M. grossa* and *M. japonica* newly sequenced here. MNCN, Museo Nacional de Ciencias Naturales (Madrid, Spain); CNP-INV, Invertebrate Collection of the Centro Nacional Patagónico (Argentina).

PCR amplification and sequencing

Partial *COI* sequences were amplified by PCR using the following primers: LCO1490 (5′-GGTCAACAAATCA TAAAGATATTGG-3′) (Folmer et al. 1994) and *COI*-H (5′-TCAGGGTGACCAAAAAATCA-3′) (Machordom et al. 2003). Amplification was carried out in a 50-μl final volume reaction consisting of 5 μl buffer containing 10 × 2 mM $MgCl_2$, 1 μl dNTP mix (10 mM), 0.4 μl of *Taq* DNA polymerase (5 U/μl) (Biotools, Madrid, Spain), 1–3 μl of genomic DNA and 0.8 μl of each primer (10 μM). Thermal cycling conditions were an initial 4-min denaturation at 94°C, followed by 40 cycles of 45 s at 94°C, 1 min at 45°C and 1 min at 72°C, ending with a final 10-min extension at 72°C. The products were visualized under blue light in 0.8% agarose gels stained with SYBR Safe (Invitrogen, Carlsbad, CA, USA), with co-migrating 100-bp or 1-kb ladder molecular weight markers. The amplification products (around 700 bp) were purified by ethanol precipitation. Both strands were sequenced using the BigDye Terminator sequencing kit and an ABI PRISM 3730 DNA Sequencer (Applied Biosystems, Grand Island, NY, USA).

Data analysis

Special alignment was unnecessary as the *COI* sequences from the analysed species did not present any gaps. Phylogenetic analyses were performed with PAUP 4.0 b10 (Swofford 2000) for maximum parsimony (MP) and maximum likelihood (ML), and with MrBayes 3.2 (Huelsenbeck 2000; Huelsenbeck and Ronquist 2001) for Bayesian inference (BI). MP parameters included a heuristic search with tree bisection-reconnection (TBR) branch swapping and ten random additions. ML was also estimated through a heuristic search, with stepwise addition, applying the TIM3 + G + I (transitional) model (Posada 2003). Two runs of 5,000,000 generations were performed for BI, sampling one tree per 1,000 replicates. The model that best fits the data (TIM3 + I + G) was found with jModelTest (Posada 2008). Branch supports for MP (1,000 replicates) and ML (150 replicates) were determined by bootstrapping (Felsenstein 1985) and by posterior probabilities (after a burn-in of 20% of the obtained trees) for BI.

Results

According to original descriptions, the three studied species of the *Malacobdella* genus are very similar in their external morphology but differ in internal morphological characters. *Malacobdella arrokeana* differs from *M. japonica* and *M. grossa* in the origin of the proboscis retractor muscle. This muscle is curved dorsally and attaches to the internal body wall in *M. arrokeana*, whereas in *M. grossa*, it originates ventrally next to the terminal sucker (Table 2). In *M. japonica*, the muscle also originates ventrally but ends freely in the parenchyma (Table 2). *M. japonica* differs from *M. arrokeana* and *M. grossa* in the position of the nerve commissure. In *M. japonica*, the posterior nerve commissure is situated around the terminal sucker, while in the other two species, the nerve commissure is situated dorsally in the posterior part of the intestine just before the anus (Table 2).

M. grossa also differs from *M. arrokeana* and *M. japonica* in the position of the excretory pores, being ventro-lateral in *M. grossa* and dorso-lateral in *M. arrokeana* and *M. japonica.* The rhynchocoel occupies most of the body length in the three species, up to 80% to 90% with the proboscis occupying two thirds of the rhynchocoel. Gonad colouration varies according to species and stage of gonad development. Generally, the gonads are dark olive green in colour in mature specimens of *M. grossa*, rosy or purple in mature *M. arrokeana* and rosy in mature *M. japonica* (Figure 1, Table 2). However, gonad colouration is always white in immature specimens (Figure 1A).

