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INVASION NOTE

Reconstructing routes of invasion of *Obama nungara* (Platyhelminthes: Tricladida) in the Iberian Peninsula

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Abstract Among other factors, globalization has promoted the spread of alien organisms, posing a great risk to Earth's biodiversity. Land planarians of the family Geoplanidae especially benefit from human-mediated transport. Many species become established in new areas, where they represent threats to the native soil fauna. *Obama nungara* is a species described from Brazil, but with many well-established populations in Europe. In this study, specimens from Argentina, Brazil, Portugal and Spain were morphologically and molecularly studied to establish the potential origin of the invasive events within the Iberian Peninsula. Analyses of the mitochondrial lineages (haplotype networks) of these populations revealed previously unknown relationships and biogeographical patterns that suggest an Argentine origin for the Iberian populations. Furthermore, comparative analysis of Argentine, Iberian and Brazilian populations revealed three well-defined and distinct *O. nungara* clades. Our findings suggest two independent introductions of different populations from Argentina that gave rise to the different Iberian populations. This population diversity suggests hidden biodiversity of alien land planarians in invaded areas and their invasive and adaptive potential.

Keywords Biodiversity · Biological invasions · Distribution · Haplotype networks · Neotropical land planarian

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Introduction

An increase in international trade in the last several decades and a lack of adequate control for alien species has led to the accidental transport of fauna and flora (Kaluza et al. 2010). The spread of invasive species has caused a reduction or extinction of native species, has damaged local ecosystems and has had a negative impact on global biodiversity (Wilcove et al. 1998). The introduction of land planarians (Platyhelminthes, Geoplanidae), recognized as top predators of the invertebrate soil fauna, pose a risk to the native invertebrates in those areas in which they have been accidentally introduced (Murchie and Gordon 2013). For instance, *Platydemus manokwari* Beauchamp, 1963, categorized as one of the 100 worst invasive species (Invasive Species Specialist Group ISSG 2015), has caused a rapid decline in terrestrial invertebrates (land snails, earthworms, isopods) in areas where it has been introduced. The "New Zealand" flatworm *Arthurdendyus triangulatus* (Dendy, 1895) was introduced in the UK, Ireland and Faroe Islands, causing a serious risk to native earthworms (Murchie and Gordon 2013).

The introduction of land planarians into Europe has become increasingly frequent and a matter of concern since the first species of geoplanid, *Paraba multicolor* (Graff, 1899), was found in Hamburg, Germany (Kraepelin 1901). Today, more than 30 species of land planarians have been reported in different countries outside their native range and 21 of them have been found in Europe (Justine et al. 2018). Of these, six species have been reported for Spain, including representatives of the subfamilies Bipaliinae, Rhynchodeminae and Geoplaninae (Vila-Farré et al. 2011; Mateos et al. 2013; Lago-Barcia et al. 2015; Carbayo et al. 2016).

Recently, Lago-Barcia et al. (2015) recorded *Obama marmorata* (Schultze and Müller, 1856) in anthropized areas in Argentina and the Iberian Peninsula (IP), mainly in greenhouses and plant nurseries. Previously, *O. marmorata* had only been recorded in southern Brazil (Froehlich 1959). Subsequently, Carbayo et al. (2016) amended the identity of those specimens found in Argentina and Spain by Lago-Barcia et al. (2015) as a new species, *Obama nungara* Carbayo, Alvarez-presas, Jones and Riutort, 2016, with records in Brazil, Argentina, Spain and the United Kingdom, and noted the co-occurrence of *O. nungara* and *O. marmorata* in southern Brazil.

Accurate knowledge of the distribution and origin of introduced populations is imperative to designing effective policy to control introduced species. The presence of *O. nungara* in Europe is significant because it is a member of the subfamily Geoplaninae, which is restricted to the Neotropics with few records outside this region. This species exhibits a ample niche, with earthworms as its main food resource, but also feeding on gastropods and other land planarians. It shows a high tolerance for environmental disturbance (Boll and Leal-Zanchet 2016). Thus, this species has great potential to become invasive. In this work, we studied new specimens of *O. nungara* from Argentina, Brazil, Portugal and Spain, integrating morphological and molecular data, to elucidate the putative origin of the Iberian populations.

Methods

Specimens were collected by hand from gardens, parks, backyards, greenhouses and human-disturbed native forests in Argentina, Brazil, Portugal and Spain (Table 1). They were collected during the day and night. Live specimens were photographed, then euthanized in boiling water and fixed in 10% formaldehyde or in Bouin's solution for histological analysis. Before fixation, a small fragment of each specimen was preserved in absolute ethanol for molecular analysis.

Morphological study

Histological sections were cut from body fragments of selected flatworms from Argentina, Brazil and Spain for species identification (Table 1). The tissues were first gradually dehydrated in increasingly concentrated solutions of ethanol, immersed in Butanol, and subsequently embedded in Paraplast$^{®}$ Tissue Embedding Medium. They were then serially sectioned at 6–8 μm thick with a microtome. Sections were stained using Azan (specimens from Spain) and Masson (specimens from Argentina and Brazil) trichrome methods. Sections were observed by optical microscope and the copulatory apparatus reconstructed for identification purposes. Histological features of the reproductive system and other anatomical features were compared between different populations and with the specimens studied by Lago-Barcia et al. (2015) and Carbayo et al. (2016).

