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Morphology and DNA barcodes reveal the presence of the non-native land planarian *Obama marmorata* (Platyhelminthes : Geoplanidae) in Europe

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Abstract. We report for the first time the occurrence of *Obama marmorata* in the Iberian Peninsula as an introduced species from the Neotropical Region. The species is also reported for the first time in Argentina. The identification was made on the basis of morphological evidence. The divergence levels of a fragment of the mitochondrial cytochrome *c* oxidase I gene from Argentinean and Iberian samples were studied. The morphology of samples from the two regions was the same and matched the original description of the species. A DNA barcoding matrix was constructed using new sequences from *O. marmorata* and sequences of related species taken from GenBank. Among the Geoplanidae, interspecific divergence ranged between 3.3 and 14.4%, while intraspecific divergence percentages were 0–1.2%, signalling the presence of a DNA barcoding gap. All *O. marmorata* sequences, irrespective of their geographic origin, form a well supported clade with an intraspecific divergence of 0–0.9 (average = 0.4%). These results indicate the utility of DNA barcoding to discover allochthonous species in this group of organisms.

Additional keywords: Argentina, exotic species, Geoplaninae, Spain, terrestrial flatworm.

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Introduction

Triclads are free-living Platyhelminthes Minot, 1876 with a worldwide distribution, ubiquitous in marine, freshwater and terrestrial habitats. While this group occurs in almost all aquatic environments on the six continents, land planarians (Geoplanidae Stimpson, 1857) are most abundant in tropical and subtropical forest regions. Land planarians are oviparous flatworms that live in humid environments. These animals and their cocoons, containing several embryos, are associated with plants or their rhizomes. Dispersal and introduction into new areas may successfully occur through unintentional human-mediated dispersal via the shipment of plants around the world or through deliberate introduction for biological control (Justine *et al*. 2014). Land planarians are generalist polyphagous predators of many soil invertebrates, such as earthworms, snails and arthropods (Ogren 1995; Sugiura 2010). Non-indigenous introduced species may be a threat to the native fauna and negatively impact agriculture; when optimal environmental conditions occur, they may be a serious conservation concern (Iwai *et al*. 2010; Breugelmans *et al*. 2012; Justine *et al*. 2014).

For Europe, most species of the Microplaninae Pantin, 1953 and Rhynchodeminae Graff, 1896 have been recognised as native, while some are non-native, introduced from different regions of the world. In the Iberian Peninsula some species are considered introduced, e.g. *Bipalium kewense* Moseley, 1878 (Filella-Subirà 1983) and *Caenoplana coerulea* Moseley, 1877 (Breugelmans *et al*. 2012; Mateos *et al*. 2013; Álvarez-Presas *et al*. 2014). The origin of other species is still unknown, such as that of *Rhynchodemus sylvaticus* (Leidy, 1851), tentatively considered as introduced by Vila-Farré *et al*. (2011).

The Geoplaninae is the most species-rich group with a Neotropical distribution but there are also a few records for Europe (*Paraba multicolor* (Kraepelin, 1901); *Obama* sp. (Álvarez-Presas *et al*. 2014)). Within the subfamily, the genus

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Obama Carbayo *et al*. 2013 includes 32 species, mostly described for Brazil. *Obama marmorata* (Schultze & Müller, 1857) was originally described from Blumenau, Santa Catarina State, Brazil, and recorded later in Rio do Testo, very near the type locality (Froehlich 1959). Here, we report for the first time the occurrence of *O. marmorata* in gardens and greenhouses of Asturias (Spain), indicating an introduction of the species. The specific identification was made on morphological grounds. The levels of divergence of a fragment of the cytochrome *c* oxidase I (COI) gene between Argentinean and Spanish samples were studied to determine the reliability of using DNA barcoding on this species. Since no authoritative barcodes (Kvist *et al*. 2010) for *O. marmorata* currently exist, newly collected specimens from the central eastern region of Argentina (where it is recorded for first time), near the south-east of Brazil (where the species was described) were included. Moreover, analyses of the divergence at intra- and interspecific levels, and the extent of the barcoding gap are analysed for the first time for this group of geoplanids.

Materials and methods

Sample collection

In total, 20 specimens (12 from Spain and 8 from Argentina) were studied (Table 1). They were manually collected during daylight hours from under flower pots, bricks and fallen logs. The flatworms were observed, photographed *in vivo* and then killed using boiling water. Each specimen was divided into two tissue portions for histology and DNA extraction, respectively. All individuals were studied molecularly, and four individuals (one from Spain and three from Argentina) were used for morphological studies.

Morphological analysis

The parts destined for histological studies were fixed in Bouin's fluid or 10% formaldehyde, and subsequently preserved in 70% ethanol. The samples were progressively dehydrated in an ascending series of ethanol, cleared in xylene and embedded in Paraplast. In the laboratory, photographs of the external morphology were taken from the specimens cleared in xylene using a stereomicroscope with a Zeiss AxioCam Icc 1 camera attached. Transverse serial sections (6–8 µm thick) of the anterior body and prepharyngeal region and sagittal serial sections of the pharynx and copulatory apparatus were stained with Azan and Masson trichrome methods (Romeis 1989). Reconstructions of the copulatory apparatus were carried out from the histological sections from both Iberian and Argentinean specimens and compared to descriptions in the literature.

The voucher specimens were deposited in the Invertebrate Collection of the Museo Nacional de Ciencias Naturales (MNCN, Spain) and in the Helminthological Collection of the Museo de La Plata (MLP, Argentina).