The *COI* fragments sequenced for *M. arrokeana*, *M. grossa* and *M. japonica* were 658 bp in length. These sequences were deposited in GenBank under accession numbers KF597241 to KF597264. The different phylogenetic analyses resulted in the same topology, with high support within the ingroup (Figure 2). The divergences, measured as uncorrected distances (percentage of nucleotide substitutions), between the three species were as follows: *M. arrokeana-M. grossa* 11.73%, *M. arrokeana-M. japonica* 10.62% and *M. grossa-M. japonica* 10.97%. There was a clear gap between these interspecific divergences and the intraspecific ones: the mean intraspecific divergence within the ingroup species was 0.18% for *M. arrokeana* (range 0% to 0.92%), 0.13% for *M. grossa* (range 0% to 0.61%) and 0.02% for *M. japonica* (range 0% to 0.15%). It is noteworthy that within the *R. sanguineus* sequences selected as the outgroup (Table 1), the divergence between one (GenBank accession number

Table 2 Characteristic features of the analysed *Malacobdella* species

Features	*M. arrokeana* (Ivanov et al. 2002)	*M. japonica* (Takakura 1897)	*M. grossa* (Müller 1776)
Excretory pores	Dorso-lateral (40% of length)	Dorso-lateral (25% of length) (Yamaoka 1940)	Ventro-lateral
Proboscis length	Most of the rhynchocoel, 80 to 90	Two thirds of the rhynchocoel length (Yamaoka 1940)	Two thirds of the rhynchocoel length
Proboscis retractor muscle	Curved dorsally and attached to the body muscular wall	Originates ventrally and ends freely in the parenchyma (Yamaoka 1940)	Originates ventrally next to the terminal sucker (Riepen 1933)
Ovary colour (mature)	White or purple (Teso et al. 2006)	Rosy (Yamaoka 1940)	Olive green or yellowish green (Gibson 1968)
Testicle colour (mature)	Pale rose (Teso et al. 2006)	White (Yamaoka 1940)	Rosy or pinkish hue (Gibson 1968)
Nerve commissure	Dorsal in the posterior part of the intestine just before the anus	Far behind the anus along the posterior margin of the sucker (Yamaoka 1940) (posterior around the terminal sucker)	Dorsal in the posterior part of intestine, above the anus
Host specificity	High (only in *Panopea abbreviata*)	High (only in *Spisula sachalinensis*)	Low (27 species)
Geographic distribution	South Atlantic Ocean (from Uruguay to north Patagonian gulfs)	North Pacific Ocean (northern Japan)	North Atlantic Ocean (Europe and North America) North Pacific Ocean (North America)