Table 1 Species analysed, with their locality and GenBank accession numbers

Species	Locality	GenBank—accession number
Family Geoplanidae		
Pasipha sp. GEO15A	Vivero Kato, Buenos Aires (Argentina) (Outgroup)	MG639898*
Obama nungara Carbayo, Alvarez-Presas, Jones & Riutort, 2016	Torroella de Fluvià, Girona (Spain)	KJ659666
	Torroella de Fluvià, Girona (Spain)	KJ659665
	Bordils, Girona, (Spain)	KJ659664
	Gavà, Barcelona, (Spain)	KJ659656
Obama nungara GEO50E	Reborio, Muros de Nalón, Asturias (Spain)	MF155841*
Obama nungara GEO65E	Vila do Conde, Oporto (Portugal)	MF155842*
Obama nungara GEO42E	Reborio, Muros de Nalón, Asturias (Spain)	MF155843*
Obama nungara GEO43E	Reborio, Muros de Nalón, Asturias (Spain)	MF155844*
Obama nungara GEO52E	Reborio, Muros de Nalón, Asturias (Spain)	MF155845*
Obama nungara GEO62E	Reborio, Muros de Nalón, Asturias (Spain)	MF155846*
Obama nungara GEO45E	Reborio, Muros de Nalón, Asturias (Spain)	MF155847*
Obama nungara GEO05E	Reborio, Muros de Nalón, Asturias (Spain)	KM053212
Obama nungara GEO54E	Reborio, Muros de Nalón, Asturias (Spain)	MF155849*
Obama nungara GEO63E	Reborio, Muros de Nalón, Asturias (Spain)	MF155850*
Obama nungara GEO53E	Reborio, Muros de Nalón, Asturias (Spain)	MF155851*
Obama nungara GEO51E	Reborio, Muros de Nalón, Asturias (Spain)	MF155852*
Obama nungara	Treto, Cantabria (Spain)	KJ659662
Obama nungara GEO17A	Vivero Ferrari, Buenos Aires (Argentina)	KM053222
Obama nungara GEO20A	Vivero Zoológico, Buenos Aires (Argentina)	MF155854*
Obama nungara GEO26A	Vivero Los Toldos Buenos Aires (Argentina)	KM053225
Obama nungara GEO27A	Vivero Los Toldos Buenos Aires (Argentina)	KM053226
Obama nungara GEO28A	Vivero Nueve de Julio. Buenos Aires (Argentina)	KM053227
Obama nungara GEO03E	Reborio, Muros de Nalón, Asturias (Spain)	KM053210
Obama nungara GEO01E	Reborio, Muros de Nalón, Asturias (Spain)	KM053208
Obama nungara GEO14A	Vivero Kato, Buenos Aires (Argentina)	KM053220
Obama nungara GEO16A	Vivero Ferrari, Buenos Aires (Argentina)	KM053221
Obama nungara GEO48E	Reborio, Muros de Nalón, Asturias (Spain)	MF155862*
Obama nungara GEO08E	Viveros La Lloba, Piedras Blancas Asturias (Spain)	KM053215
Obama nungara GEO12E	Viveros La Lloba, Piedras Blancas. Asturias (Spain)	KM053219
Obama nungara GEO04E	Reborio, Muros de Nalón, Asturias (Spain)	KM053211
Obama nungara	Bordils, Girona (Spain)	KJ659663
Obama nungara GEO89 V	Sopelana, Vizcaya (Spain)	MF155866*
Obama nungara GEO91 V	Sopelana, Vizcaya (Spain)	MF155867*
Obama nungara GEO94 V	Sopelana, Vizcaya (Spain)	MF155868*
Obama nungara GEO41A	Córdoba (Argentina)	MF155869*
Obama nungara GEO93 V	Sopelana, Vizcaya (Spain)	MF155870*
Obama nungara GEO86 V	Sopelana, Vizcaya (Spain)	MF155871*
Obama nungara	Torroella de Fluvià, Girona (Spain)	KT714108
Obama nungara	Torroella de Fluvià, Girona (Spain)	KT714107
Obama nungara	Torroella de Fluvià, Girona (Spain)	KT714106
Obama nungara	Torroella de Fluvià, Girona (Spain)	KT714105

Table 1 continued

Species	Locality	GenBank—accession number
Obama nungara GEO90 V	Sopelana, Vizcaya (Spain)	MF155872*
Obama nungara GEO21A	Vivero Zoológico, Buenos Aires (Argentina)	MF155873*
Obama nungara GEO23A	Valeria del Mar, Buenos Aires (Argentina)	MF155874*
Obama nungara	Bordils, Girona (Spain)	KJ659654
Obama nungara GEO38A	Parque Pereyra Iraola, Buenos Aires (Argentina)	MF155875*
Obama nungara	Villasar de Mar, Barcelona (Spain)	KJ659658
Obama nungara	Bordils, Girona (Spain)	KJ659653
Obama nungara GEO22A	Vivero Zoológico, Buenos Aires (Argentina)	MF155876*
Obama nungara GEO37A	Parque Pereyra Iraola, Buenos Aires (Argentina)	MF155877*
Obama nungara GEO31A	Isla Martín García, Buenos Aires (Argentina)	MF155878*
Obama nungara GEO13A	Vivero Kato, Buenos Aires (Argentina)	MF155879*
Obama nungara GEO66B	Santiago, Rio Grande do Sul (Brazil)	MF155880*
Obama nungara GEO70B	Santiago, Rio Grande do Sul (Brazil)	MF155881*
Obama nungara GEO67B	Santiago, Rio Grande do Sul (Brazil)	MF155882*
Obama nungara GEO75B	Santiago, Rio Grande do Sul (Brazil)	MF155883*
Obama nungara GEO77B	Santiago, Rio Grande do Sul (Brazil)	MF155884*
Obama nungara	Parque Estadual da Serra do Tabuleiro, SC (Brazil)	KC608309
Obama nungara	Parque Estadual da Serra do Tabuleiro, SC (Brazil)	KC608308
Obama nungara GEO74B	Santiago, Rio Grande do Sul (Brazil)	MF155885*
Obama nungara GEO72B	Santiago, Rio Grande do Sul (Brazil)	MF155886*
Obama nungara GEO68B	Santiago, Rio Grande do Sul (Brazil)	MF155887*
Obama nungara GEO71B	Santiago, Rio Grande do Sul (Brazil)	MF155888*
Obama anthropophila Amaral, Leal-Zanchet and Carbayo, 2015	Floresta Nacional Sao Francisco de Paula, RS (Brazil)	KP962470
	Floresta Nacional Sao Francisco de Paula, RS (Brazil)	KP962468
	Floresta Nacional Sao Francisco de Paula, RS (Brazil)	KP962466
	Floresta Nacional Sao Francisco de Paula, RS (Brazil)	KP962465
	Floresta Nacional Sao Francisco de Paula, RS (Brazil)	KP962469
	Floresta Nacional Sao Francisco de Paula, RS (Brazil)	KP962467
	Floresta Nacional Sao Francisco de Paula, RS (Brazil)	KP962397
	P.N. Serra do Itajai, SC. (Brazil)	KP962432
	P.N. Serra do Itajai, SC. (Brazil)	KP962431
	P.N. Serra do Itajai, SC. (Brazil)	KP962429
Obama decidualis Amaral and Leal-Zanchet 2015	Santa Maria, RS (Brazil)	KP962391
	Portao, RS (Brazil)	KP962386
	Santa Maria, RS (Brazil)	KP962389
	Santa Maria, RS (Brazil)	KP962384
	Santa Maria, RS (Brazil)	KP962388
	Santa Maria, RS (Brazil)	KP962385
	Santa Maria, RS (Brazil)	KP962390
	Santa Maria, RS (Brazil)	KP962387
Obama josefi (Carbayo and Leal-Zanchet 2001)	Floresta Nacional Sao Francisco de Paula, RS. (Brazil)	KP962367
	Floresta Nacional Sao Francisco de Paula, RS. (Brazil)	KC608318
	Floresta Nacional Sao Francisco de Paula, RS(Brazil)	KC608317

