RECONSTRUCTING ROUTES OF INVASION OF OBAMA NUNGARA (PLATYHELMINTHES: TRICLADIDA) IN THE IBERIAN PENINSULA

Running title: Routes of invasion of Obama nungara

1Departamento de Biodiversidad y Biologia Evolutiva, Museo Nacional de Ciencias Naturales (CSIC), Madrid, Spain.

2Institut de Ciències del Mar (CSIC), Passeig Maritim 37-49, E-08003 Barcelona, Spain

3División Zoología Invertebrados, Museo de La Plata (FCNyM-UNLP), Paseo del Bosque s/n, La Plata, Buenos Aires, Argentina & CONICET Consejo Nacional de Investigaciones Científicas y Técnicas.

4Departamento de Biología, Facultad de Ciencias, Universidad Autónoma de Madrid, Cantoblanco, 28049, Madrid, Spain.

6Present address: Laboratório de Ecologia e Evolução, Escola de Artes, Ciências e Humanidades, Universidade de São Paulo, Av. Arlindo Bettio, 1000, São Paulo, SP 03828-000, Brazil.

7Corresponding author: norena@mncn.csic.es

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 Obama 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

Acknowledgements
This work was supported by the Museo Nacional de Ciencias Naturales (CSIC), funded by project CGL2011-29916 (Spanish Ministry of Economy, Industry and Competitiveness). LN, FB and CD are financed by Project N11/728 and N11/886 (FCNyM-UNLP), PIP 1122012010635CO (CONICET) and PICT 2014-0768 (ANPCyT). The authors are deeply indebted to Lourdes Alcaraz for their help and assistance in obtaining Obama's original genetic sequences. We are grateful to the staff of the greenhouses that collaborated with us.
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, *Parabu 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 Bipaliniinae, 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 anthrопized areas in Argentina and the Iberian Peninsula (IP), mainly in greenhouses and plant nurseries. Previously, *O. marmorata* had only been only 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*, 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). 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-COI F (forward 5' - GTGATGCCAGTTATGATTGTTGT-3') and COIpr-b2 (reverse 5' -...
ATGAGCAACAACATAAAGTATCATG-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 ºC (10 min), followed by 46 cycles of denaturation at 96 ºC (1 min), annealing at 61 ºC (1 min) and extension at 72 ºC (1 min), ending with a final extension step at 72 ºC (7 min). Amplified products were purified with ExoSAP-IT (Affymetrix, OH, USA) and both strands were sequenced on an ABI Prism 3730 (www.secuagen.es). DNA sequences were cleaned at the primer ends using Sequencher® 5.2 (Gene Codes Corporation, Ann Arbor, MI, USA; http://www.gene codes.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 Pasiphae 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-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 10 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 dorum is also splattered with spots irregularly disposed and anastomosed, lending a marbled appearance (Figs. 1–3). 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 substantially. Specimens from the Iberian Peninsula are slightly smaller than those from Argentina and Brazil, reaching 50mm in length and 6mm in maximum width.

Internal morphology (Figs. 1–3). 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. 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 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 Figure 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 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 unnecessary introductions of fauna and flora into foreign countries.