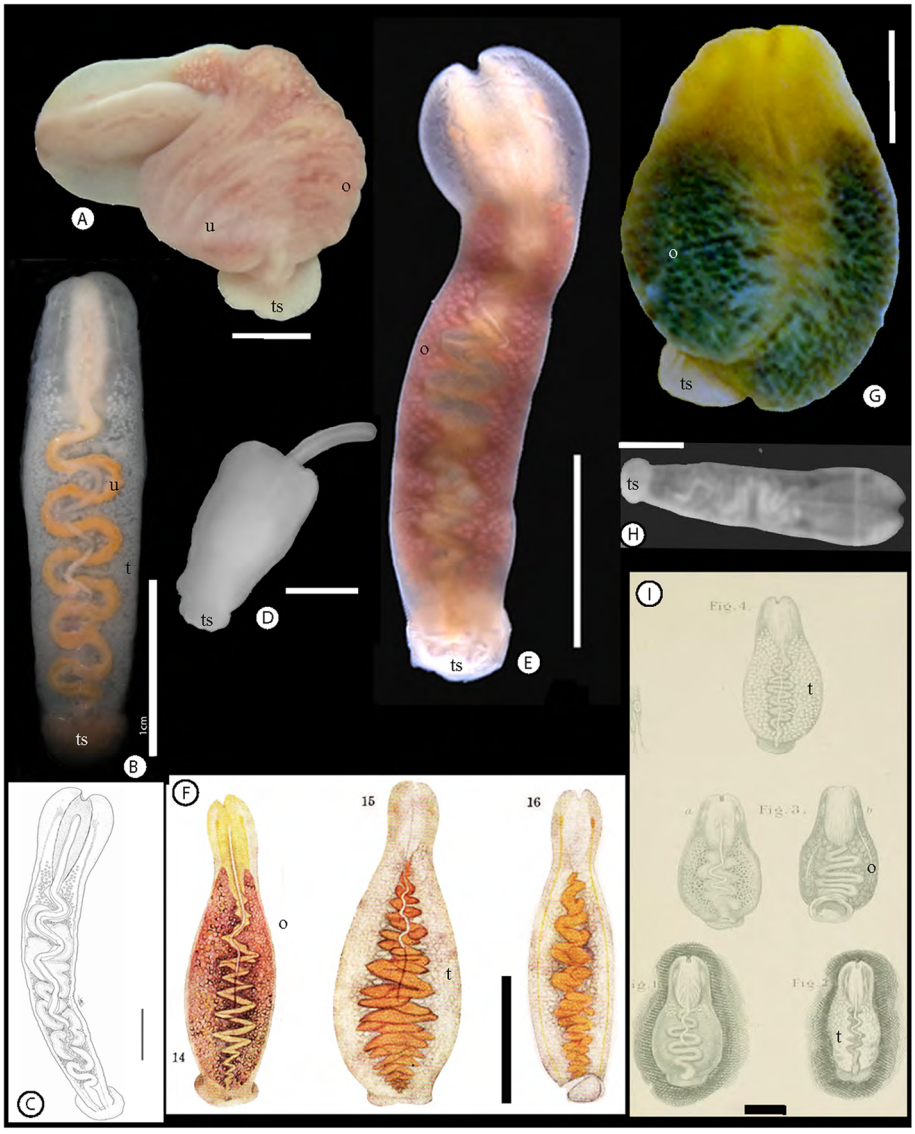

Figure 1 Specimens of the different *Malacobdella* species here analysed. *Malacobdella arrokeana*: **(A)** unrelaxed mature female, **(B)** relaxed male, **(C)** original illustration (Ivanov et al. 2002) and **(D)** immature specimen (scale bars: **A**, **B**: 10 mm; **C**: 3.5 mm, **D**: 1 mm). *Malacobdella japonica*: **(E)** relaxed mature female and **(F)** original illustration (Yamaoka 1940) (scale bars: **E**: 10 mm; **F**: 1 mm). *Malacobdella grossa*: **(G)** unrelaxed mature female, **(H)** immature specimen and **(I)** original illustration (Riepen 1933) (scale bars: **A**: 10 mm, **B**: 4 mm; **C**: 1 mm). Abbreviations: ts, terminal sucker; u, gut undulations; o, ovaries; t, testes.

HQ848580) of the sequences and the rest of the sequences of this species (GenBank AJ436938 and those collected by the authors) was more than 15% (15.75%), while the maximum divergence between the others was 100-fold lower (around 0.15%), suggesting that the *COI* sequence HQ848580 from GenBank is not actually from *R. sanguineus*. The five substitutions found among the *M. grossa* haplotypes were synonymous, as was the unique change among specimens of *M. japonica*, while of the 15 substitutions found among the haplotypes of *M. arrokeana*, three lead to amino acid changes.

Discussion

As previously mentioned, nemerteans of the *Malacobdella* genus are very difficult to identify based only on external characters. Rather, it is the internal morphology that provides the main taxonomic features used for identification, which can be differentiated only after a rigorous histological procedure. The genetic analysis performed here clearly shows that the distinguishing internal morphological differences used to separate the three species of *Malacobdella* analysed are concordant with differences in a fragment of the *COI* gene. However, previous