DNA extraction, amplification and sequencing

Tissues for molecular analysis were fixed in absolute ethanol. Total genomic DNA was purified following a standard phenol–chloroform extraction protocol (Sambrook *et al*. 1989). Primers PrLCOI (Sanna *et al*. 2009) and COIpr-b2 (Bessho *et al*. 1992*a*, 1992*b*) were used to amplify by polymerase chain reaction (PCR) fragments of the mitochondrial cytochrome *c* oxidase I (COI) gene. Standard PCR reactions were performed using the Illustra™ PuReTaq™ Ready-To-Go™ PCR beads (GE Healthcare, UK) following the manufacturer's protocol in a total volume of 25 µL, which included 0.5 µL of each primer (10 µM) and 2 µL of template DNA. PCRs consisted of an initial denaturation at 95°C for 10 min, followed by 46 cycles of denaturation at 96°C for 1 min, annealing at 51°C for 1 min and extension at 72°C for 1 min, with a final extension of 10 min at 72°C. Amplification products (324 bp) were purified using ExoSAP-IT (Affymetrix, OH) before sequencing both strands on an ABI Prism 3730. DNA sequence data were edited and aligned with Sequencher (Gene Codes Corporation) (Sequencher® 5.2 sequence analysis software, Gene Codes Corporation, Ann Arbor, MI, USA: http://www.genecodes.com). The sequences of the mitochondrial COI gene reported in this paper have been deposited in the EMBL/GenBank data libraries under accession numbers KM053208–KM053227.

Phylogenetic analysis

Phylogenetic analysis included the newly determined nucleotide COI sequences of the 20 individuals collected both in their likely native (Argentina, South America) and introduced (Spain, Europe) distribution ranges, as well as additional sequences of several representatives of the genus *Geoplana* (*sensu lato*, recently revised and subdivided into more genera by Carbayo *et al*. (2013), whose nomenclature is adopted here), available from GenBank. Five species of the subfamily Rhynchodeminae, the sister group of Geoplaninae (Álvarez-Presas *et al*. 2008, Riutort *et al*. 2012, Carbayo *et al*. 2013), were selected as outgroups for the phylogenetic analyses. jModelTest 2.1.4 (Darriba *et al*. 2012) was used to estimate the evolutionary model that best fit the nucleotide dataset, which according to the Akaike Information Criterion (AIC: Akaike 1974) was TPM3uf+I+G. The final alignment, including 69 terminal specimens, was subjected to the coalescent-based gene tree reconstruction method implemented in BEAST 1.8.0 (Rambaut and Drummond 2007) to estimate phylogenetic relationships among taxa in our dataset. The model of substitution used in this analysis was HKY+I+G, which was the best model of those directly available in BEAUti (ranked third in jModelltest analyses under the AIC). The birth–death process was chosen as the coalescent prior and a strict clock was assumed. The analysis was run for 100 million generations, sampling every 10 000 generations. Results were visualised in Tracer 1.5 (Rambaut and Drummond 2007) to check for convergence and adequate effective sample sizes of parameters in the model.

Uncorrected (p-distance) genetic distances between and within species (excluding the outgroup taxa) were calculated with MEGA 6 (Tamura *et al*. 2013). The specific labels employed here were defined according to the taxonomic labels defined by Carbayo *et al*. (2013). Four of the species (*Matuxia tuxaua*, *Geoplana vaginuloides*, *Obama carinata* and *Imbira marcusi*) showed more similar intraspecific distances than those found at an interspecific level between other species of the dataset. Therefore, each haplotype of these four species was considered a different

Table 1. Species analysed, with their locality and GenBank accession numbers
(+), sequences from present study