Table 1 continued

Species	Locality	GenBank—accession number
Obama sp.	P.N. Serra do Itajai, SC (Brazil)	KP962430
	P.N. Serra do Itajai, SC (Brazil)	KP962358
	P.N. Serra do Itajai, SC (Brazil)	KP962357
	P.N. Serra do Itajai, SC (Brazil)	KP962359
Obama marmorata (Schultze and Müller, 1857)	Parque Estadual da Serra do Tabuleiro, SC (Brazil)	KT714104
	Parque Nacional da Serra de Itajaí, SC (Brazil)	KT714103
Obama ladislavii (Graff 1899)	P.E. Serra do Tabuleiro, SC (Brazil)	KP962492
	P.E. Serra do Tabuleiro, SC (Brazil)	KP962490
	P.E. Serra do Tabuleiro, SC (Brazil)	KP962451
	P.E. Serra do Tabuleiro, SC (Brazil)	KP962450
	P.E. Serra do Tabuleiro, SC (Brazil)	KP962448
	P.E. Serra do Tabuleiro, SC (Brazil)	KP962446
	P.E. Serra do Tabuleiro, SC (Brazil)	KP962444
	P.E. Serra do Tabuleiro, SC (Brazil)	KP962489
	P.E. Serra do Tabuleiro, SC (Brazil)	KP962480
	P.E. Serra do Tabuleiro, SC (Brazil)	KP962455
Obama burmeisteri (Schultze and Müller, 1857)	P.E. Cantareira, SP. (Brazil)	KP962378
	Sao Paulo, SP. (Brazil)	KP962354
	P.E. Cantareira, SP. (Brazil)	KP962371
	Sao Paulo, SP. (Brazil)	KP962355
	P.E. Cantareira, SP. (Brazil)	KP962373
	Sao Paulo, SP. (Brazil)	KP962362
	P.E. Cantareira, SP. (Brazil)	KP962377
Obama carinata (Riester, 1938)	P.E. Intervales, SP. (Brazil)	KP962376
	P.E. Intervales, SP. (Brazil)	KP962375
	P.E. Intervales, SP. (Brazil)	KP962372
	P.E. Cantareira, SP. (Brazil)	KP962363
	P.E. Cantareira, SP. (Brazil)	KP962364
	P.E. Cantareira, SP. (Brazil)	KP962365
	P.E. Intervales, SP. (Brazil)	KP962370
	P.E. Cantareira, SP. (Brazil)	KP962361
	P.E. Intervales, SP. (Brazil)	KP962369
	P.E. Intervales, SP. (Brazil)	KP962368

GEOXX code for the processed specimen; letter A for specimens from Argentina; B specimens from Brazil; E for specimens from Asturias, Spain; V specimens from Vizcaya, Spain

*Sequences from present study

The specimens analysed in this work were deposited in the collections of the Museo Nacional de Ciencias Naturales, Madrid (Spain) and Museo de La Plata (Argentina).

Mitochondrial DNA study and haplotype network analysis

Genomic DNA was extracted following a standard phenol–chloroform extraction protocol (Sambrook et al. 1989). A fragment of the cytochrome oxidase I

(COI) gene was amplified using the newly designed primer Obama-COIF (forward 5′-GTGATGCCAGTTATGATTGGTGGT-3′) and COIpr-b2 (reverse 5′-ATGAGCAACAACATAATAAGTATCATG-3′) (Bessho et al. 1992). The PCR was performed in a total volume of 25 µl containing 0.125 µl of *Taq* polymerase (5U/µl, 5 PRIME), 2.5 µl of reaction buffer (5 PRIME), 0.5 µl of each primer (10 µM), 2 µl of dNTPs (10 µM) and 2 µl of DNA. The thermocycling program began with an initial denaturation step at 95 °C (10 min), followed by 46 cycles of denaturation at 96 °C (1 min), annealing at 61 °C (1 min) and extension at 72 °C (1 min), ending with a final extension step at 72 °C (7 min). Amplified products were purified with ExoSAP-IT (Affymetrix, OH, USA) and both strands were sequenced on an ABI Prism 3730 (www.secugen.es). DNA sequences were cleaned at the primer ends using Sequencher® 5.2 (Gene Codes Corporation, Ann Arbor, MI, USA; http://www.genecodes.com), aligned with MAFFT (Katoh and Standley 2013) and manually checked with BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html).

A total of 121 sequences (each corresponding to a 710 bp fragment) were used for molecular analyses and included sequences from specimens collected for this study and those obtained from GenBank of related *Obama* species (Table 1). A *Pasipha* sp. (Specimen GEO15) sequence was used as an outgroup.

Haplotype networks were constructed using the software PopART (http://popart.otago.ac.nz). Specimens from Argentina, Brazil, Portugal and Spain (Table 1) were included in this analysis. TCS methods (Clement et al. 2000) were used to infer relationships among samples.

A COI gene tree was reconstructed by Maximum Likelihood (ML), and implemented in W-IQ-TREE (Trifinopoulos et al. 2016). W-IQ-TREE includes an evolutionary model selection process similar to jModelTest (Darriba et al. 2012), and an extended process using the FreeRate heterogeneity model. The latter model estimates rates freely and moderates the discrete Gamma model. W-IQ-TREE also uses the Bayesian information criterion to select the best-fit model. For phylogenomic data, W-IQ-TREE determines the best-fit partitioning scheme using a fast implementation of Partition Finder (Lanfear et al. 2012).

The RAxML generated tree was used as the input in a Bayesian Poisson Tree Processes (bPTP) analysis, implemented on the bPTP server (http://species.h-its.org/bptp/; Zhang et al. 2013). PTP is a single-locus species delimitation method that only uses nucleotide substitution information and implements a model that assumes that gene tree branch lengths are generated by two independent Poisson process classes (within- and among-species substitution events). The bPTP analysis was run using 100,000 MCMC generations, with a thinning of 100 and a burn-in of 10%.

This approach does not require ultrametrization of trees or other species delineation models, such as the Generalized Mixed Yule Coalescent (GMYC) model (Fujisawa and Barraclough 2013). With the PTP model, speciation or branching events are modelled in terms of the number of substitutions (represented by branch lengths), and therefore, only requires a phylogenetic input tree.