References

1. Álvarez-Presas M, Amaral S., Carbaya F, Leal-Zanchet AM, Riutort M (2015) Focus on the details: morphological evidence supports new cryptic land flatworm (Platyhelminthes) species revealed with molecules. Org Divers Evol 15:379–403.
2. Bessho Y, Ohama T, Osawa S (1992) Planarian mitochondria I. Heterogeneity of cytochrome c oxidase subunit I gene sequences in the freshwater planarian, Dugesia japonica. J Mol Evol 34:324–330.
3. Boll PK, Leal-Zanchet AM (2016) Preference for different prey allows the coexistence of several land planarians in areas of the Atlantic Forest. Zoology 119(3):162-168.
4. Carbaya F, Álvarez-Presas M, Jones H, Riutort M (2016) The true identity of Obama (Platyhelminthes: Geoplanidae) flatworm spreading across Europe. Zool J Linn Soc 177:5–28.
5. Clement M, Posada D, Crandall KA (2000) TCS: a computer program to estimate gene genealogies. Mol Ecol 9:1657-1660.
6. Darriba D, Taboada GL, Doallo R, Posada D (2012) jModelTest 2: more models, new heuristics and parallel computing. Nature Methods 9:772.
7. Ducey PK, West L-J, Shaw G, De Lisle J (2005) Reproductive ecology and evolution in the invasive terrestrial planarian Bipalium adventitium across North America. Pedobiologia 49: 67—377
8. Fujisawa T, Barracough TG (2013) Delimiting Species Using Single-locus Data and the Generalized Mixed Yule Coalescent (GMYC) Approach: A Revised Method and Evaluation on Simulated Datasets. Syst Biol 62:707–724. https://doi.org/10.1093/sysbio/syt033
9. Froehlich CG (1959) On Geoplanids from Brazil. Bol Fac Fil Cie Let Univ S Paulo, Ser Zool 22:201–242.
10. Invasive Species Specialist Group ISSG (2015) The Global Invasive Species Database. Version 2015.1. http://www.iucngisd.org/gisd/. Downloaded on May 31 2018.
11. Justine J, Winsor L, Gey D, Gros P, Thevenot J (2014) The invasive New Guinea flatworm Platydemus manokwari in France, the first record for Europe: time for action is now. PeerJ 2:e297.
12. Justine J, Winsor L, Barrière P, Fanai C, Gey D, Han AWK, La Quay, Lefeuvre J, Meyer J, Philippart D, Robinson DG, Tatsia F. (2015) The invasive land planarian Platydemus manokwari (Platyhelminthes, Geoplanidae): records from six new localities, including the first in the USA. PeerJ 3:e1037.
13. Justine J, Winsor L, Gey D, Gros P, Thévenot J. (2018) Giant worms chez moi! Hammerhead flatworms (Platyhelminthes, Geoplanidae, Bipalium spp., Diversibilium spp.) in metropolitan France and overseas French territories. PeerJ 6:e4672. https://doi.org/10.7717/peerj.4672
14. Kaluza P, Kölzsch A, Gastner MT, Blasius M. (2010) The complex network of global cargo ship movements. J R Soc Interface 7:1093-1103. https://doi.org/10.1098/rsif.2009.0495
15. Katoh K, Standley DM (2013) MAFFT multiple sequence alignment software version 7: improvements in performance and usability (outlines version 7). Mol Biol Evol. 30:772–780.
16. Kraepelin K (1901) Über die durch den Schiffsverkehr in Hamburg eigenschleppten Tiere. Mitt naturh Mus Hamb 18:183–209.
17. Lago-Barcia D, Fernández-Álvarez FA, Negrete L, Brusa F, Damborenea C, Grande C, Noreña C (2015) Morphology and DNA barcodes reveal the presence of the non-native land planarian Obama marmorata (Platyhelminthes: Geoplanidae) in Europe. Invertebr Syst 29:12–22.
18. Lanfear R, Calcott B, Ho SY, Guindon S (2012) Partitionfinder: combined selection of partitioning schemes and substitution models for phylogenetic analyses. Mol Biol Evol 29:1695-1701.
19. Mateos E, Tudó A, Álvarez-Presas M, Riutort M (2013) Planàries terrestres exòtiques a la Garrotxa. Annals de la delegación de la Garrotxa de la ICHN 6:67–73.
20. Murchie AK, Gordon AW (2013) The impact of the “New Zealand flatworm”, Arthurdendyus triangulatus, on earthworm populations in the field. Biol Invasions 15:569-586.
21. Sambrook J, Fritsch E, Maniatis T (1989) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory: New York.
22. Trifinopoulos J, Nguyen LT, von Haeseler A, Minh BQ (2016) W-IQ-TREE: a fast online phylogenetic tool for maximum likelihood analysis. Nucleic Acids Res 44:232-235.
23. Vila-Farré M, Sluys R, Mateos E, Jones HD, Romero R (2011) Land planarians (Platyhelminthes: Tricladida: Geoplanidae) from the Iberian Peninsula: new records and description of two new species, with a discussion on ecology. J Nat Hist 45:869–891. https://doi.org/10.1080/00222933.2010.536267
24. Wilcove DS, Rothstein D, Dubow J, Phillips A, Losos E (1998) Quantifying threats to imperilled species in the United States. Bioscience 48:607-615.
25. Zhang J, Kapli P, Pavlidis P, Stamatakis A (2013) A general species delimitation method with applications to phylogenetic placements. Bioinformatics 29:869–2876.
Legends

Figure 1+2: 1. *Obama nungara* from the Iberian Peninsula (GEO04) and 2. from Argentina (GEO21). 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. Abbreviations: 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.

Figure 3+4: 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. Abbreviations: 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.

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

Figure 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.
Table 1: Species analysed, with their locality and GenBank accession numbers. (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). *