Species	Locality	GenBank accession number
Rhynchodeminae		
Arthurdendyus triangulatus (Dendy, 1895)		DQ666027
Arthurdendyus testaceus (Hutton, 1880)	Marlborough (Australia)	AF178305
Australoplana sp.	Australia	DQ666028
Dolichoplana striata Moseley, 1877	Igrejinha, RS (Brazil)	KC608226
Endeavouria septemlineata (Hyman, 1939)	Parque Ecológico do Tietê, São Paulo, SP (Brazil)	KC608233
Geoplaninae		
Cratera crioula (Froehlich, 1955)	Parque Estadual Serra da Cantareira, SP (Brazil)	KC608323–KC608324
Cratera pseudovaginuloides (Riester, 1938)	Parque Nacional da Serra dos Órgãos, RJ (Brazil)	KC608251–KC608252
Cratera sp. 1 [*sensu* Carbayo *et al*., 2013]	Parque Nacional Serra da Bocaina, SP (Brazil)	KC608284–KC608287
Cratera sp. 2 [*sensu* Carbayo *et al*., 2013]	Parque Estadual Intervales, SP (Brazil)	KC608281
Cratera sp. 4 [*sensu* Carbayo *et al*., 2013]	Parque Nacional Serra da Bocaina, SP (Brazil)	KC608285
Cratera sp. 5 [*sensu* Carbayo *et al*., 2013]	Parque Estadual do Desengano, RJ (Brazil)	KC608278
Cratera tamoia (Froehlich, 1955)	Parque Nacional da Serra dos Órgãos, RJ (Brazil)	KC608246, KC608254
Geoplana chita Froehlich, 1956	São Sebastião, SP (Brazil)	KC608329–KC608330
Geoplana hina Marcus, 1951	Parque Nacional Saint Hilaire / Lange, PR (Brazil)	KC608261
Geoplana quagga Marcus, 1951	Parque Ecológico do Tietê, São Paulo, SP (Brazil)	KC608236
Geoplana quagga Marcus, 1951	Parque Nacional Saint Hilaire / Lange, PR (Brazil)	KC608265
Geoplana sp. 3 [*sensu* Carbayo *et al*., 2013]	Parque Nacional Serra da Bocaina, SP (Brazil)	KC608282
Geoplana vaginuloides (Darwin, 1844) (OTU1)	Parque Nacional Saint Hilaire / Lange, PR (Brazil)	KC608262
Geoplana vaginuloides (Darwin, 1844) (OTU2)	Parque da Previdência, São Paulo, SP (Brazil)	KC608247
Imbira marcusi (Carbayo *et al*., 2013) (OTU1)	São Sebastião, SP (Brazil)	KC608241
Imbira marcusi (Carbayo *et al*., 2013) (OTU2)	Parque Nacional Serra da Bocaina, SP (Brazil)	KC608291
Matuxia matuta (Froehlich, 1955)	Parque Estadual do Desengano, RJ (Brazil)	KC608277
Matuxia tuxaua (Froehlich, 1955) (OTU1)	Parque Nacional da Serra dos Órgãos, RJ (Brazil)	KC608253
Matuxia tuxaua (Froehlich, 1955) (OTU2)	Parque Estadual Serra da Cantareira, SP (Brazil)	KC608302
Notogynaphallia goetschi (Riester, 1938)	Parque Estadual Serra da Cantareira, SP (Brazil)	KC608294
Obama burmeisteri (Schultze & Müller, 1857)	Parque Ecológico do Tietê, São Paulo, SP (Brazil)	KC608234
Obama burmeisteri (Schultze & Müller, 1857)	Parque Estadual Serra da Cantareira, SP (Brazil)	KC608297
Obama carinata (Riester, 1938) (OTU 1)	Parque Estadual Intervales, SP (Brazil)	KC608304
Obama carinata (Riester, 1938) (OTU 2)	Parque Estadual Intervales, SP (Brazil)	KC608307
Obama josefi (Carbayo & Leal-Zanchet, 2001)	Floresta Nacional de São Francisco de Paula, RS (Brazil)	KC608317–KC608318
Obama ladislavii (Graff, 1899)	Parque Nacional da Serra de Itajaí, SC (Brazil)	KC608258
Obama ladislavii [*sensu* Froehlich, 1959]	Parque Nacional da Serra de Itajaí, SC (Brazil)	KC608256, KC608259–KC608260
Obama marmorata (Schultze & Müller, 1857) (GEO1–GEO7)	Reborio, Muros de Nalón, Asturias (Spain). 43°33′12″N, 6°05′34″W	KM053208–KM053214 (+)
Obama marmorata (Schultze & Müller, 1857) (GEO8–GEO12)	Greenhouse La Lloba, Piedras Blancas, Asturias (Spain). 43°32′55″N, 6°00′15″W	KM053215–KM053219 (+)
Obama marmorata (Schultze & Müller, 1857) (GEO14)	Greenhouse Kato, Buenos Aires (Argentina). 34°54′29″S, 58°09′20″W	KM053220 (+)
Obama marmorata (Schultze & Müller, 1857) (GEO16–GEO18)	Greenhouse Ferrari, Buenos Aires (Argentina). 34°56′55″S, 57°57′38″W	KM053221–KM053223 (+)
Obama marmorata (Schultze & Müller, 1857) (GEO25–GEO27)	Los Toldos, Buenos Aires (Argentina). 35°01′01″S, 61°03′04″W	KM053224–KM053226 (+)
Obama marmorata (Schultze & Müller, 1857) (GEO28)	Nueve de Julio, Buenos Aires (Argentina). 35°27′16″S, 60°54′35″W	KM053227 (+)
Obama sp. 6 [*sensu* Carbayo *et al*. 2013]	Parque Estadual da Serra do Tabuleiro, SC (Brazil)	KC608308–KC608309
Paraba franciscana (Leal-Zanchet & Carbayo, 2001)	Floresta Nacional de São Francisco de Paula, RS (Brazil)	KC608312, KC608335
Paraba multicolor (Graff, 1899) (OTU1)	Parque Estadual Serra da Cantareira, SP (Brazil)	KC608299
Paraba multicolor (Graff, 1899) (OTU2)	Parque Nacional Serra da Bocaina, SP (Brazil)	KC608271
Paraba phocaica (Marcus, 1951)	Parque Nacional Serra da Bocaina, SP (Brazil)	KC608289–KC608290
Paraba rubidolineata (Baptista & Leal-Zanchet, 2005)	Floresta Nacional de São Francisco de Paula, RS (Brazil)	KC608333