Results

Morphological identification

External morphology. Live specimens of *Obama nungara* from Argentina, Brazil and Spain are lanceolate, with the anterior region of the body gradually narrowing towards the tip, which is blunt, and the posterior region ending abruptly. The colour pattern of the dorsal surface varies from light to dark brown with a thin median stripe of yellowish to light brown pigment. The dorsum is also splattered with spots irregularly disposed and anastomosed, lending a marbled appearance (Figs. 1, 2, 3). The ventral surface is light gray to whitish. The eyes are monolobated, contour the anterior tip and extend uniserially 3–4 mm from the tip, following marginally for 3–6 mm in two–three irregular rows. They become dorsal pluriserial and trilobated, occupying 60–75% of body width in their maximum extension (between 15 and 30% of body length). Posteriorly, they are more isolated reaching the posterior end. After fixation, the length of specimens is 32–70 mm (mean: 52 mm), and maximum width is 4–8 mm (mean: 6 mm). Mouth and gonopore distance from the anterior tip is 55–66% (mean: 61%) and 69–86% (mean: 77%) relative to body length. However, the external aspect of the specimens of the studied populations did not differ

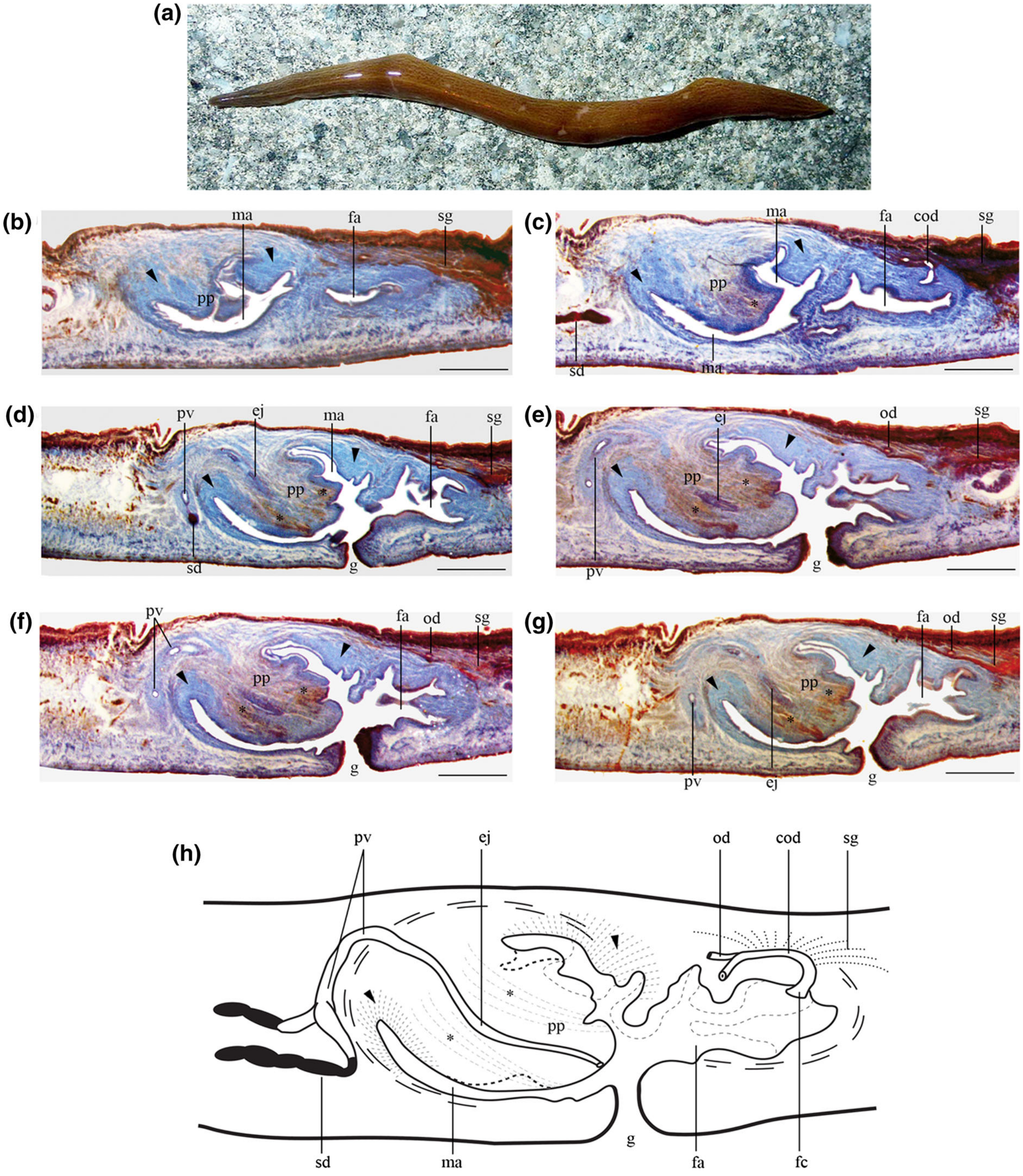
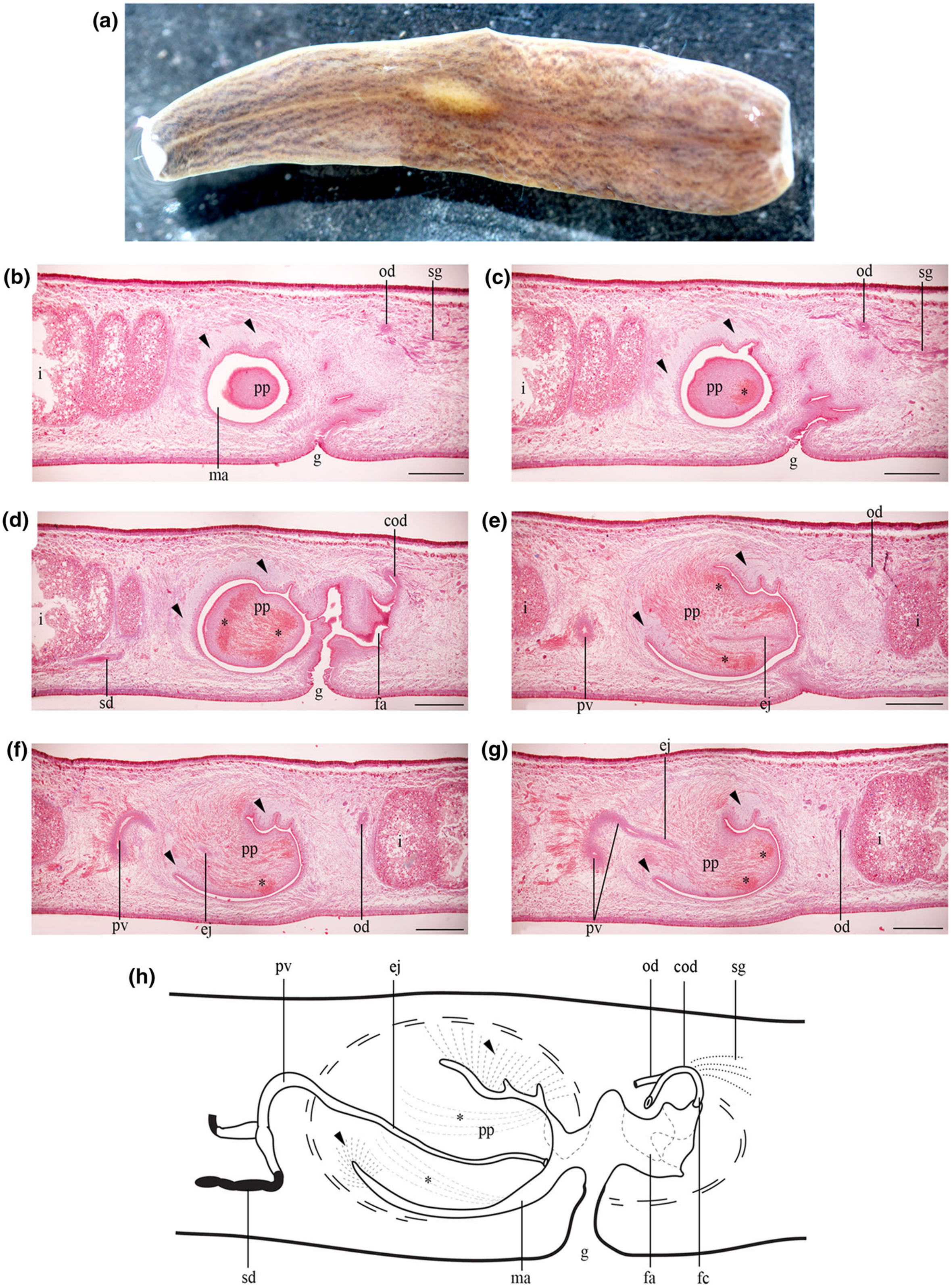