| Species | Localities | GenBank – accession number |
|---------|------------|----------------------------|
| *Pasphe* sp. GEO15A | | |
| *Obama nungara* Carbayo, Alvarez-Presas, Jones & Riutort, 2016 | Torroella de Fluvíà, Girona Spain. | KJ659666 |
| | | |
| | | |
| | Vivero Kato, Buenos Aires (Argentina). | MG639898* |
| | | |
| | Torroella de Fluvíà, Girona Spain. | KJ659665 |
| | | |
| | | |
| | Vivero Ferrari, Buenos Aires (Argentina). | MF155841* |
| | | |
| | Vivero Zoológico, Buenos Aires (Argentina). | MF155842* |
| | | |
| | Vivero Los Toldos. Buenos Aires (Argentina). | MF155843* |
| | | |
| | Vivero Los Toldos. Buenos Aires (Argentina). | MF155845* |
| | | |
| | Vivero Nueve de Julio. Buenos Aires (Argentina). | MF155846* |
| | | |
| | Reborio, Muros de Nalón, Asturias (Spain). | MF155847* |
| | | |
| | Reborio, Muros de Nalón, Asturias (Spain). | MF155849* |
| | | |
| | Reborio, Muros de Nalón, Asturias (Spain). | MF155850* |
| | | |
| | Reborio, Muros de Nalón, Asturias (Spain). | MF155851* |
| | | |
| | Reborio, Muros de Nalón, Asturias (Spain). | MF155852* |
| | | |
| | Vivero Ferrari, Buenos Aires (Argentina). | KJ659663 |
| | | |
| | Vivero Zoológico, Buenos Aires (Argentina). | KM053222 |
| | | |
| | Vivero Los Toldos. Buenos Aires (Argentina). | KM053225 |
| | | |
| | Vivero Los Toldos. Buenos Aires (Argentina). | KM053226 |
| | | |
| | Vivero Nueve de Julio. Buenos Aires (Argentina). | KM053227 |
| | | |
| | Reborio, Muros de Nalón, Asturias (Spain). | KM053210 |
| | | |
| | Reborio, Muros de Nalón, Asturias (Spain). | KM053208 |
| | | |
| | Reborio, Muros de Nalón, Asturias (Spain). | KM053209 |
| | | |
| | Reborio, Muros de Nalón, Asturias (Spain). | KM053211 |
| | | |
| | Bordils, Girona, Spain | KJ659663 |
| | | |
| | Sopelana, Vizcaya (Spain). | MF155866* |
| | | |
| | Sopelana, Vizcaya (Spain). | MF155867* |
| | | |
| | Sopelana, Vizcaya (Spain). | MF155868* |
| | | |
| | Córdoba (Argentina). | MF155869* |
| | | |
| | Sopelana, Vizcaya (Spain). | MF155870* |
| | | |
| | Sopelana, Vizcaya (Spain). | MF155871* |
| | | |
| | Torroella de Fluvíà, Girona Spain. | KT714108 |
| | | |
| | Torroella de Fluvíà, Girona Spain. | KT714107 |
| | | |
| | Torroella de Fluvíà, Girona Spain. | KT714106 |
| | | |
| | Torroella de Fluvíà, Girona Spain. | KT714105 |
| | | |
| | Sopelana, Vizcaya (Spain). | MF155872* |
| | | |
| | Vivero Zoológico, Buenos Aires (Argentina). | MF155873* |
| | | |
| | Valeria del Mar, Buenos Aires (Argentina). | MF155874* |
| | | |
| | Bordils, Girona (Spain). | KJ659654 |
| | | |
| | Parque Pereyra Iraola, Buenos Aires (Argentina). | MF155875* |
| | | |
| | Villas de Mar, Barcelona (Spain). | KJ659658 |
| | | |
| | Bordils, Girona (Spain). | KJ659653 |
| **Obama nungara** | Location | Accession Number |
|-------------------|----------|------------------|
| GEO22A            | Vivero Zoológico, Buenos Aires (Argentina) | MF155876* |
| GEO37A            | Parque Pereyra Iraola, Buenos Aires (Argentina) | MF155877* |
| GEO31A            | Isla Martin Garcia, Buenos Aires (Argentina) | MF155878* |
| GEO13A            | Vivero Kato, Buenos Aires (Argentina) | MF155879* |
| GEO66B            | Santiago, Rio Grande do Sul (Brazil) | MF155880* |
| GEO70B            | Santiago, Rio Grande do Sul (Brazil) | MF155881* |
| GEO67B            | Santiago, Rio Grande do Sul (Brazil) | MF155882* |
| GEO75B            | Santiago, Rio Grande do Sul (Brazil) | MF155883* |
| GEO77B            | Santiago, Rio Grande do Sul (Brazil) | MF155884* |
| GEO2438           | Parque Estadual da Serra do Tabuleiro, SC (Brazil) | KC608309 |
| GEO2439           | Parque Estadual da Serra do Tabuleiro, SC (Brazil) | KC608308 |
| GEO74B            | Santiago, Rio Grande do Sul (Brazil) | MF155885* |
| GEO72B            | Santiago, Rio Grande do Sul (Brazil) | MF155886* |
| GEO68B            | Santiago, Rio Grande do Sul (Brazil) | MF155887* |
| GEO71B            | Santiago, Rio Grande do Sul (Brazil) | MF155888* |
| **Obama anthropophila** | Floresta Nacional São Francisco de Paula, RS (Brazil) | KP962470 |
| **Obama decidualis** | Santa Maria, RS (Brazil) | KP962391 |
| **Obama josefi** | Floresta Nacional São Francisco de Paula, RS (Brazil) | KP962367 |
| **Obama sp.**     | P.N. Serra do Itajai, SC (Brazil) | KP962430 |
| **Obama marmorata** | Parque Estadual da Serra do Tabuleiro, SC (Brazil) | KT714104 |
| **Obama ladislavii** | P.E. Serra do Tabuleiro, SC (Brazil) | KP962492 |
| **Obama burmeisteri** | P.E. Cantareira, SP (Brazil) | KP962378 |
| Location                                      | Accession Number |
|-----------------------------------------------|------------------|
| P.E. Cantareira, SP. (Brazil)                 | KP962377         |
| 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         |

*Obama carinata* (Riester, 1938)