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Phylogenetic and morphological study of the genus *Potamolithus* (Truncatelloidea: Tateidae) in hotspots of diversity at the Paranaense Forest, Argentina, with the addition of six new species

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ABSTRACT

The Atlantic Forest is a highly fragmented biodiversity hotspot, which in Argentina is represented by the Paranaense Forest, covering a wide area of the province of Misiones. In conservation studies, groups of vertebrates, plants and arthropods are generally used together but other invertebrates scarcely used. In the present work, we aim to provide information on new species of freshwater gastropods, from protected and unprotected areas. Specimens were collected in a protected area (Yabotí Biosphere Reserve) and in two unprotected areas (Alba Posse city and Centro de Investigaciones Antonia Ramos -CIAR-). The shell, radula, internal anatomy and the molecular information of the partial sequence of mitochondrial cytochrome c oxidase subunit I (COI) were analysed. The phylogenetic trees were obtained using Maximum Likelihood and Bayesian Inference analyses. Altogether, the anatomical, shell, and genetic studies allow us to differentiate six new species of the genus *Potamolithus* from the others present in Argentina and the surrounding areas. These new species raise the number of freshwater gastropods for the malacological province Río Uruguay to 64. Three of the new species have been found within a protected area, while other three at unprotected areas. The presence of invasive species, such as *Limnoperna fortunei* (Dunker 1857) and *Melanoides tuberculata* (Müller, 1774), as well as the possible creation of a hydroelectric dam, may modify the environment inhabited by the new endemic fauna.

1. Introduction

The Atlantic Forest has been ranked as the 35th world's biodiversity hotspot area (Mittermeier et al., 2004) where the original vegetative cover has been reduced by at least 70 percent. These critical areas occupy less than 2% of the earth's land surface (7–8% of the original forest remains) and harbour around 7% of the World's flora and fauna (Leal and Câmara 2003). Habitat fragmentation associated with other human disturbances (e.g. illegal logging, plant and animal poaching, and the introduction of alien species) have led to the loss of biodiversity (loss of ecosystems, populations, genetic variability, species and ecological processes that maintain local diversity) (Leal and Câmara 2003; Marini and Garcia 2005).

The Paranaense Forest, a subdivision of the Atlantic Forest, occupies the northeast region of Argentina (mainly, the province of Misiones and the northeast region of Corrientes), the southeast of Brazil and the east of Paraguay (Giraudo and Povedano 2004; Giraudo et al., 2005; Negrete et al., 2019). This ecosystem is one of the most threatened ones in the world (Giraudo et al., 2005). Most

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of the studies for conservation have been carried out on vertebrates, plants or arthropods (Avigliano et al., 2019). However, in recent years in this sector of the Paranaense Forest have been carrying out studies on molluscs and other invertebrates providing information not only on new species, but also on possible impacts that may affect the genetic diversity of the species present in the before mentioned area (Gutiérrez Gregoric and Rumi 2008; Gutiérrez Gregoric 2012; Gutiérrez Gregoric and de Lucía 2016; Gurovich et al., 2017; 2018; Negrete and Brusa 2017; Vogler et al., 2019).

The orography of the Misiones Province is rather pronounced and marked by a central ridge that acts as a watershed between the two great international rivers: Paraná and Uruguay—respectively of the Misionerean and Uruguay River malacological provinces as defined by Núñez et al. (2010). The Uruguay River is considered one of the global hotspots of freshwater gastropod diversity according to Strong et al. (2008), within the category of "Large rivers and their first and second order tributaries". This hotspot is represented mainly by the Tateidae Thiele, 1925 (genus *Potamolithus*, with 12 endemic species of this basin), and to a lesser extent by Chilinidae Gray, 1870 (six endemic species of the genus *Chilina* Gray, 1828) and Ampullariidae Gray, 1824 (endemic genus *Felipponea* Dall, 1919 with three species) (Gutiérrez Gregoric and de Lucía 2016).

Tateidae is the second family of freshwater gastropods with more species in the world (43 genera and 490 species) with Gondwanan distribution (Lydeard and Cummings 2019). Most of the species are native to Australia, New Zealand, New Caledonia, and other islands of the South Pacific (Ponder, 2019). Only two genera have been registered outside these areas: *Potamopyrgus* Stimpson, 1865, originally from New Zealand and adjacent islands, which behaves in an invasive manner worldwide (Ponder, 1988; Bowler, 1991; Shimada and Urabe 2003; Hamada et al., 2013; Collado, 2014), and *Potamolithus* Pilsbry & Rush, 1896, the only native genus belonging to this family that may be found in southern South America (Argentina, Chile, Uruguay and southern Brazil) (Wilke et al., 2013; Haase and Zielske 2015; Zielske and Haase 2015; Ponder, 2019; Collado et al., 2019a, 2019b).

Potamolithus is represented by 41 species, out of which 15 have Paysandú (Uruguay) as type locality; 32 are present at the Del Plata basin (Argentina, Uruguay and Brazil); five present only in Brazil (one at the San Francisco basin and four at the South Atlantic basin), while four species inhabit both Argentine Patagonia and southern Chile (Pilsbry, 1899; Pilsbry, 1911; Pilsbry, 1924; Pilsbry, 1925; Davis and Silva 1984; Simone and Moracchioli, 1994; López Armengol, 1985; López Armengol and Darrigran, 1998; Rumi et al., 2008; Núñez, 2017; de Lucía and Gutiérrez Gregoric, 2017a; 2017b; Collado et al., 2019a). Most species of this genus have descriptions only of their shells, and only a few present anatomical or molecular studies (Davis and Silva, 1984; López Armengol, 1985; Simone and Moracchioli, 1994; Silva andVeitenheimer-Mendes, 2004; Wilke et al., 2013; de Lucía and Gutiérrez Gregoric, 2017a; 2017b; Núñez, 2017). In terms of species level, the majority of Tateidae are difficult to identify by using shells alone; anatomical features or molecular data are often necessary for a more accurate identification (Collado et al., 2019a; Ponder, 2019).

In Argentina, *Potamolithus* has the most species (N = 25) of freshwater gastropods (Rumi et al., 2008; Gutiérrez Gregoric and de Lucía 2016; Núñez, 2017; de Lucía and Gutiérrez Gregoric, 2017a, 2017b). Most of them may be found in the Uruguay River and its tributaries. In the Misiones Province there are 15 species of *Potamolithus* (Fig. 1 and supplementary material SM.1) (Pilsbry, 1911; Parodiz, 1965; Parodiz, 1966; López Armengol, 1985; Rumi et al., 2008; Núñez et al., 2010; Núñez, 2017) of which 14 are found in the basin of the Uruguay River (seven endemic, four shared with the basin of the Paraná River, and three shared with the basin of the Paraná River and the Río de la Plata River), and one in the basin of the Paraná River. Of these 14 species, only two have been described using anatomical and shell characters (Núñez, 2017), in four cases head-foot, radula and pallial organs data have been included, while all the rest have been described using only the external shell morphology. None of these species present molecular data.

In the present study, we describe six new species of the genus *Potamolithus* in the Paranaense Forest, with the knowledge that many species in conservation areas remain undescribed.

2. Material and methods

2.1. Studied material

Specimens were collected in five hydrological systems from the Misiones Province: the Yabotí Miní stream (26°57′38″S, 53°49′30″W) in 2005; Uruguay River: Alba Pose city (27°33′59″S, 54°40′31″W) in 2010 and the Moconá Falls (27°8′43″S, 53°53′23″W) in 2011 and 2016; the Oveja Negra stream (27°08′14″S, 53°55′27″W) in 2015 and 2016; the Pepirí Miní stream (27°08′42″S, 53°55′41″W) and the Acaraguá stream (27°26′48″S, 54°55′41″W) in 2016 (Fig. 1). In addition, material from the Malacological Collection in the Museum of La Plata of the National University of La Plata, Buenos Aires Province, Argentina (MLP-Ma); photographs of lectotypes, paralectotypes and syntypes of The Academy of Natural Sciences of Philadelphia, USA (ANSP) and the Natural History Museum, London (NHMUK) have been analysed. Photographs of the holotype of *Potamolithus peristomatus misionum* Parodiz, 1966 were requested from the Carnegie Museum of Natural History, USA (CMNH), but the material was not found. Photographs of the holotype of *Potamolithus tricostatus* Brot, 1867 were requested from the Natural History Museum, Geneva (MHN), but the material was not found. The specimens used have been deposited in the Malacological Collection of MLP-Ma and the National Collection of Invertebrates of the Argentine Museum of Natural Sciences Bernardino Rivadavia, Buenos Aires, Argentina (MACN-In).