Fig. 1 *Obama nungara* from the Iberian Peninsula (GEO04): **a** Dorsal view of a live specimen. **b**–**g** Selected sagittal sections of the copulatory apparatus. **h** Schematic reconstruction of the copulatory apparatus, in sagittal view. In all figures, anterior end of the body is on the left. cod common ovovitelline duct, *ej* ejaculatory duct, *fa* female atrium, *fc* female canal, *g* gonopore, *ma* male atrium, *od* ovovitelline duct, *pp* penis papilla, *pv* prostatic vesicle, *sd* sperm duct, *sg* shell glands Cyanophil fine granular secretion is indicated by arrowheads, and erythrophil fine granules are indicated by asterisks. Scale bars: 500 lm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◀**Fig. 2** *Obama nungara* from Argentina (GEO21). **a** Dorsal view of a fixed specimen. **b–g** Selected sagittal sections of the copulatory apparatus. **h** Schematic reconstruction of the copulatory apparatus, in sagittal view. In all figures, anterior end of the body is on the left. *cod* common ovovitelline duct, *ej* ejaculatory duct, *fa* female atrium, *fc* female canal, *g* gonopore, *ma* male atrium, *od* ovovitelline duct, *pp* penis papilla, *pv* prostatic vesicle, *sd* sperm duct, *sg* shell glands Cyanophil fine granular secretion is indicated by arrowheads, and erythrophil fine granules are indicated by asterisks. Scale bars: 500 µm

substantially. Specimens from the Iberian Peninsula are slightly smaller than those from Argentina and Brazil, reaching 50 mm in length and 6 mm in maximum width.

Internal morphology (Figs. 1, 2, 3). The cutaneous musculature exhibits the three typical layers of Geoplaninae (circular, diagonal, and longitudinal muscle). The specimens from the Iberian Peninsula show a thicker cutaneous musculature than those from Argentina and Brazil, with the ventral musculature slightly thicker than the dorsal. Parenchymatic musculature is composed of a dorsal layer, with decussate fibers, and supra- and sub-intestinal transverse muscle layers. The pharynx is cylindrical. Posterior to the pharynx, the sperm ducts are dilated and full of spermatozoa, constituting spermiducal vesicles. In the proximity of the penis bulb, the sperm ducts run to the sagittal plane and open into the paired tubular portion of the ventro-dorsally oriented prostatic vesicle. The prostatic vesicle, which is extrabulbar, follows as an unpaired C-shaped part. After a very short section inside the penis bulb, the prostatic vesicle communicates with the ejaculatory duct, which runs proximally straight and distally sinuous. The individuals from the Iberian Peninsula show a more symmetrical penis papilla than those from Argentina and Brazil and the ejaculatory duct is almost straight throughout its extension. The penis papilla, in some specimens from all populations studied herein, is bent to the left, with the dorsal insertion posteriorly displaced, but never reaching the level of the gonopore. On the Iberian specimens, the penis papilla may reach the opening of the gonopore, contrary to the South American individuals where it never reaches the gonopore. The stroma of the penis papilla is pierced by conspicuous erythrophil fine granules of two types, pinkish and erythrophil granules. The latter are more intensely stained and are organized in discrete bundles that traverse the lining epithelium of the papilla. The male atrium presents dorsal folds, one of which separates it from the female atrium. Abundant cyanophil fine granular secretion surrounds the ventro- and dorso-anterior wall of the male atrium, extending to the penis insertions.

The ovovitelline ducts arise from the ovaries, and posteriorly to the gonopore, bend dorso-medially and join each other to form a common glandular ovovitelline duct. This duct runs backwards and opens into a short female canal, which is antero-ventrally flexed. The female atrium exhibits a narrow lumen owing to its highly folded walls. Folds in the distal region of the male and female atrium depend on the state of maturity of the studied individuals.

Haplotype network analysis and mitochondrial DNA phylogeny

The haplotype network analysis recognized 21 haplotypes of *Obama nungara*. Three main networks were obtained presenting 18–21 bases that varied between them (Fig. 4). Network 1 (coincident with clade 1) included five haplotypes of specimens from Argentina (marked in blue) and one haplotype of specimens from Spain (marked in green), with the latter haplotype (haplotype 6) being the most frequent. Haplotypes in this network present a minimum of four variable characters between them.

Network 2 (coincident with clade 2 and marked in orange) was formed by eleven specimens from Brazil, nine from the present study and two obtained from the Gen Bank database. This network showed five haplotypes presenting one or two variable bases. Haplotype 9 is the most frequent.