Table 2. Mean p-distance percentages at inter- and intraspecific levels

n.a.: not applicable, since only one specimen was available for this species

	Interspecific mean p-distances																														Intraspecific mean p-distances
Paraba phocaica																															0
Obama burmeisteri	10.4																														0.39
Cratera pseudovaginuloides	8.3	6.5																													0
Cratera tamoia	12	9.4	8.3																												0.79
Cratera crioula	9.4	6.7	5.5	6.5																											0
Cratera sp. 1	12.8	9.1	9.6	6.7	6.1																										1.2
Matuxia tuxaua (OTU 1)	10.6	11.2	10.6	10.2	9.4	9.6																									n.a.
Matuxia tuxaua (OTU 2)	10.6	11	9.8	9.8	9.4	11.2	8.3																								n.a.
Matuxia matuta	12.2	10.4	11.4	14	10.6	12.8	10.2	13.4																							n.a.
Cratera sp. 4	10.2	6.5	7.9	9.3	7.9	9.6	7.9	10.2	5.9																						n.a.
Obama sp. 6	7.9	10.8	7.1	10.6	9.1	10.8	9.4	12.2	9.4	8.3																					0
Obama josefi	7.7	9.6	7.1	9.8	6.7	9.3	8.7	8.5	10.2	7.5	7.3																				0.39
Obama ladislavii [*sensu* Froehlich 1959]	10	9.5	7	8.5	7.3	9.1	7.2	9.6	10.1	8.1	8.1	5.6																			0.26
Obama carinata (OTU 1)	9.1	8.9	9.4	9.6	8.7	10.6	9.8	9.8	10.2	7.9	9.4	8.9	10																		n.a.
Obama carinata (OTU 2)	11	10.4	9.1	10.8	8.3	12.4	9.8	8.3	13.4	9.8	11.4	8.9	8.8	7.1																	n.a.
Geoplana vaginuloides (OTU 1)	10.6	11.2	7.9	9.8	9.1	10	10.2	11	11.8	10.2	7.9	10	9.3	9.8	11																n.a.
Geoplana vaginuloides (OTU 2)	7.1	10	7.1	10.6	7.9	10.4	7.9	9.4	11.4	9.4	6.3	6.5	6.8	7.9	9.1	7.9															n.a.
Geoplana chita	8.7	10	5.5	8.3	7.5	10	9.4	10.2	11.4	8.3	7.1	6.5	7.7	8.3	9.1	6.3	5.1														n.a.
Paraba multicolor	9.3	8.1	8.1	9.4	10.2	10.2	12.6	11.4	12	10.2	9.3	8.5	9	9.4	11.8	10.4	9.3	9.6													0.79
Geoplana quagga	8.7	11.6	10.6	12.8	11.8	14.4	9.8	9.1	12.2	9.8	9.8	9.3	9.6	8.7	10.6	9.8	7.9	9.8	10.6												0.39
Paraba franciscana	8.7	9.8	8.3	9.3	6.7	10.4	7.9	9.4	11	8.3	8.3	9.3	7.6	9.8	9.1	9.8	7.5	8.3	9.6	7.5											0
Imbira marcusi (OTU 1)	8.7	10	9.1	11.2	9.1	12.4	11.8	11.8	11.8	10.2	7.9	8.9	9.2	9.1	9.8	10.6	7.1	7.5	9.4	8.7	7.5										0
Imbira marcusi (OTU 2)	9.1	8.5	8.7	10	6.3	10.4	11.4	9.4	9.8	9.1	7.9	7.3	8	6.3	7.9	9.8	7.1	8.3	8.3	9.1	8.7	4.3									n.a.
Geoplana sp. 3	11	11.2	9.1	10.8	9.4	10.8	11.4	13	12.2	10.6	10.6	11.4	10.9	13	11.8	11.4	10.6	9.1	12.6	10.2	9.4	9.8	11								n.a.
Cratera sp. 5	8.3	8.5	7.5	9.3	7.9	9.6	11	11.4	11.4	7.9	7.9	9.3	9.7	10.6	10.6	7.5	8.7	7.1	9.3	11.4	8.7	8.7	8.7	9.4							n.a.
Geoplana hina	11.4	10	9.8	7.5	7.1	7.5	11.4	10.2	12.6	10.2	11	9.4	8.1	11	11.8	11.4	10.6	9.8	9.6	13.4	10.2	11.4	9.8	11	8.3						n.a.
Obama ladislavii (Graff, 1899)	11.8	6.9	6.3	10.2	7.9	10.4	9.4	11	10.6	9.1	8.3	9.4	6.6	9.4	8.7	9.8	9.8	9.1	8.1	11.4	9.4	11	8.3	12.2	8.3	9.4					n.a.
Cratera sp. 2	11.8	10.4	9.1	9.8	9.1	10.8	11.4	9.8	11.4	11	11.4	10.4	10.1	11	11	11	11.4	11	9.4	9.1	8.7	9.8	9.1	10.6	11.4	9.8	10.2				n.a.
Paraba rubidolineata	11.8	9.6	9.8	10	9.1	10	10.6	11	11	9.4	9.8	10.2	10	9.1	8.3	12.2	8.7	11	10.6	9.8	8.7	11	9.4	9.1	11.4	12.2	10.6				n.a.
Notogynaphallia goetschi	8.3	9.3	8.7	11.6	9.8	11.6	12.2	11.8	9.8	9.4	5.9	7.7	8.4	10.2	11.4	11	9.1	9.4	9.3	11.8	10.2	7.9	6.7	12.2	7.1	9.8	8.3	11			n.a.
Obama marmorata	8.1	10	6.3	10.5	8.3	10.7	10.6	11.2	9.5	8.1	3.3	6.5	7.4	8.4	9.9	7.9	5.6	5.7	8.2	9.6	8.2	6.9	6	10.5	8.4	10.1	8.3	10	6.4		0.40

Operational Taxonomic Unit (OTU) in the genetic divergences analysis (Tables 1, 2).

Results

Morphological analysis

Order **Tricladida** Lang, 1881

Superfamily **GEOPLANOIDEA** Stimpson, 1857

Family **GEOPLANIDAE** Stimpson, 1857

Subfamily **Geoplaninae** Stimpson, 1857

Genus ***Obama*** Carbayo, Álvarez-Presas, Olivares, Marques, Froehlich & Riutort, 2013

Geoplana marmorata Schultze & Müller, 1857: 19–38.
Obama marmorata (Schultze & Müller 1857) Carbayo, Álvarez-Presas, Olivares, Marques, Froehlich, & Riutort, 2013: 520, 523.
Type species: *Geoplana fryi* Graff, 1899.

Voucher specimens

Twelve specimens from Asturias, Spain: One mature specimen from Reborio, Muros de Nalón; 22.viii.2013. Anterior region: sagittal sections on 50 slides (7 µm) with pharynx; copulatory apparatus: sagittal sections (7 µm) on 51 slides (MNCN 4.01/995 to MNCN 4.01/1.095).

Eight specimens from Buenos Aires Province, Argentina. Three of them sectioned: MLP 6750: La Plata City, Kato Greenhouse; 11.v.2011; anterior region 1 (cephalic region): transverse sections (7 µm) on 13 slides; anterior region 2 (post-cephalic region): sagittal sections (7 µm) on 20 slides; prepharyngeal region: transverse sections (7 µm) on 5 slides; pharynx: sagittal sections (7 µm) on 15 slides; copulatory apparatus: sagittal sections (7 µm) on 15 slides. MLP 6752: La Plata City, Ferrari Greenhouse; 16.ix.2011; anterior region 1: transverse sections (7 µm) on 12 slides; anterior region 2: sagittal sections (7 µm) on 23 slides; prepharyngeal region: transverse sections (7 µm) on 5 slides; pharynx: sagittal sections (7 µm) on 24 slides; copulatory apparatus: sagittal sections (7 µm) on 26 slides. MLP 6754: Los Toldos City; 1.i.2013; anterior region 1: transverse sections (7 µm) on 12 slides; anterior region 2: sagittal sections (7 µm) on 28 slides; prepharyngeal region: transverse sections (6 µm) on 6 slides; pharynx: sagittal sections (8 µm) on 18 slides; copulatory apparatus: sagittal sections on 32 slides (7 µm). The other five are preserved in 70% ethanol: MLP 6751, 6753, 6755, 6756 and 6757.