2.2. Morphological studies

The specimens have been analysed under a Leica MZ6 stereoscopic microscope and drawn with the aid of a *camera lucida*. The shells have been drawn and photographed in their umbilical, ventral, lateral, apical, and dorsal views. Some shells and opercula have been observed using the scanning electron microscope (SEM) (JEOL/JSMT 6360 LV, Tokyo, Japan) of the Museum of La Plata, with backscattered electron (BSE) and secondary scanning electron (SE) microscopy. Two techniques have been used to decalcify the shell and proceed to analyse the external and internal anatomy: some specimens have been immersed in hydrochloric acid (1N); and others in modified Raillet-Henry solution (distilled water 93%, acetic acid 2%, formalin 5% and 6 g of sodium chloride per l of solution) (Paraense 1976). The specimens of the MLP have been drawn, measured, and photographed and those with dry tissue inside the radula have been removed.

We have taken measurements of eight variables of the shell according to Parodiz (1951), Hershler and Landye (1988) and Núñez (2017): total length (TL), total width (TW), spire length (LS), spire base (SB), body whorl length (LBW), whorls number (NW), aperture length (LA), aperture width (WA). To compare and establish relationships between measures, we have analysed the following proportions: LBW/TL; TL/TW; LS/TL; SB/TW; LA/TL. To verify the existence of differences in the set of dependent variables and to identify the variables that distance populations, we have performed a discriminant analysis (DA). The DA is indicated in order to explore

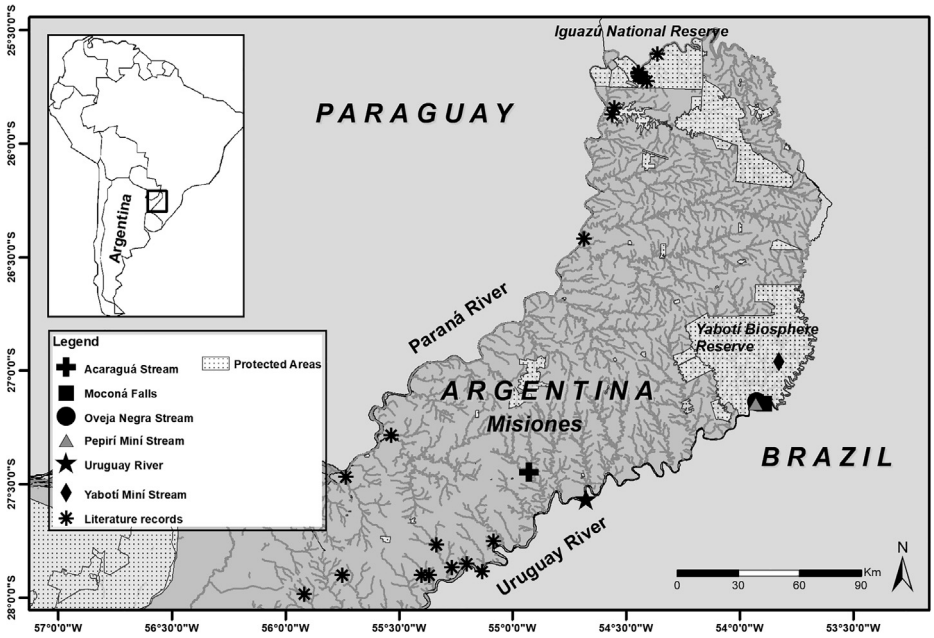

Fig. 1. Sampling localities and reported. Protected area: Yabotí Biosphere Reserve (■ Yabotí Miní stream; ◆ Moconá Falls; ● Oveja Negra stream; △ Pepirí Miní stream). Unprotected area: ★ Alba Posse city; ✚ Acaraguá stream located within Centro de Investigaciones Antonia Ramos (CIAR); ✳ records of the literature of *Potamolithus* spp. See more information in supplementary material S.M.1.

the differences of different groups of observations that were previously defined to, then, allow the most probable classification of new observations in one of the groups, for example to evaluate if the morphometric identity corresponds to the taxonomic identity. For this purpose, the variables highly correlated were removed following the Pearson value, considering the limit $r \geq 0.95$. The statistical analysis was performed using the program Past (version 3.5, Hammer et al., 2001).

The radulae was prepared according to the protocol of Holznagel (1998), and observed in SEM, with backscattered electron (BSE) and secondary scanning electron (SE) microscopy. Those bulbs that have been preserved in the Raillet-Henry solution, prior to treatment with the Holznagel protocol, have been placed in alcohol for a week. The measurements have been made from the images obtained in the SEM. We followed Davis et al. (1986) for radular measurements and Hershler & Ponder (1998) for descriptions of the teeth. The anatomical characterization and measurements were performed following Davis et al. (1986), López Armengol (1985); Hershler and Landye (1988) and Hershler and Ponder (1998). The following anatomical measures were taken from the drawings: ctenidium length (CL); osphradium length (OL); ctenidium number filaments; width of mantle border (WMB); mantle cavity length (MCL = WBM + CL); OL/CL percentage ratio; visceral mass length (VML) (total magnitude of the segments that extend from the posterior end to the anterior end through the midpoint of the visceral mass); CL/VML percentage ratio. The position and distance between the nuchal node (females) and the base of the penis (males) with respect to the right eye, as well as the angle of the nuchal node, or the base of the penis with respect to the midline of the neck were calculated following Davis et al. (1986). For measurements of the nervous system, we have followed Davis et al. (1976). The nervous system concentration (RPG) was also calculated: length of the pleuro-supraesophageal connective/(length of the suprasophageal ganglion + length of the pleuro-suprasophageal connective + length of the right pleural ganglion). In the descriptions of the new species, only the characteristics that differ from the general description of a typical *Potamolithus*, or some unique characteristic of that species are commented.

2.3. Molecular studies

A fragment of tissue was dissected from the foot of the snails collected in 2015 (one specimen) and 2016 (13 specimens) for DNA extraction, using the CTAB method (Winnepennickx et al., 1993) in those larger specimens and with the commercial kit Qiagen in samples that present a very small size. Partial sequences of the mitochondrial cytochrome c oxidase subunit I (COI) gene were amplified with the universal primers by Folmer et al. (1994). The polymerase chain reaction (PCR) amplification was performed following Gutiérrez Gregoric et al. (2013, 2014). The PCR products were purified using a Pure DNA-Clean Up kit (PB-L Productos Bio-Lógicos) and the two DNA strands of the gene were directly sequenced in Macrogen Inc., Korea. The original sequences were edited in BioEdit v7.0.5.3 (Hall, 2001) and then aligned together with homologous COI sequences downloaded from GenBank using Clustal Xv2.0.12 (Larkin et al., 2007). The uncorrected genetic distance (p-distance) and the corrected distance (with the Kimura-2-parameters model) were calculated using MEGA X (Kumar et al., 2018). The information and access numbers of the Genbank collection are provided in supplementary material SM.2. Some entities belonging to the genus *Potamolithus* but mistakenly included in the genus *Heleobia* Stimpson, 1865 (Cochliopidae family) (Koch et al., 2015; Collado et al., 2019a, 2019b) were incorporated into the molecular analysis. Phylogenetic trees were obtained using Maximum Likelihood (ML) and Bayesian inference (BI) methods. The ML analysis was performed using PhyML (Guindon et al., 2010) via the ATGC bioinformatics platform (http://www.atgc-montpellier.fr/). The best nucleotide substitution model selected by using the SMS program (Lefort et al., 2017) and the Akaike Information Criterion was the GTR + G model. The statistical support of the nodes was evaluated using 1,000 bootstrap replicates. The BI was performed using MrBayes v3.1.2 (Ronquist et al., 2012) after selecting the best evolutionary model in jModelTest

2.1.7 (Darriba et al., 2012) under the Akaike Information Criterion (TPM3uf + I + G). This analysis was run for 5 million generations, sampling trees every 5,000 generations and using a burn-in period of 20%.

3. Results

Taxonomy.
Superfamily Truncatelloidea Gray, 1840.
Family Tateidae Thiele, 1925.

3.1. Genus Potamolithus Pilsbry and Rush, 1896

3.1.1. Description

3.1.1.1. Shell and operculum. Small shell (<10 mm), relatively solid, trochoid, globose or conic, complete peristome in sexually mature specimens. Operculum corneous, elliptical, with submarginal nucleus, paucispiral, lacking pegs or internal white smear.

3.1.1.2. Radula. Taenioglossa. Central tooth broad, with several anterior and basal cups. The basal cusps arise from the outer side of the face of the tooth, and the inner ones are larger than the outer ones.

3.1.1.3. Head-foot and non-genital anatomy. Cephalic tentacles long, thin, pointed, usually pigmented. Snout usually pigmented, lips unpigmentated, and no pigmentation around eyes. Eye lobe generally well developed. A nuchal node, generally displaced to right of head, may be present in females of some species (function unknown). Ctenidium with triangular filaments. Osphradium elongate oval, usually located in front of the middle zone of ctenidium. Hypobranchial gland poorly visible and covers almost entire rectum. Intestine curved, faecal pellets oriented transversely, anus near edge of mantle cavity on right side. Renal organ behind pallial cavity. Pericardium behind mantle cavity and connecting ctenidium by short blood vessel.