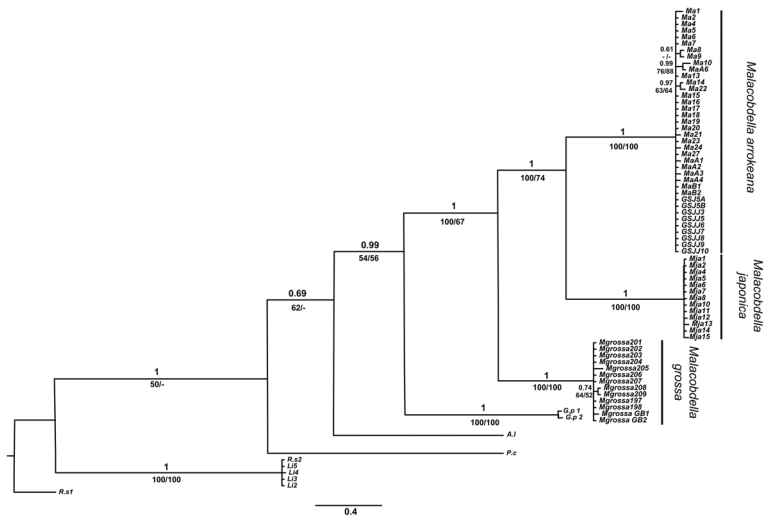

Figure 2 Haplotype phylogeny estimated by Bayesian analysis of the *Malacobdella* species studied. The numbers above the branches represent the posterior probabilities; the numbers below the branches represent the bootstrap percentages of the MP and ML analysis, respectively. When a certain node was not recovered by one of the methods, a hyphen was added. Ma, GSJ, *M. arrokeana*; Mja, *M. japonica*; *Mgrossa*, *M. grossa*; Li and R.s, *Ramphogordius sanguineus*, from Argentina and GenBank respectively; A.l, *Amphiporus lactifloreus*; P.c, *Paradrepanophorus crassus*; G.p, *Geonemertes pelaensis*.

DNA barcoding studies with nemerteans of the genera *Tetrastemma* and *Cerebratulus* revealed a lack of concordance between morphological and molecular characters (see Strand and Sundberg 2005; Sundberg et al. 2010). These authors argued that this difference could be the result of intraspecific variation or changes in the external morphology during development (Cantell 1975). They also concluded that the morphological characters used to describe *Tetrastemma* and *Cerebratulus* species are inadequate to identify evolutionary lineages. Our results showed that the divergence found between the sequences of the *Malacobdella* species (10% to 11% between the three species) are consistent with their status as distinct species. The values here found for intra- and interspecific divergences are in agreement with values found for other nemertean groups (e.g. Sundberg et al. 2010; Chen et al. 2010; Andrade et al. 2012; Kvist et al. 2013). The only exception was the divergence obtained between samples of *R. sanguineus*, 100-fold higher than other intraspecific values, suggesting that the *COI* sequence from GenBank (HQ848580) is not actually from *R. sanguineus*.

The phylogenetic analysis presented here showed that *M. arrokeana* and *M. japonica* are more closely related to each other than to *M. grossa*. Further studies using more molecular markers could clarify the phylogeny of the *Malacobdella* genus, taking into account the high host specificity of the majority of the species *versus* the cosmopolitan distribution and low host specificity of *M. grossa*. In this sense, the sequence of this last species from many hosts throughout its entire distribution would clarify whether *M. grossa* is indeed a single cosmopolitan species or a complex of species.

Based on the available literature by Takakura (1897), Yamaoka (1940), Riepen (1933), Gibson (1967, 1968, 1994), Gibson and Jennings (1969), Kozloff (1991), Ivanov et al. (2002) and the present work, the positions of the proboscis muscle and the nerve commissure appear to be good diagnostic characters for identifying species within the genus *Malacobdella* (since they are unique characteristic autapomorphies).

Several authors have concluded that nemertean species identification based only on morphological characters could be unreliable (see Strand and Sundberg 2005; Sundberg et al. 2009; Thornhill et al. 2008; Chen et al. 2010; Fernández-Álvarez and Machordom 2013; Kvist et al. 2013). More comprehensive future studies using the results presented here may strengthen species identification of other *Malacobdella* species by DNA barcoding.

Conclusions

Analysis of the *COI* (DNA barcoding) sequences presented in this work is clearly a powerful tool for species identification, at least of the three *Malacobdella* species studied. These molecular data are congruent with identifications based on internal morphological characters used in the original descriptions and re-descriptions of these three species and could be used to delineate between species in this genus with a similar external morphology.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
The work presented here was carried out in collaboration among all authors. JEFA, GB and AM defined the research theme and wrote the manuscript. JEFA, HK, MS and PS collected the samples in Argentina, Japan and Sweden, respectively, and took the photos. PS and MS provided *COI* sequences from *M. grossa*. JEFA and AM carried out the laboratory work (DNA extraction, PCR amplification, edition and sequences analysis), analysed the data and interpreted the results. JEFA prepared the tables and figures. PS, MS and HK improved the manuscript and made the English revision. All authors read and approved the final manuscript.

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