Morphological description

Live specimens measured 55–70 mm in length and 7 mm in maximum width at rest. After being preserved, the body

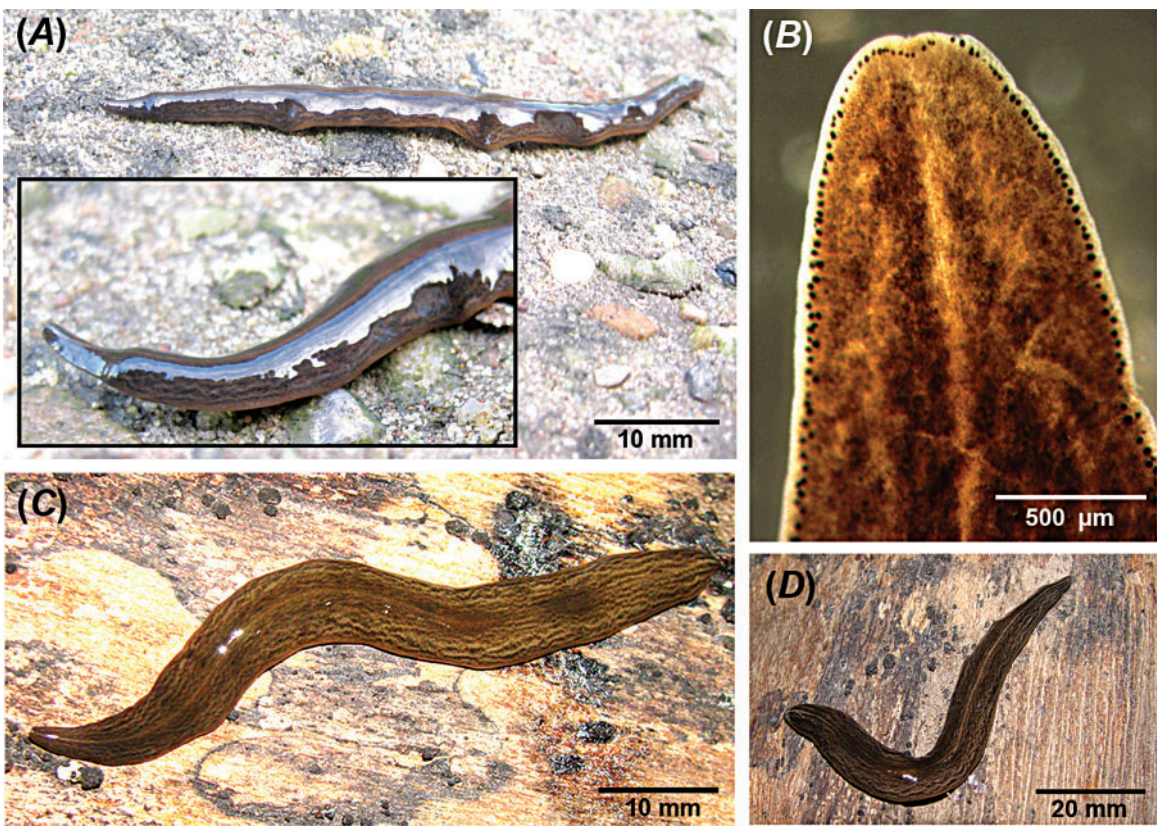

Fig. 1. Photographs of Iberian (*A*, *B*) and Argentinean (*C*, *D*) *Obama marmorata* individuals. *A*, lateral view of a live specimen, with a magnification of the anterior body region; *B*, detail of arrangement of eyes from the cephalic region in a cleared specimen; *C*, *D*, dorsal aspect of live specimens with light (*C*) and dark (*D*) ground colour.