3.1.1.4. Reproductive system. Male. Testis, in general, branched and lobed. Posterior vas deferens leaves the testis, reaches the wall of the mantle cavity and enters the prostate. Anterior vas deferens can have a spiral or wavy shape, as well as a thicker one, equal or finer than the posterior vas deferens. Anterior vas deferens leaves the prostate, then crosses the wall of the cavity of the mantle and enters the wall of the body, close to the columellar muscle, and goes to the neck, until it enters through the base of the penis, where it becomes thinner. Penis located on right side of neck, near centre. Distally, an eversible terminal papilla is usually present. In a few species, no preputial collar or terminal papilla may be distinguished. *Female*. Ovary, in general, branched and lobed. Oviduct, in general, first makes contact with seminal receptacle and then with bursa copulatrix, to finally contact the ventral groove of pallial oviduct. Pallial oviduct divided into albumen and capsule glands, opening close to anus. Oviparous. Egg capsules hemispherical, smooth, attached to rocks or shells of *Potamolithus*, or other freshwater snails such as *Chilina* or *Heleobia*.

3.1.1.5. Nervous system. Typical of Truncatelloidea. Ganglia separated by connectives and elongated commissures. Cerebral and pleural ganglia abutting or fused, cerebral-pleural connective not distinguished. Ganglia may have a slight grey pigmentation. Left pleural ganglion separated from subesophageal ganglion by short pleuro-subesophageal connective (Fig. 2).

3.1.1.6. Ecology. Microphagous. Inhabit lotic environments, preferably rocky, but have been found in sand even on aquatic vegetation (eg. *Schoenoplectus californicus* (C.A.Mey.) Soják).

3.2. Potamolithus rauli de Lucía & Gutiérrez Gregoric sp. nov

(Fig. 3) lsid:zoobank.org:act:8EB37616-313B-4652-B181-89ADEA8C32A4

3.2.1. Etymology

The specific epithet is dedicated to Raúl, the father of the first author of this paper.

3.2.2. Type-material

Holotype: MLP-Ma 15053: Acaraguá Stream (27°26′48″S, 54°55′41″W), Misiones Province, Argentina. *Coll.* F. Brusa, L. Negrete and A. Zivano, 2016.

Paratypes: MLP-Ma 15054 and MACN-In 43333. Same locality.

Additional material: MLP-Ma 15055. Same locality.

Distribution: Only known from type locality.

3.2.3. Diagnosis

Shell small, globose, thin. TL 1.42-2.63 mm, 3.19–3.89 whorls. Gill filaments: 14–18. Penis with small left expansion on the preputial collar. Females without nuchal node.

3.2.4. Description

3.2.4.1. Shell and operculum (supplementary material SM.3 and SM.4) (n = 31). Globose, thin, small, light brown periostracum that turns green towards the opening. TL 1.42-2.63 mm (holotype: 2.16 mm), TW 1.37-2.43 mm (holotype: 2.10 mm); 3.19–3.89 whorls. Protoconch 1.51 whorls, without ornamentation Short spire (11% of TL). Aperture slightly pyriform (73% of TL). Flat columella (slightly concave), with an adcolumellar area (area to the right of columella).

3.2.4.2. Radula (supplementary material SM.5) (n = 3). Radular ribbon: medium size (33% of TL shell), length 0.74 mm, width 0.11 mm. Total rows 81 (in development 18). Central tooth: 13 anterior cusps and 3–4 pairs of basal cusps, trapezoidal shape (width of the anterior surface 15.31 μm; width of the basal surface 21.98 μm), dorsal surface with slight concavity, with deep excavation at the base (60% of the height of the tooth), the basal process that joins the central tooth to the U-shaped lingual membrane and with a length equal to the lateral margin; Lateral tooth: 11–12 cusps, longer and dagger-shaped mesocone (pointed); Internal marginal tooth: 26–28 cusps equally-developed, being the sixth cusp from the outside longer than others; External marginal tooth: 25–32 cusps equally-developed.

3.2.4.3. Head-foot and non-genital anatomy (supplementary material SM.6) (n = 7). Very dark grey/black head except on the lips; dark grey tentacle with a darker midline (black). Mantle dorsally dark grey, lighter on the sides, ventrally unpigmentated. Female without a nuchal node. Ctenidium occupies 27% of VML, with 14–18 filaments. Osphradium occupies 38% of CL.

3.2.4.4. Reproductive system (supplementary material SM.6) (n = 7). Male. Long penis (occupies 31% of VML), white, with a left expansion on the preputial collar, with long, fine terminal papilla (sometimes with black pigmentation inside). Distance from penis to eyes 0.13 mm. Anterior vas deferens wavy and similar in thickness to posterior vas deferens. *Female*. Seminal receptacle spherical-oval, small, very short canal, placed ventrally against the wall of bursa copulatrix. Bursa copulatrix oval-piriform, positioned

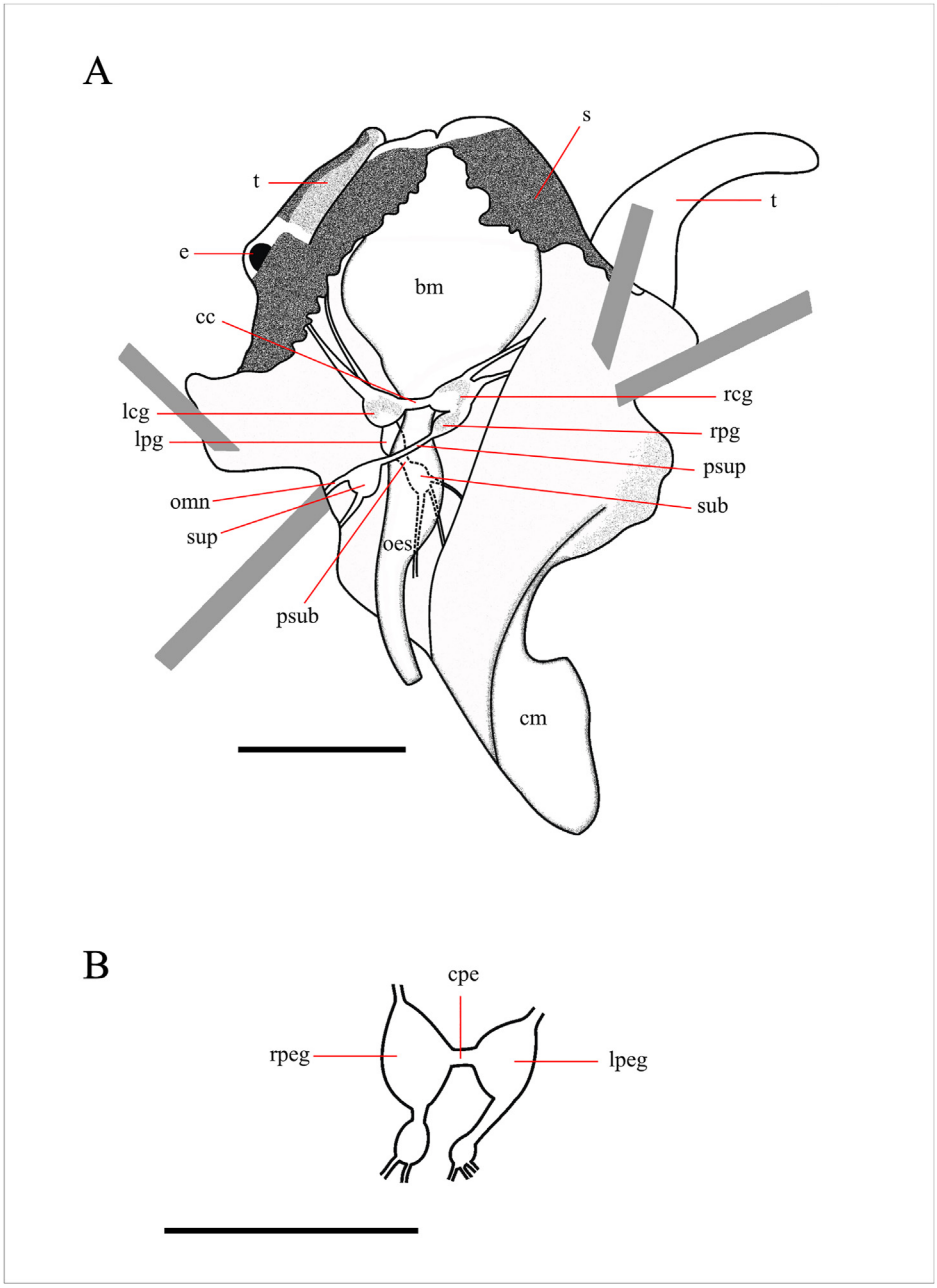

Fig. 2. General scheme of the nervous system of *Potamolithus nelidae* sp. nov. (A) Dorsal view of the central nervous system; (B) Anterior view of the pedal ganglion complex. Abbreviations: cc, cerebral commissure; cm, columellar muscle; cpe, commissure pedal; e, eye; lcg, left cerebral ganglion; lpeg, left pedal ganglion; lpg, left pleural ganglion; oes, oesophagus; omn, osphradium-mantle nerve; psub, pleuro-subsophageal connective; psup, pleuro-supraesophageal connective; rcg, right cerebral ganglion; rpeg, right pedal ganglion; rpg, right pleural ganglion; s, snout; sub, subesophageal ganglion; sup, supraesophageal ganglion; t, tentacle. The grey bars are needles. The right tentacle is in ventral view (without pigmentation). Dotted line: ganglia below the oesophagus. Scale bars: 1 mm.

over middle part of albumin gland, approximately half length of albumen gland, bursal duct enters bursa anteriorly. Albumin gland not present in mantle cavity, approximately same size as capsule gland.