Finally, network 3 (coincident with clade 3) included specimens from Spain, Portugal (both marked in green), and from Argentina (marked in blue). This group showed eleven haplotypes. Haplotype 17 included only specimens from Argentina and haplotype 12 was shared by specimens from Argentina and Spain (Fig. 4). Haplotypes in this network varied by one or two bases. The haplotypes 16 and 21 were the most frequent, both formed by specimens from different regions of the Iberian Peninsula.

ML analyses including all sequences from Table 1 yielded a tree (Fig. 5) with the best-fit model TPM2+I+G4, chosen according to the Bayesian Inference Criterion (BIC). The *O. nungara* sequences were grouped in a well-supported clade (99%). This clade was sister to a clade that included *O. josefi*, *O.*

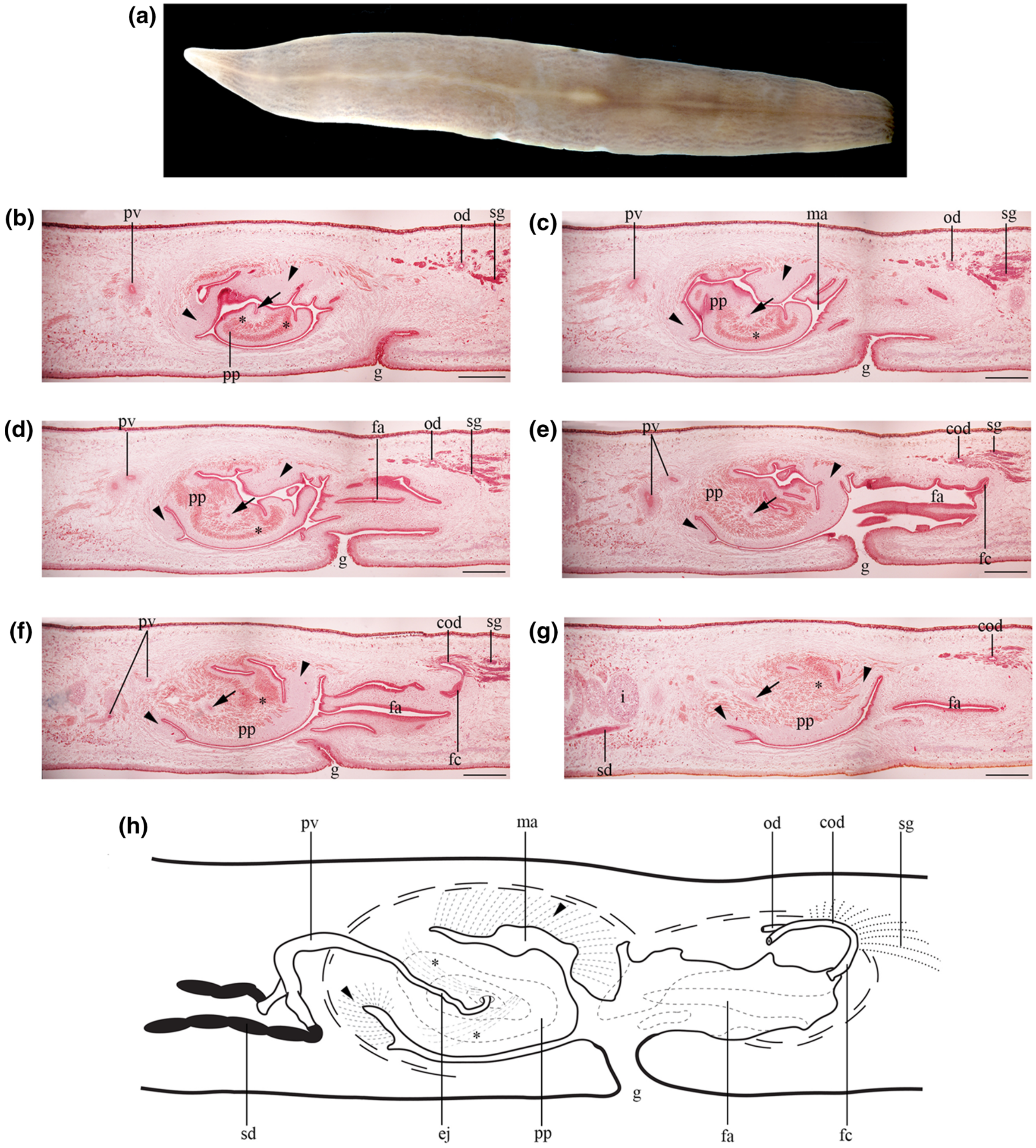


Fig. 3 *Obama nungara* from Brazil (GEO66). **a** Dorsal view of a fixed specimen. **b**–**g** Selected sagittal sections of the copulatory apparatus. **h** Schematic reconstruction of the copulatory apparatus, in sagittal view. In all figures, anterior end of the body is on the left. *cod* common ovovitelline duct, *ej* ejaculatory duct, *fa* female atrium, *fc* female canal, *g* gonopore, *ma* male atrium, *od* ovovitelline duct, *pp* penis papilla, *pv* prostatic vesicle, *sd* sperm duct, *sg* shell glands. Cyanophil fine granular secretion is indicated by arrowheads, and erythrophil fine granules are indicated by asterisks. Scale bars: 500 µm

decidualis, *O. anthropophila*, and a clade formed by *O. marmorata* and 4 specimens of *Obama* sp. (KP962357, KP962359, KP962430). *O. ladislavii* was the sister group to these two main clades. Although *O. burmeisteri* and *O. carinata*, showed well-supported monophyletic clades with bootstrap