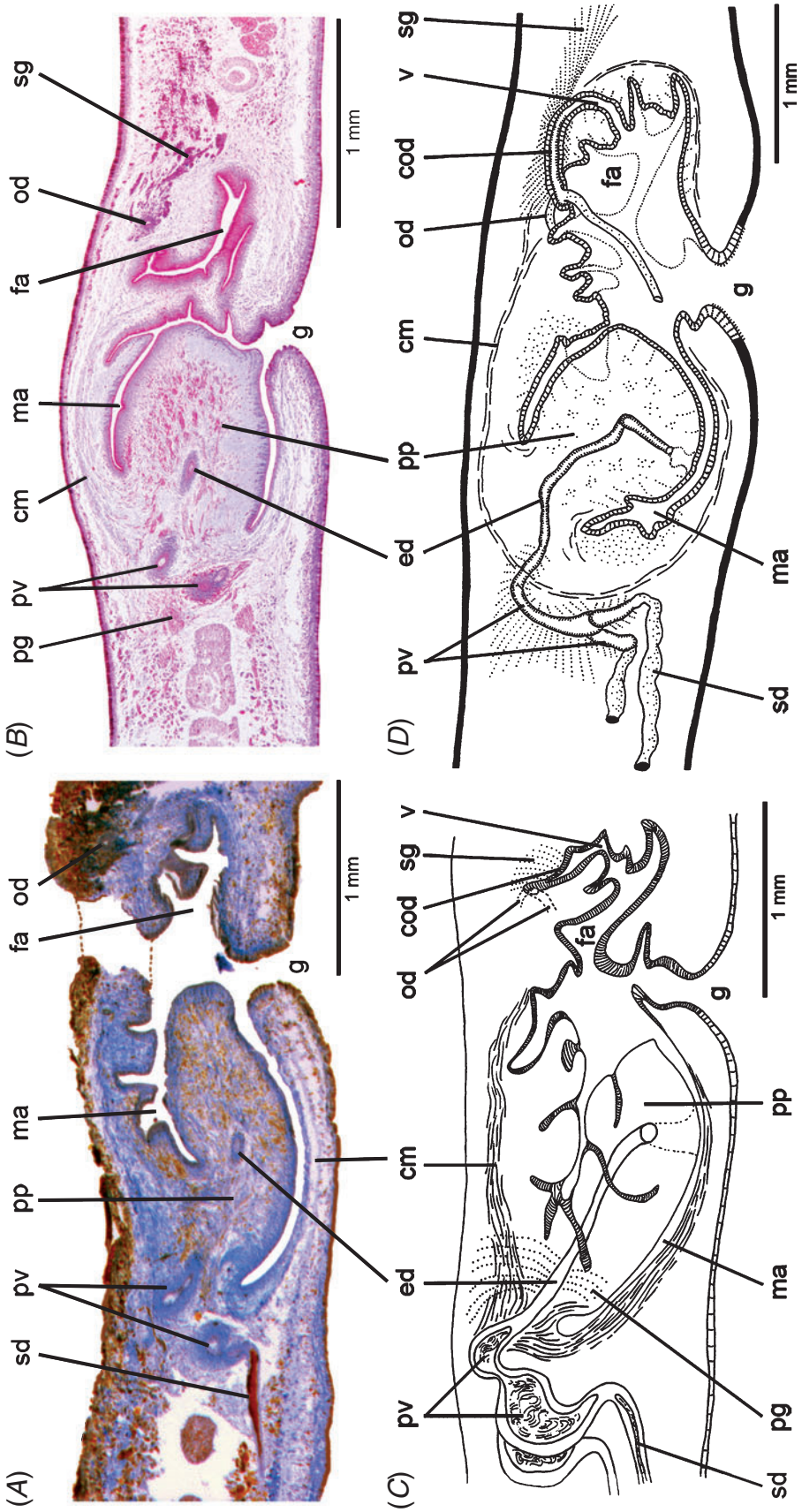

Fig. 2. Sagittal sections and schematic reconstructions of the copulatory apparatus in Iberian (*A*, *C*) and Argentinean (*B*, *D*) *Obama marmorata* specimens. Abbreviations: cm, common muscle coat; cod, common glandular ovovitelline duct; ed, ejaculatory duct; fa, female atrium; g, gonopore; ma, male atrium; od, ovovitelline duct; pg, prostatic glands; pp, penis papilla; pv, prostatic vesicle; sd, sperm duct; sg, shell glands; v, vagina.

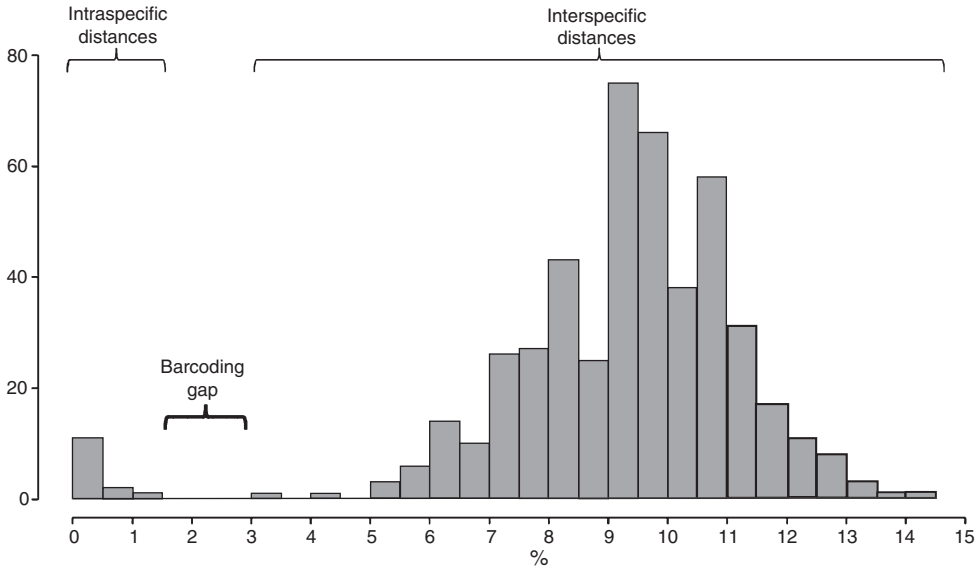

Fig. 3. The uncorrected genetic intra- and interspecific p-distances across the entire dataset. The gap between 1.2 and 3.3% level of divergence indicates the presence of a barcoding gap.

measurements were reduced to 30–52 mm in length and 2.5–6 mm in maximum width. The dorsal ground colour goes from light to dark brown with numerous dark brown irregular stripes that lend a marbled appearance. A median stripe lighter than the rest of the body is present, but it is less visible in the darkest specimens (Fig. 1). The ventral surface is light grey. The mouth and gonopore are situated in the posterior third of the body in the preserved animals. The distance from the mouth and the gonopore to the anterior tip varied from 46% to 68% and 75% to 80% of the body length, respectively. The anterior tip is blunt and progressively widens until the first third of the body, where the margins become parallel until the posterior narrowing. The eyes are initially marginal and uniserial encircling the anterior region. About 2–3 mm behind the anterior tip, the ocelli are spread in two or three rows, for ~2–3 mm, along the body margins. Posteriorly, the eyes are dorsal and pluriserial, occupying 1/3 of the dorsal surface on each lateral side (10–15 rows of eyes), at 8–9 mm from the anterior tip. The eyes (Fig. 1*B*) gradually become less numerous and at the prepharyngeal level they are more scattered (4–6 rows on each side); posterior to the copulatory apparatus the eyes become scattered and continue to the posterior tip.