3.2.4.5. Genetic information. Two sequences of the COI gene of 658 bp were obtained and registered at the GenBank under the identification numbers MT299927 and MT299928.

3.2.4.6. Remarks. This new species presents no differences between males and females in terms of body coloration, number of gill filaments or shell. *Potamolithus rauli* sp. nov. is like *Potamolithus karsticus* (Simone and Moracchioli, 1994) and *Potamolithus troglobius* Simone and Moracchioli, 1994, regarding its small size; it is larger than *Potamolithus elenae* (de Lucía and Gutiérrez Gregoric, 2017a; 2017b), and smaller than *Potamolithus ribeirensis* (Pilsbry, 1911), from the Feitoria river, Brazil (Fig. 4; Taxonomic key). It differs from these four in the shape of the shell, colour of the head, number of gill filaments, and shape of the penis, vas deferens and radula characters (supplementary material SM.7). Because of its small size, the nervous system was not analysed.

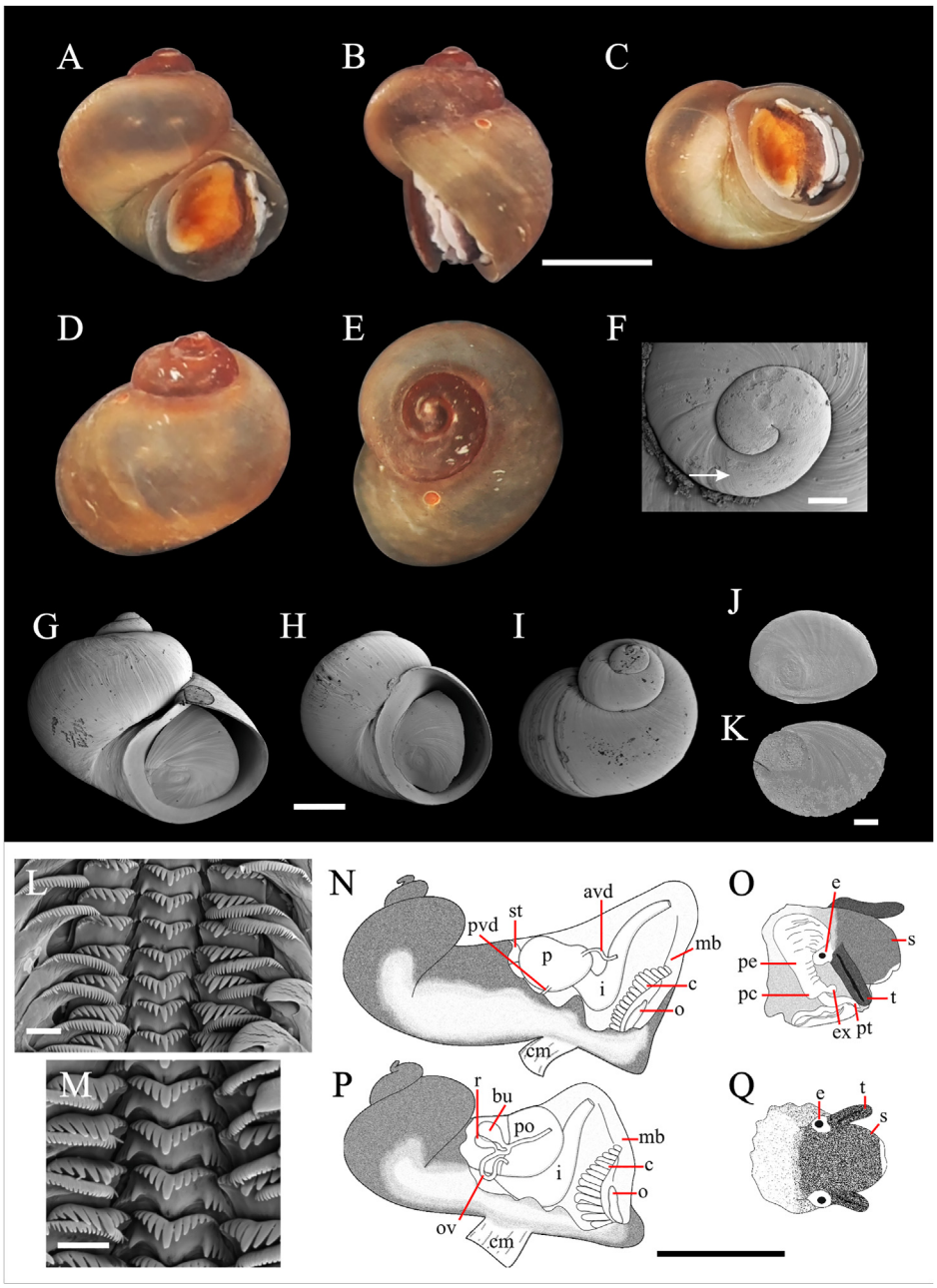

Fig. 3. *Potamolithus rauli* sp. nov. (A–E) Holotype (MLP-Ma 15053) shell; (F–M) Paratypes (MLP-Ma 15054); (F–I) Shell (images of the SEM-BSE-); (J–K) Operculum: internal view, external view (images of the SEM-BSE-); (L–M) Radula (images of the SEM-BSE-); (N–O) Male; (P–Q) Female. Abbreviations: avd, anterior vas deferens; bu, bursa; c, ctenidium; cm, columellar muscle; e, eye; ex, expansion on preputial collar; i, intestine; mb, mantle border; o, osphadium; ov, oviduct; p, prostate; pc, prepucial collar; pe, penis; po, pallial oviduct; pt, terminal papilla; pvd, posterior vas deferens; r, receptacle; s, snout; st, stomach; t, tentacle. Arrow: limit of protoconch. Scale bars: A-E, N-Q = 1 mm; F = 100 μm; G-I = 500 μm; J-K = 200 μm; L-M = 10 μm.

3.3. *Potamolithus nezibrus de Lucía & Gutiérrez Gregoric sp. nov*

(Fig. 5) lsid:zoobank.org:act:93CB3E86-9722-4AF3-98E2-F04BBC4FAA3C

3.3.1. *Etymology*

Dedicated to Lisandro Negrete, Agustina Zivano and Francisco Brusa who have collected the material analysed.

3.3.2. *Type-material*

Holotype: MLP-Ma 15056 (male). Oveja Negra Stream (27°08′14″S, 53°55′27″W), Misiones Province, Argentina. *Coll.* F. Brusa, L. Negrete and A. Zivano, 2016.

Alotype: MLP-Ma 15057 (female). Same locality.

Paratypes: MLP-Ma 15058 and MACN-In 43334. Same locality.

Additional material: MLP-Ma 15059, MLP-Ma 15060 and MACN-In 43342. Same locality.

Distribution: Only known from type locality.