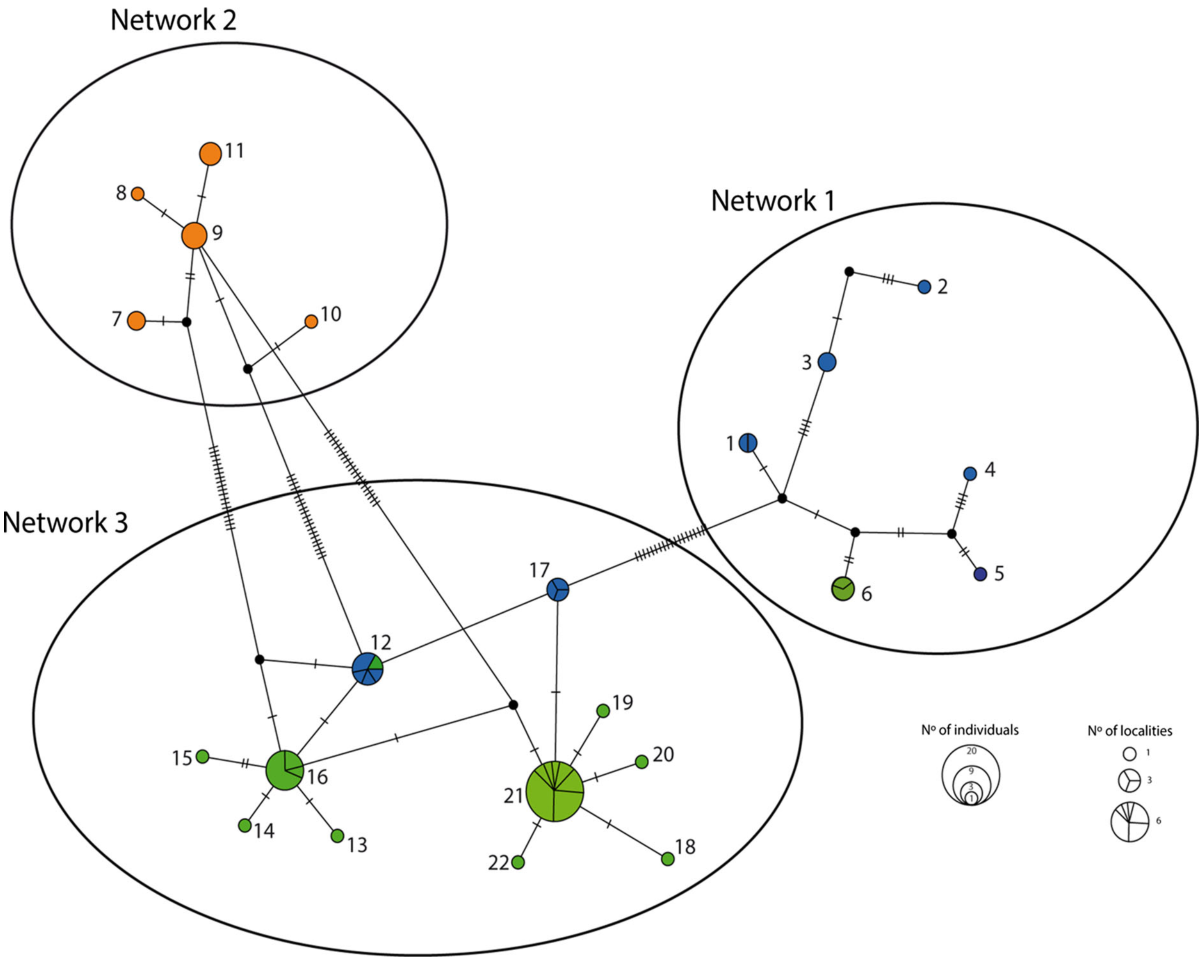


Fig. 4 TCS network inferred from *Obama nungara* mitochondrial sequences from Argentina, Brazil, Iberian Peninsula. Sequence nodes from Argentina are in blue, Brazil in orange and Iberian Peninsula in green

values of 100% and 81%, respectively, their relationships with the other clades were not well-supported.

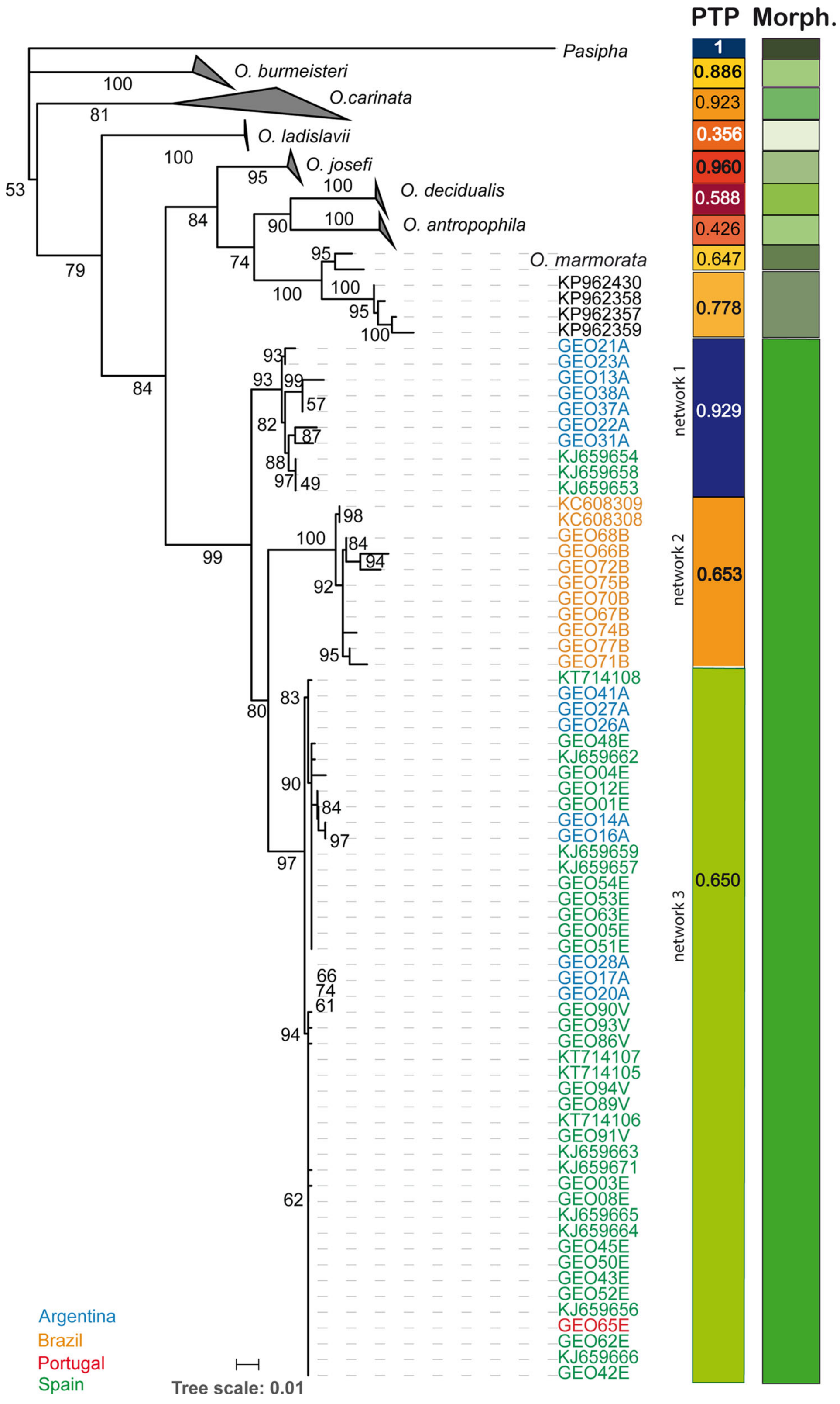
The 66 sequences that formed the *Obama nungara* clade, clustered into three well-differentiated lineages (bootstrap values of 93, 100 and 97%, respectively). Two lineages included sequences from the Iberian Peninsula and Argentina and the third included Brazilian sequences. *Obama nungara* clade 1 (Fig. 5) included specimens from Argentina and from two different localities in Spain; *O. nungara* clade 2 consisted exclusively of Brazilian specimens; and *O. nungara* clade 3 comprised specimens from different localities in Argentina and the Iberian Peninsula.