The creeping sole occupies 95% of the body width on the ventral surface. The cutaneous musculature is composed of the three typical layers of the Geoplaninae, which are organised in circular, diagonal and an inner longitudinal layer, the latter being the thickest. The parenchymatic musculature consists of supraintestinal and subintestinal transverse muscle fibres. The pharynx is cylindrical with a richly folded border (Fig. 2). The mouth is located at the posterior third of the pharyngeal pouch. The pharynx joins the intestine through a short oesophagus. The male reproductive system consists of dorsal testes arranged in three or four rows on each side of the body, starting anterior to the ovaries and extending to the proximity of the pharynx. The sperm ducts are dorsal to the ovovitelline ducts, and they are distally expanded with their lumen full of spermatozoa. They bend dorso-medially to open into the forked proximal portion of the prostatic vesicle. The distal extrabulbar portion of the prostatic vesicle is an unpaired canal with an inverted U shape. The distal part of the prostatic vesicle penetrates the musculature of the penis bulb and communicates with the ejaculatory duct (Fig. 2). The prostatic vesicle is lined with a ciliated, columnar epithelium and receives abundant glandular secretion. The ejaculatory duct runs almost straight and centrally through the penis papilla in its proximal portion; the rest of the ejaculatory duct is sinuous and turns to the left, following the curvature of the papilla and opens at the ventral side of the penis papilla (Fig. 2*C, D*). The penis papilla is asymmetrical and provided with irregular folds, occupying the entire cavity of the male atrium. Dorsal folds separate the male and female atrium. The oviducts arise from the dorsal surface of the ovaries. At about the level of the gonopore, the oviducts bend dorso-medially and join together to form a common glandular ovovitelline duct, which receives the secretion of the shell glands. The common duct runs backwards and opens into a short vagina that joins with the female atrium. The lumen of the female atrium is narrow due to its highly folded walls (Fig. 2*B*).

Molecular data and phylogenetic analysis

Uncorrected genetic distances across the entire dataset ranged from 0 to 1.2% (average 0.4%) at an intraspecific level, while divergences across species ranged between 3.3 and 14.4% (average 9.4%) (Fig. 3). Therefore, there is a barcoding gap between 1.2 and 3.3% levels of divergence (Fig. 3). However, the proximity between the higher intraspecific and the lower interspecific values indicate that the usage of DNA barcoding in geoplanids should be undertaken with caution because recent

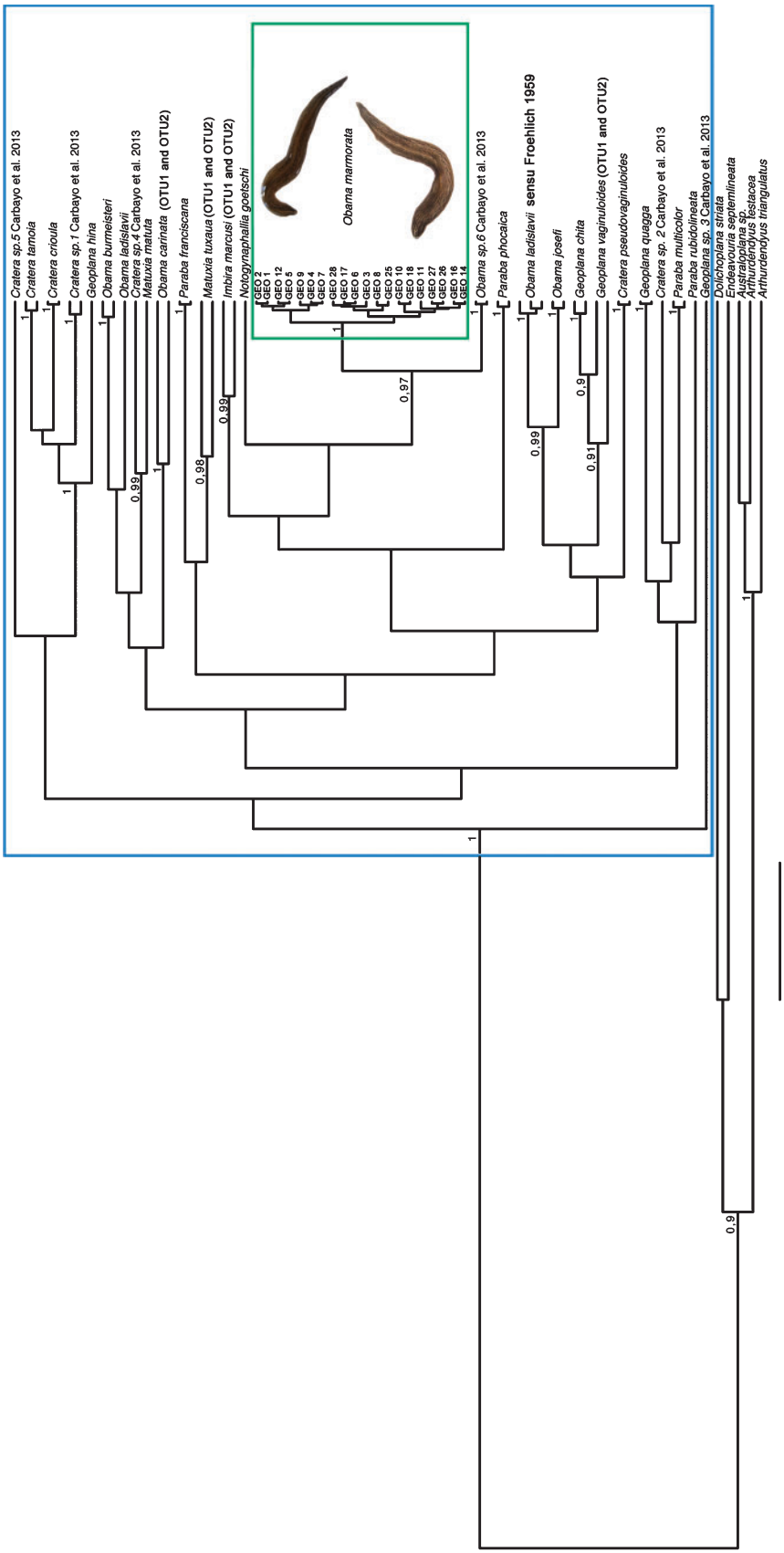

Fig. 4. Coalescent-based Bayesian tree generated by BEAST 1.8.0 from the COI matrix. Posterior probabilities above 0.9 are shown.