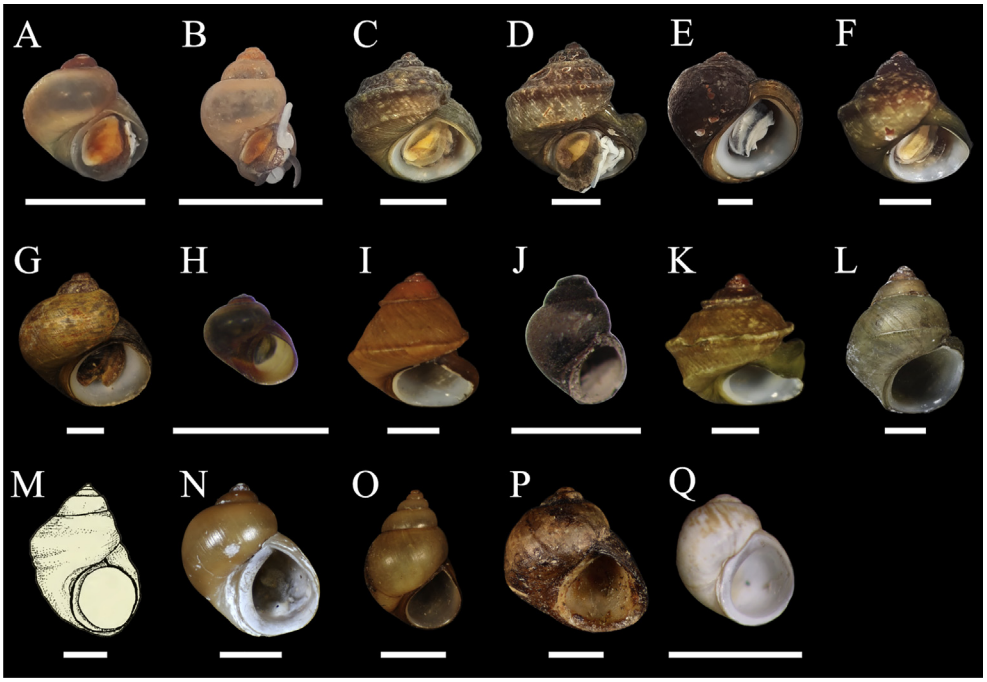

Fig. 4. Shells of the six new species and other similar species compared here. (A) *Potamolithus rauli* sp. nov. (holotype MLP-Ma 15053); (B) *Potamolithus nezibrus* sp. nov. (holotype MLP-Ma 15056); (C) *Potamolithus tricarinatus* sp. nov. (holotype MLP-Ma 15061); (D) *Potamolithus recurvatus* sp. nov. (holotype MLP-Ma 15064); (E) *Potamolithus grossus* sp. nov. (holotype MLP-Ma 15066); (F) *Potamolithus nelidae* sp. nov. (holotype MLP-Ma 15075); (G) *Potamolithus doeringi* (lectotype ANSP 103074); (H) *Potamolithus elenae* (holotype MLP-Ma 14202); (I) *Potamolithus hidalgoi* (lectotype ANSP 69687); (J) *Potamolithus karsticus* (holotype MZUSP 27942); (K) *Potamolithus microthauma* (lectotype ANSP 69689); (L) *Potamolithus peristomatus* (lectotype NHMUK 1854.12.4.338); (M) *Potamolithus peristomatus misionum* (original drawing); (N) *Potamolithus ribeirensis* (ANSP 353484); (O) *Potamolithus simplex* (lectotype ANSP 63433); (P) *Potamolithus tricostatus* (syntypes ANSP 27086); (Q) *Potamolithus troglobius* (holotype MZUSP 27947). Scale bars: 2 mm. Images taken from MLP-Ma (A, B, C, D, E, F, H); ANSP (G, I, K, N, O, P); Simone 2006 (J, Q); NHMUK (L); The Nautilus 1966 (M); with permission.

3.3.3. Diagnosis

Small shell, conic, thin. TL 1.67-2.16 mm, 3.95–4.68 whorls. Gill filaments: 13–17. Penis without terminal papilla or preputial collar. Females without nuchal node.

3.3.4. Description

3.3.4.1. Shell and operculum (supplementary material SM.3 and SM.4) (n = 22). Conic, thin, small. Periostracum light brown. TL 1.67-2.16 mm (holotype: 1.90 mm), TW 1.35-1.72 mm (holotype: 1.39 mm); 3.95–4.68 whorls. Medium spire (22% of TL). Aperture oval (50% of TL). Concave columella.

3.3.4.2. Radula (supplementary material SM.5) (n = 3). Radular ribbon: medium size (26% of the TL shell), length 0.54 mm, width 0.09 mm. Total rows 78 (in development 18). Central tooth: 11–13 anterior cusps and 2 pairs of basal cusps on each side, trapezoidal shape (width of the anterior surface 12.16 μm; width of the basal surface 19.26 μm), dorsal surface with concavity, with deep excavation (57%) at the base of the tooth; Lateral tooth: 12–13 cusps, longer and dagger-shaped mesocone (pointed); Internal marginal tooth: 27–32 cusps equally-developed, being the sixth cusp from the outside longer than others; External marginal tooth: 31 cusps.

3.3.4.3. Head-foot and non-genital anatomy (supplementary material SM.6) (n = 12). Black head and tentacles. In some specimens, lips are dark grey in dorsal area. Mantle dorsally black or dark grey, lighter on the sides, ventrally unpigmented. Female without a nuchal node. Ctenidium occupies 19% of VML, with 13–17 filaments. Osphradium occupies 44% of CL.

3.3.4.4. Reproductive system (supplementary material SM.6) (n = 12). Male. Long penis (occupies 39% of VML), white, with internal black spots on some specimens, without a preputial collar or differentiated terminal papilla. Distance from penis to eyes 0.22 mm. Anterior vas deferens leaves prostate in a spiral way (in some, after leaving the prostate and making a few turns, it makes a U before crossing the final wall of mantle cavity) and of a thickness similar to the posterior vas deferens. *Female*: Seminal receptacle spherical-oval, small, duct very short, placed postero-ventrally against the wall of bursa copulatrix. Bursa copulatrix oval, positioned over posterior of albumin gland, approximately same length as albumin gland, bursal duct enters bursa anteriorly. Albumin gland not present in the mantle cavity, approximately same size as capsule gland.

3.3.4.5. Genetic information. Two sequences of the COI gene of 658 bp were obtained and registered in the GenBank under the identification numbers MT299929 and MT299930.

3.3.4.6. Remarks. The limit of protoconch is not observed because it is eroded. There are no differences between males and females in terms of body coloration, the number of gill filaments or the shell. Because of its size, the nervous system could not be analysed, nor the length of the ovary, or the testicle be measured. *Potamolithus nezibrus* sp. nov. is small like *P. rauli* sp. nov., *P. karsticus, P. troglobius*, *P. elenae* and *P. ribeirensis* of the Feitoria river, Brazil (Fig. 4; Taxonomic key). However, it differs from them in the shape of the shell, body colour, number of gill filaments, shape of the penis, and the radula characters (supplementary material SM.7).

Potamolithus nezibrus sp. nov. has a similar shell and penis (shape, lack of the preputial collar, and terminal papilla) to that described by Núñez (2017) for *P. simplex* (Pilsbry, 1911). Furthermore, these two species are found in the same stream. However,