Species delimitation. Mitochondrial species delimitation results (bPTP) are summarized in Fig. 4. Within the main clade, bPTP values also indicated three groups. Representatives of Argentina, together with specimens from Spain, showed a bPTP value of 0.929, thus representing one group. All of the Brazilian specimens of *O. nungara*, constituted a second group with a bPTP value of 0.653. Specimens from various localities in the Iberian Peninsula (Portugal and Spain) and Argentina formed the last group, with a bPTP value of 0.650.

Discussion

Morphological identification

The external features and the internal morphology, mainly in regards to the copulatory apparatus, of the specimens from the Iberian Peninsula and those from Argentina and Brazil, agree with the original


PTP
Morph.
Pasipha
O. burmeisteri
O.carinata
O. ladislavii
O. josefi
O. decidualis
O. antropophila
O. marmorata
KP962430
KP962358
KP962357
KP962359
GEO21A
GEO23A
GEO13A
GEO38A
GEO37A
GEO22A
GEO31A
KJ659654
KJ659658
KJ659653
KC608309
KC608308
GEO68B
GEO66B
GEO72B
GEO75B
GEO70B
GEO67B
GEO74B
GEO77B
GEO71B
KT714108
GEO41A
GEO27A
GEO26A
GEO48E
KJ659662
GEO04E
GEO12E
GEO01E
GEO14A
GEO16A
KJ659659
KJ659657
GEO54E
GEO53E
GEO63E
GEO05E
GEO51E
GEO28A
GEO17A
GEO20A
GEO90V
GEO93V
GEO86V
KT714107
KT714105
GEO94V
GEO89V
KT714106
GEO91V
KJ659663
KJ659671
GEO03E
GEO08E
KJ659665
KJ659664
GEO45E
GEO50E
GEO43E
GEO52E
KJ659656
GEO65E
GEO62E
KJ659666
GEO42E
1
0.886
0.923
0.356
0.960
0.588
0.426
0.647
0.778
0.929
0.653
0.650
network 1
network 2
network 3
100
81
53
100
95
100
100
90
84
74
79
95
100
100
95
100
84
93
93
99
57
82
87
88
97
49
99
98
100
84
94
92
95
80
83
90
84
97
97
66
74
61
94
62
Argentina
Brazil
Portugal
Spain
Tree scale: 0.01

◀**Fig. 5** Best tree of the molecular phylogeny from the ML analyses according to Bayesian Inference Criterion (bootstrap values are indicated on the nodes). Species clusters corresponding to bPTP analyses in column 1, morphological delineations in column 2

description of *Obama nungara* (Carbayo et al. 2016). The colour pattern of the Iberian specimens varies between beige with dark striations to a dark brown background splattered with almost black striations lending a marbled appearance to the dorsum of *O. nungara*. The ventral region is whitish milky. This heterogeneity in the dorsal colour pattern has also been observed in Brazilian and Argentina populations.

However, the populations showed some differences in size (with the Spanish specimens smaller than the South American ones), and in the thickness of the cutaneous musculature (which was more developed in some specimens from Sopelana, Vizcaya, Spain). This feature could be due to diverse maturity stages. Also, the individuals from the Iberian Peninsula showed a more symmetrical penial papilla than those from Argentina and Brazil, which reach the opening of the gonopore, contrary to the South American individuals in which the penial papilla never reached the gonopore. Moreover, the ejaculatory duct in specimens from the Iberian Peninsula was straight. These small differences could represent a slight morphological divergence between the studied specimens of the Iberian Peninsula and those of South America.

Haplotype network and mitochondrial DNA analysis

The haplotype network analyses indicate the relationships among mitochondrial lineages of different *O. nungara* populations and reveal an Argentine origin of the Iberian Peninsula populations, at least in two different colonization events. The Brazilian clade presents the greatest differences with the other two clades, as it does not share haplotypes with any other clade outside of Brazil. *Obama nungara* seems to be widely distributed within Argentina, and thus a comprehensive sampling from this country is needed to better clarify relationships among populations, since the founding populations are currently unknown.

Despite the morphological similarity between individuals from different clades, the molecular differences are remarkable. The question arises whether the three identified clades are comprised of different populations of the same species or three different cryptic species. A similar situation occurs with *Platydemus manokwari*, with two different COI haplotypes, differentiated by 15 variable bases, and few morphological differences (Justine et al. 2015). Contrary to Justine et al. (2015), who found only two haplotypes for specimens of *P. manokwari* from different countries and only one population with genetic diversity, we found high levels of genetic diversity in *O. nungara* with twenty-one haplotypes.

Several species of land planarians have been recognized as introduced and are now found in different parts of the world (Álvarez-Presas et al. 2015, Justine et al. 2015, 2018). Many of these are from the Southern Hemisphere or from tropical Asia, and some show invasive traits. Successful colonization by land planarians is due to their life history traits such as hermaphroditism, sperm storage, and large capsules containing multiple offspring, which facilitates colonization, allowing only a few individuals to establish new populations (Ducey et al. 2005). *Obama nungara* has been accidentally introduced in the Iberian Peninsula and could have expanded to Europe due to the increase in the commercial exchange amongst European countries and with South America, *e.g.* Brazil and Argentina (the source of potential new introductions). There is little information on the biological life history of *O. nungara*, but we know the potential adaptive capacity of this species based on the plasticity of its food habits, as a generalist predator of soil invertebrates, such as earthworms, gastropods and other land planarians (Boll and Leal-Zanchet 2016). Invasion success is linked to the diet breadth of the introduced species, and thus *O. nungara* could be a successful invader, becoming a threat to the diversity of native invertebrates.

Conclusions

The similarities between specimens from Argentina and the Iberian Peninsula strongly indicate that the origin of the Iberian populations is Argentina. We believe that identifying routes of introduction of invasive species is key to controlling alien species dispersal. Through the use of haplotype networks, such introductions can be identified as demonstrated in the present study. With the routes identified, special measures can be applied in commerce and transport policies to avoid unintentional introductions of fauna and flora into foreign countries.

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