speciation events may have occurred. Some species, represented by more than one sequence, included highly divergent branches, with p-distance values well above mean intraspecific values reported for other species: *Matuxia tuxaua* (Froehlich, 1955) (8.3%), *Geoplana vaginuloides* (Darwin, 1844) (7.9%), *Obama carinata* (Riester, 1938) (7.1%) and *Imbira marcusi* Carbayo *et al*., 2013 (4.3%) (Table 2), suggesting the potential existence of additional cryptic species. In *O. marmorata*, an average intraspecific divergence ranging from 0 to 0.9% (0.4% on average) was found. The entire range of divergence percentages is covered by the Argentinean specimens (0–0.9%, average 0.3%), whereas the p-distance values of the Iberian specimens ranged from 0 to 0.6% (average 0.3%). The mean p-distance found between the Iberian and Argentinean samples was 0.3%.

The Coalescent Bayesian Tree is depicted in Fig. 4. All samples morphologically assigned to *Obama marmorata* are grouped in a well supported clade (Bayesian Posterior Probabilities (BPP) = 1). *O. marmorata* and *Obama* sp. 6 *sensu* Carbayo *et al*. (2013) were recovered as sister groups (BPP = 0.97). Only some other terminal branches received BPP values above 0.9.

Discussion

Specific identification was performed on the basis of the comparative study of the morphology. Regarding the colour pattern, the specimens studied agree with the typical marble pattern of *O. marmorata* (Schultze and Müller 1857; Froehlich 1959). The internal morphology agrees with the description provided by Froehlich (1959). Only small differences in the prostatic vesicle between the European and the Argentinean specimens were observed, with the forked proximal portions being more dilated in the specimen from Spain. However, these differences may well be due to preservation artefacts or different degrees of maturity of the specimens.

Although relationships within Geoplaninae are largely unresolved, our phylogenetic hypothesis based on analyses of mitochondrial COI sequences corroborates, with high statistical support, our assignment of the Argentinean and Spanish individuals to the same species (*O. marmorata*) of the Geoplaninae in general and to *Obama* in particular (Fig. 4).

The comparison of COI sequences found no significant differences among the Argentinean and Spanish individuals of *O. marmorata* at the specific level: four haplotypes were found in total and the divergence between the two groups is low (0.3% on average). *O. marmorata* is the sister group to *Obama* sp. 6 *sensu* Carbayo *et al*. (2013) (Fig. 4), with the mean distance between the two species being 3.3% (Table 2). Our results support the recognition of *Obama* sp. 6 as an undescribed species (Álvarez-Presas *et al*. 2014), closely related to *O. marmorata*. The presence of a barcoding gap between intra- and interspecific divergence levels suggests restrictions to gene flow among these two clades, which would be consistent with their recognition as independent species. With the exception of four species, all haplotypes associated with a single taxonomic label have intraspecific genetic distances below 1.2%. These four species are *Matuxia tuxaua*, *Geoplana vaginuloides*, *Obama carinata* and *Imbira marcusi.* P-distance values between the specimens with these taxonomic labels range from 4.3 to 8.3%, values associated with interspecific divergences in other species. Moreover, our results (Fig. 4) confirm that *G. vaginuloides* is paraphyletic as Carbayo *et al*. (2013: fig. 3) previously reported. Future studies should re-evaluate taxon delineation and individual assignment of samples to these four species.

DNA barcoding has been successfully used as a tool for identifying animal species, including alien species. It is particularly useful in morphologically similar taxa, or in groups that require specialised methodologies for their identification (e.g. Ward *et al*. 2009; Fernández-Álvarez and Machordom 2013). Extensive molecular databases are necessary to successfully apply barcoding methods to identify specimens. Land planarians are under-represented, but recently some progress has been made (Álvarez-Presas *et al*. 2012) and our results, in accordance with the assertions of Álvarez-Presas and Riutort (2014), suggest that this could be a fruitful approach, especially if the studies provide a link between morphological and molecular datasets in an integrative approach to taxonomy.

O. marmorata had previously been reported only in Brazil (Schultze and Müller 1857; Froehlich 1959). As Geoplaninae are Neotropical taxa, the presence of *O. marmorata* in Argentina may be due to an extensive natural distribution of this species. However, the presence of *O. marmorata* in the Iberian Peninsula is certainly due to an unintentional introduction by commercial trade from its native range; the occurrence of this species in other areas of Europe is to be expected.

Land planarians are top predators of invertebrates such as earthworms, arthropods, slugs and snails, affecting population dynamics of soil species when they are introduced to a new area (Haria *et al*. 1998; Fiore *et al*. 2004; Iwai *et al*. 2010). Previous studies have shown that environmental temperatures are a critical factor in the expansion of land planarians (Justine *et al*. 2014). However, because the biology and environmental tolerance ranges of *O. marmorata* in its native area are unknown, the impact that it can cause in areas where it has been introduced is very difficult to assess. Further studies are needed to elucidate whether *O. marmorata* is an introduced or invasive species in the Iberian Peninsula, what its environmental requirements are and what its dispersal potential is. This information is necessary to assess the potential risks posed to the native fauna and flora.

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