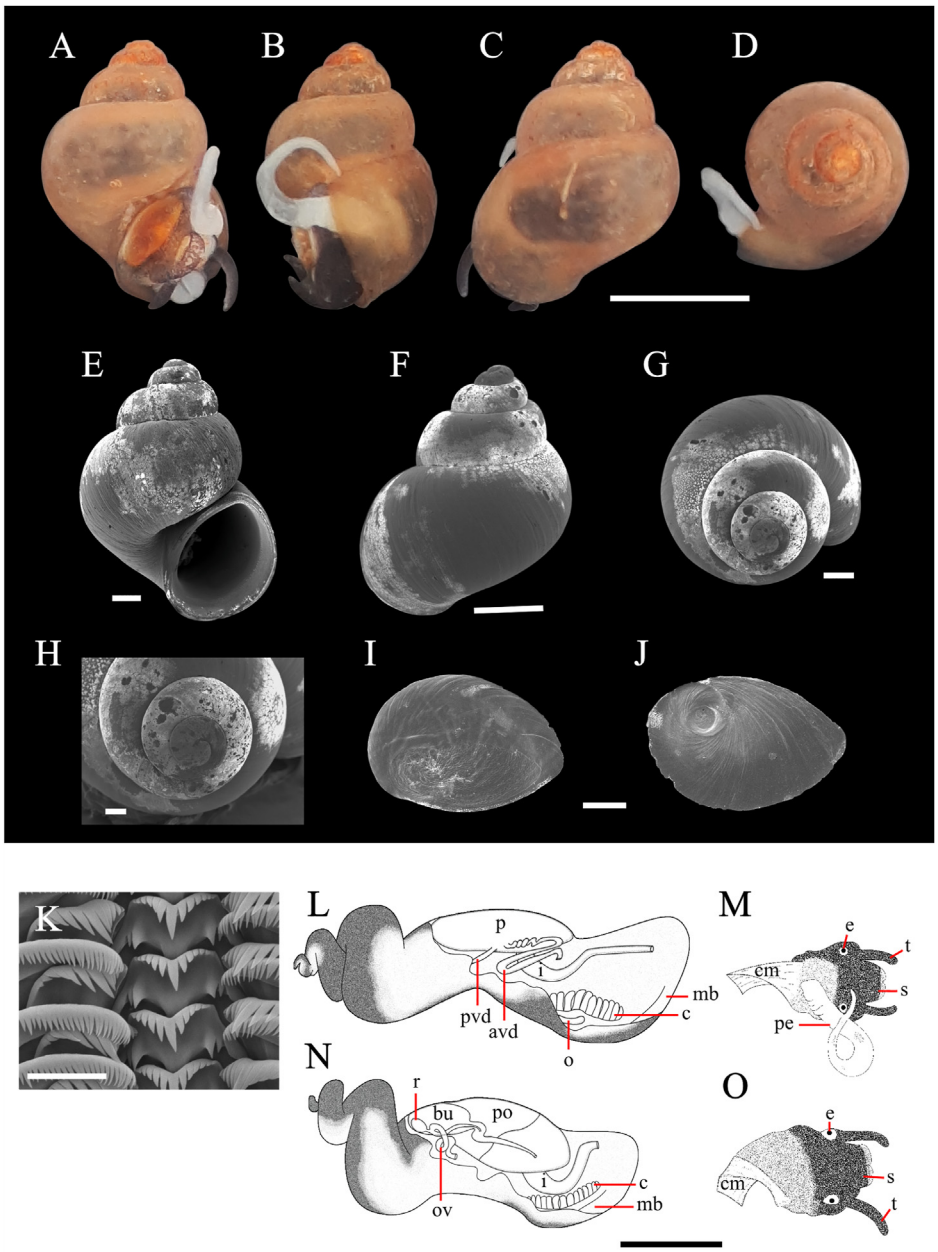

Fig. 5. *Potamolithus nezibrus* sp. nov. (A–D) Holotype (MLP-Ma 15056) shell; (E–K) Paratypes (MLP-Ma 15058); (E–H) Shell (images of the SEM-SE-); (I–J) Operculum: internal view, external view (images of the SEM-SE-); (K) Radula (image of the SEM-BSE-); (L–M) Male; (N–O) Female. Abbreviations: avd, anterior vas deferens; bu, bursa; c, ctenidium; cm, columellar muscle; e, eye; i: intestine; mb, mantle border; o, osphadium; ov, oviduct; p, prostate; pe, penis; po, pallial oviduct; pvd, posterior vas deferens; r, receptacle; s, snout; t, tentacle. Scale bars: A-D, L-O = 1 mm; E, G, I-J = 200 μm; F = 500 μm; H = 100 μm; K = 10 μm.

they are different species due to the size of the shell, the internal anatomy, and the genetic information.

3.4. *Potamolithus tricarinatus de Lucía & Gutiérrez Gregoric sp. nov*

(Fig. 6) lsid:zoobank.org:act:9516A0C4-7E11-4343-8910-60F085E6D834

3.4.1. *Etymology*

For having three keels.

3.4.2. *Type-material*

Holotype: MLP-Ma 15061. Acaraguá Stream (27°26′48″S, 54°55′41″W), Misiones Province, Argentina. *Coll.* F. Brusa, L. Negrete and A. Zivano, 2016.

Paratypes: MLP-Ma 15062 and MACN-In 43335. Same locality.

Additional material: MLP-Ma 15063 and MACN-In 43343. Same locality.

Distribution: Only known from the type locality.

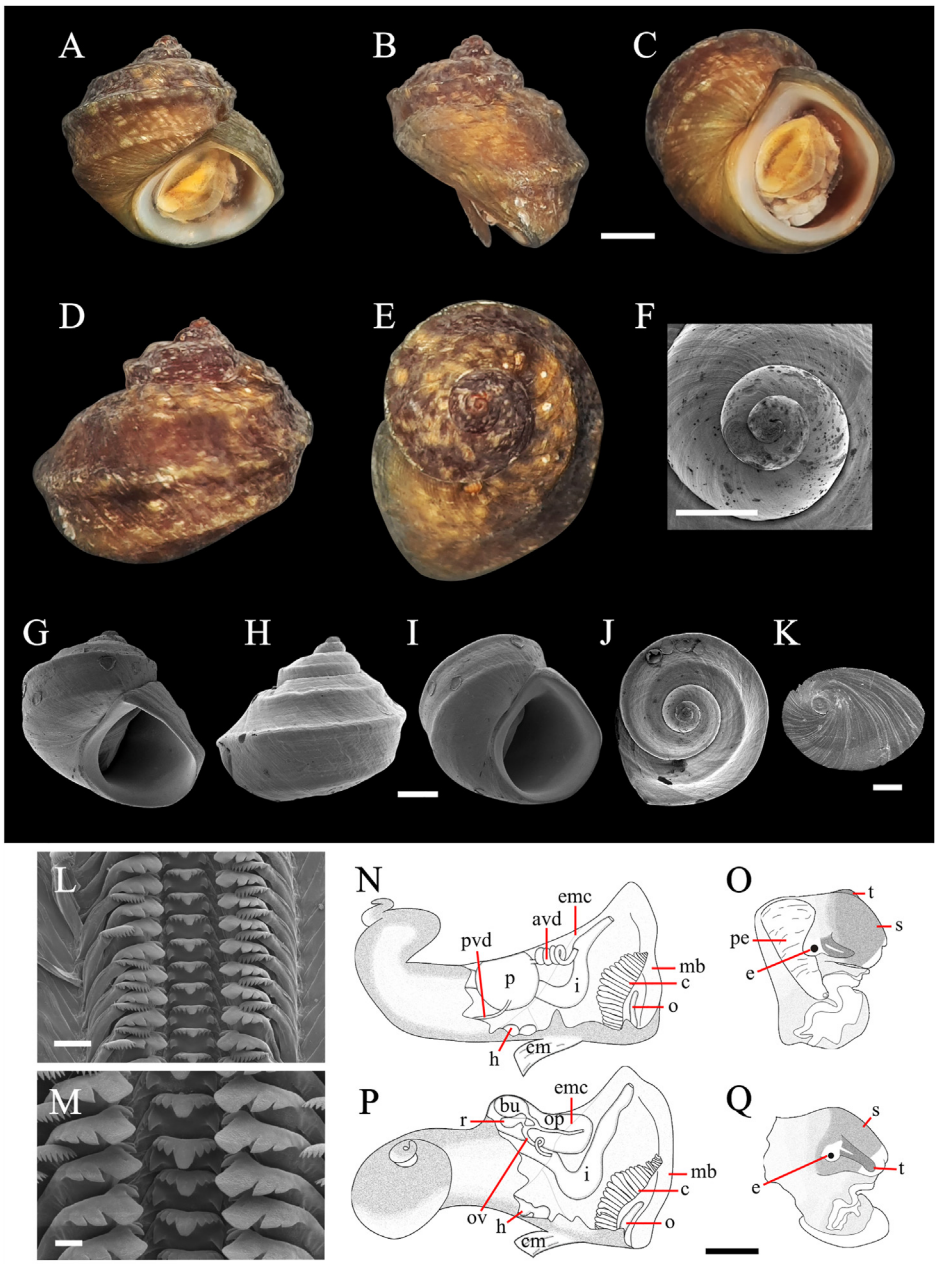

Fig. 6. *Potamolithus tricarinatus* sp. nov. (A–E) Holotype (MLP-Ma 15061) shell; (F–M) Paratypes (MLP-Ma 15062), (F–J) Shell (images of the SEM-SE-); (K) Operculum (images of the SEM-SE-); (L–M) Radula (images of the SEM-SE-); (N–O) Male; (P–Q) Female. Abbreviations: avd, anterior vas deferens; bu, bursa; c, ctenidium; cm, columellar muscle; emc, posterior end of mantle cavity; e, eye; h, heart; i, intestine; mb, mantle border; o, osphadium; ov, oviduct; p, prostate; pe, penis; po: pallial oviduct; pvd, posterior vas deferens; r, receptacle; s, snout; t, tentacle. Scale bars: A-E, G-J, N-Q = 1 mm; F = 500 μm; K = 500 μm; L = 50 μm; M = 20 μm.

3.4.3. Diagnosis

Thick trochoid shell, with three keels (upper, middle and basal), TL 3.50-5.83 mm and 3.15–5.86 whorls. Gill filaments: 31–40. Female without nuchal node.

3.4.4. Description

3.4.4.1. Shell and operculum (supplementary material SM.3 and SM.4) (n = 30). Trochoid, thick, dark brown in first whorls with lighter spots (no defined pattern), in last whorls it turns to a greenish-brown hue, peristome dark green. TL 3.50-5.83 mm (holotype: 4.83 mm), TW 3.87-6.25 mm (holotype: 5.04 mm); 3.15–5.86 whorls. Three keels (upper, middle and basal) out of which the middle keel is more pronounced and forms two grooves: one above and one below it. Short spire (12% of TL). Aperture pyriform (69% of TL). Columella slightly concave. In those individuals with incomplete peristome, keels are more marked in aperture, deforming it. Specimens with incomplete peristome are slightly smaller than those with complete peristome.

3.4.4.2. Radula (supplementary material SM.5) (n = 2). Radular ribbon: large size (40% of TL shell), length 2.11 mm, width 0.27 mm. Total rows 80 (in development 24). Central tooth: 7–9 anterior cusps and 3 pairs of basal cusps on each side, trapezoidal shape (width of the anterior surface 45.70 μm; width of the basal surface 72.37 μm), flat dorsal surface, with superficial excavation (33.30%) at the base of the tooth; Lateral tooth: 5–7 cusps, with more

developed mesocone and parallel edges taking a square shape; Internal marginal tooth: 16–17 cusps; External marginal tooth: 18–22 cusps.

3.4.4.3. Head-foot and non-genital anatomy (supplementary material SM.6) (n = 6). Dark grey head (except lips). Dorsally grey tentacles darker than head, with thin white line in middle, ventrally unpigmented. Dark grey foot. Mantle dorsally dark grey, lighter on sides, ventrally unpigmented. Female without nuchal node. Ctenidium occupies 34% of VML, with 31–40 filaments. Osphradium occupies 36% of CL.

3.4.4.4. Reproductive system (supplementary material SM.6) (n = 6). *Male*: White penis (occupies 25% of the VML), terminal papilla may present a slightly grey internal pigmentation. Distance from the penis to eyes 0.26 mm. Anterior vas deferens leaves the prostate spirally and is thicker than the posterior vas deferens. *Female*: Seminal receptacle oval, large, short canal, placed antero-ventrally against the wall of bursa copulatrix. Bursa copulatrix oval, placed on middle part of albumin gland, slightly smaller than albumin gland, bursal duct enters bursa anteriorly. Albumin gland not present in mantle cavity, slightly smaller than capsule gland. Specimens with incomplete peristome (juveniles) present the ovary already formed, but with the pallial oviduct still in a state of formation.

3.4.4.5. Nervous system (n = 1). Cerebral and pleural ganglia with mild pigmentation. The pleuro-subesophageal connective corresponds to 23% of the pleuro-suprasophageal connective. RPG ratio 0.42. Cerebral ganglion 0.31 mm; cerebral commissure 0.26 mm; right pleural ganglion 0.29 mm, left pleural ganglion 0.21 mm; supraesophageal ganglion 0.18 mm; subesophageal ganglion 0.18 mm; pedal ganglion 0.39 mm; commissure pedal 0.13 mm.

3.4.4.6. Genetic information. Two sequences of the COI gene of 658 bp were obtained and registered in the GenBank under the identification numbers MT299931 and MT299932.

3.4.4.7. Remarks. The limit of protoconch is not observed because it is eroded. *Potamolithus tricarinatus* sp. nov. presents no differences between males and females in terms of body coloration, number of gill filaments or shell. *Potamolithus tricostatus* (ANSP 27086, Syntype) has three little developed keels, its type material has not been located, and the type locality is not well defined (a province of Argentina, Entre Ríos has been mentioned by Brot (1867)) (Fig. 4; Taxonomic key). *Potamolithus microthauma* Pilsbry, 1896 (ANSP 69689, Lectotype) also has three keels on the last whorls (being the middle keel the most developed), one keel on the penultimate whorl and the outer lip folds back to the dorsal area. These characteristics are not present in *P. tricarinatus* sp. nov. Only the shell of *P. tricostatus* and *P. microthauma* is known.

3.5. *Potamolithus recurvatus de Lucía & Gutiérrez Gregoric sp. nov*

(Fig. 7) lsid:zoobank.org:act:DB6399F0-B5C2-4C1D-8DC4-BCF70695E12B

3.5.1. Etymology

For presenting, the outer lip turned to the dorsal area.

3.5.2. Type-material

Holotype: MLP-Ma 15064. Uruguay River (27°33′59″S, 54°40′31″W), Alba Posse, Misiones Province, Argentina. *Coll.* D. E. Gutiérrez Gregoric, V. Núñez and R. Vogler, 2010.

Paratypes: MLP-Ma 15065. Same locality.

Distribution: Only known from the type locality.

3.5.3. Diagnosis

Thick trochoid shell, with three keels (upper, middle and basal), TL 5.53-5.74 mm and 3.42–4.35 whorls. Outer lip turned to dorsal area. Gill filaments: 37–43. Female with nuchal node.

3.5.4. Description

3.5.4.1. Shell and operculum (supplementary material SM.3 and SM.4) (n = 3). Trochoid, thick, dark brown, TL 5.53-5.74 mm (holotype: 5.74 mm), TW 5.10-6.44 mm (holotype: 5.60 mm); 3.42–4.35 whorls. Three keels (upper, middle and basal) out of which the middle cavity is more pronounced and forms two grooves one above and one below it. Short spire (12% of TL). Aperture oval (61% of TL). Outer lip turned to dorsal area. Concave columella.

3.5.4.2. Radula (supplementary material SM.5) (n = 1). Radular ribbon: large size (43% of TL shell), length 2.44 mm, width 0.28 mm. Total rows 86 (in development 29). Central tooth: 9–10 anterior cusps, 3 left basal cusps and 2 right basal cusps, trapezoidal shape (width of the anterior surface 43.67 μm; width of the basal surface 78.12 μm), flat dorsal surface, without excavation (20%) of the tooth base; Lateral tooth: 7 cusps, with more developed mesocone and parallel edges taking a square shape; Internal marginal tooth: 18 cusps; External marginal tooth: 24–25 cusps.

3.5.4.3. Head-foot and non-genital anatomy (supplementary material SM.6) (n = 2). Black head, lips unpigmented, except in female specimen that presented a black dorsal spot on lip; white tentacles with medium black band. Dark grey foot. Mantle dorsally black, lighter on sides, ventrally light grey. Female with nuchal node (0.55 mm) triangular on neck, distance from node to eyes 0.63 mm. Ctenidium occupies 35% of VML, with 37–43 filaments. Osphradium occupies 25% of CL.

3.5.4.4. Reproductive system (supplementary material SM.6) (n = 2). *Male*: White penis (occupies 38% of VML) with slight pigmentation in middle part, especially in preputial collar and in terminal papilla. Distance from penis to eyes 0.74 mm. Anterior vas deferens leaves the spiral prostate and is as wide as the posterior vas deferens. *Female*: Seminal receptacle oval, duct very short, placed antero-ventrally against the wall of bursa copulatrix. Bursa copulatrix spherical, positioned on posterior half of albumen gland, approximately same length as albumen gland, bursal duct enters bursa in anterior half. Albumen gland not present in mantle cavity, slightly smaller than capsule gland.

3.5.4.5. Nervous system (n = 1). Cerebral and pleural ganglia with mild pigmentation. Pleuro-subesophageal connective corresponds to 13% of the pleuro-suprasophageal connective. RPG ratio 0.46. Cerebral ganglion 0.29 mm; cerebral commissure 0.28 mm; right pleural ganglion 0.24 mm, left pleural ganglion 0.22 mm; suprasophageal ganglion 0.21 mm; osphradium-mantle nerve 0.37 mm; subesophageal ganglion 0.24 mm; pedal ganglion 0.32 mm; commissure pedal 0.10 mm.

3.5.4.6. Genetic information. No DNA available.

3.5.4.7. Remarks. Potamolithus recurvatus sp. nov. and *P. tricarinatus* sp. nov. are similar in terms of the trochoid shape of the shell, the size, the presence of three keels, and the radula. However, they show differences in the colour of the head, and tentacles and the shape of the penis (Table 1). In addition, the females of the *P. recurvatus* sp. nov. have a nuchal node not present in

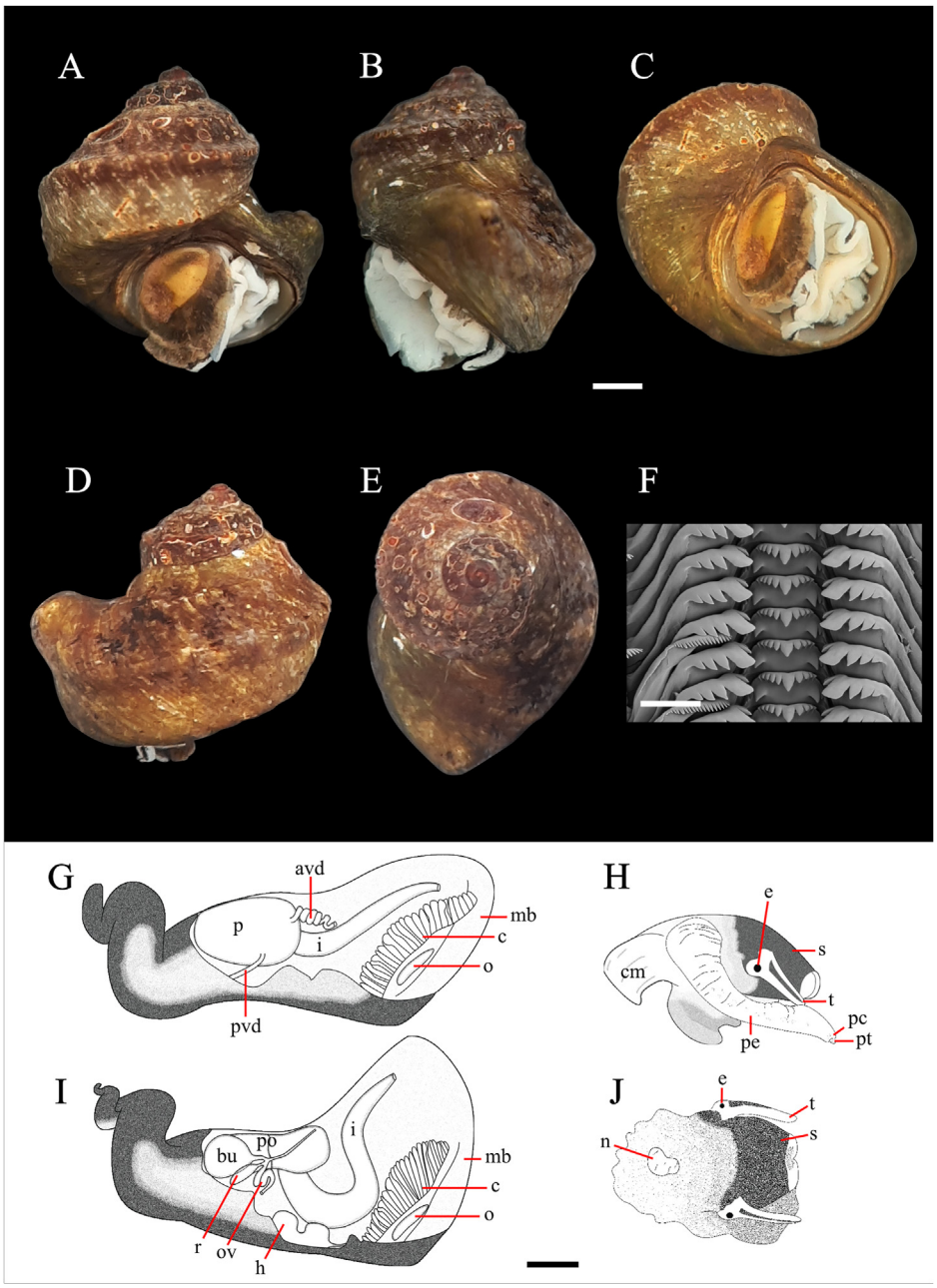

Fig. 7. *Potamolithus recurvatus* sp. nov. (A–E) Holotype (MLP-Ma 15064) shell; (F) Paratype (MLP-Ma 15065) Radula (image of the SEM-BSE-); (G–H) Male; (I–J) Female. Abbreviations: avd, anterior vas deferens; bu, bursa; c, ctenidium; cm, columellar muscle; e, eye; h, heart; i, intestine; mb, mantle border; n, nuchal node; o, osphadium; ov, oviduct; p, prostate; pc, prepucial collar; pe, penis; po, pallial oviduct; pt, terminal papilla; pvd, posterior vas deferens; r, receptacle; s, snout; t, tentacle. Scale bars: A-E, G-J = 1 mm; F = 50 μm.

P. tricarinatus sp. nov. (Fig. 4; Taxonomic key). *Potamolithus microthauma* (ANSP 69689, Lectotype) has three keels and the outer lip folds towards the dorsal area, similar to what happens in *P. recurvatus* sp. nov. However, *P. recurvatus* sp. nov. differs from *P. microthauma* in the size and shape of the keels. Although *P. tricostatus* (ANSP 27086, Syntype) has three keels, these are poorly developed, which differs from *P. recurvatus* sp. nov. Only the shell of *P. tricostatus* and *P. microthauma* is known.

3.6. *Potamolithus grossus de Lucía & Gutiérrez Gregoric sp. nov*

(Fig. 8) zoobank.org:act:BEAF2304-272B-44C9-88B8-B2B9D6FF512D

3.6.1. *Etymology*

Relating to the very thick shell.

3.6.2. *Type-material*

Holotype: MLP-Ma 15066. Pepirí Miní Stream (27°08′42″S; 53°55′41″W), Misiones Province, Argentina. *Coll.* F. Brusa, L. Negrete and A. Zivano, 2016.

Paratypes: MLP-Ma 15067, MLP-Ma 15069, MLP-Ma 15071, MLP-Ma 15073, MACN-In 43336. Yabotí Miní Stream (26°57′38″S, 53°49′30″W); Moconá Falls (Uruguay river) (27°8′43″S, 53°53′23″W); Pepirí Miní Stream (27°08′42″S, 53°54′28″W), Misiones Province, Argentina.

Table 1
Comparison of the six new species of *Potamolithus*.

	Potamolithus rauli	*Potamolithus nezibrus*	*Potamolithus tricarinatus*	*Potamolithus recurvatus*	*Potamolithus grossus*	*Potamolithus nelidae*
Shell shape	Globose	Conic	Trochoid	Trochoid	Globose	Trochoid
Total length	2.01	1.94	5.04	5.65	7.07	5.26
Aperture shape	Slightly pyriform	Oval	Pyriform	Oval	Pyriform	Pyriform
Keel	No	No	Yes (3)	Yes (3)	No	Yes (1)
Head colouring	Dark grey	Black	Dark grey	Black	Black	Black
Nuchal node	No	No	No	Yes	Yes	Yes
Gill filaments	14–18	13–17	31–40	37–43	35–40	33–42
Radular formula	13/3–4; 11; 26–28; 24-32	13/2-2; 12–13; 27-32; 31	7-8/3; 5–6; 12–16; 15-21	9-10/3-2; 7; 18; 24-25	13-18/5; 12–14; 22-29; 22-29	7-11/3; 6–9; 12-18; 20-28
Lateral tooth mesocone	Dagger shaped	Dagger shaped	Square shape	Square shape	Blunt	Square shape
Anterior vas deferens	Wavy	Spiral	Spiral	Spiral	Wavy	Spiral-Wavy
Expansion on preputial collar	Yes	No	No	No	No	No

Additional material: MLP-Ma 15068, MLP-Ma 15070, MLP-Ma 15072, MLP-Ma 15074; MACN-In 43337 and MACN-In 43338. Same as paratypes.

Distribution: Same as paratypes.

3.6.3. Diagnosis

Large shell, globose, with a very thick wall, callus in the labrum and adcolumellar area of wide development, TL 5.78-8.22 mm, 4.14–5.27 whorls. Gill filaments: 34–41. Female with very developed and oval nuchal node.

3.6.4. Description

3.6.4.1. Shell and operculum (supplementary material SM.3 and SM.4) (n = 64). Globose, large, thick, callus in outer lip, greenish-brown, under sutures have a green colour. TL 5.78-8.22 mm (holotype: 8.06 mm); TW 5.78-8.39 mm (holotype: 8.11 mm); 4.14–5.27 whorls. Generally, it tends to be wider than longer. Dorsal area of shell irregular with undulations that do not form grooves or keels. Peristome brown in some individuals, while in others, dark green. Short spire (10% of TL). Aperture piriform (73% of TL). With outer lip and adcolumellar area thickened in those who have complete peristome. In some, outer lip begins to deform and separate from aperture once it is thickened. Columella flat and wide. Well-marked growth lines.

3.6.4.2. Radula (supplementary material SM.5) (n = 12). Radular ribbon: medium size (23% of TL shell), length 1.57 mm, width 0.22 mm. Total rows 109 (in development 23). Central tooth: 13–17 anterior cusps, with 3 or 4 pairs of basal cusps, trapezoidal shape (width of the anterior surface 26.41 μm; width of the basal surface 44.32 μm), dorsal surface with great concavity, with superficial excavation (35.6%) at the base of the tooth, basal process that joins central tooth to the U-shaped lingual membrane and that is longer than the lateral margin; Lateral tooth: 13–16 cusps, slight dorsal concavity, square face with basal tongue underneath, with more developed mesocone and parallel edges, with blunt tip (may be due to wear); Internal marginal tooth: 22–30 cusps; External marginal tooth: 26–36 cusps.

3.6.4.3. Head-foot and non-genital anatomy (supplementary material SM.6) (n = 15). Black head except for lips. Dark grey tentacles dorsally lightening towards tip, with medium black line. Foot with black and dark grey areas. Mantle dorsally black, lighter on sides, ventrally unpigmented. Female with developed nuchal node (0.90 mm), oval and flattened, with stretch marks (like penises), attached from left side of node to neck and folded to right side. Distance from node to eyes 1.93 mm. Ctenidium occupies 29% of VML, with 34–41 filaments. Osphradium occupies 44% of CL.

3.6.4.4. Reproductive system (supplementary material SM.6) (n = 15). Male: White penis (occupies 41% of VML), inside terminal papilla and on preputial collar it presents black scores. Distance from penis to eyes 0.30 mm. Anterior vas deferens wavy and similar in thickness to the posterior vas deferens. *Female*: Seminal receptacle oval-piriform, duct short, placed antero-ventrally against the wall of bursa copulatrix. Bursa copulatrix spherical-pyriform, positioned over mid-posterior part of albumen gland, approximately half length of albumen gland, bursal duct enters bursa anteriorly. Albumen gland not present in the mantle cavity, in some specimens slightly longer than capsule gland and slightly shorter in others.

3.6.4.5. Nervous system (n = 4). Cerebral and pleural ganglia with mild pigmentation. Pleuro-subsophageal connective corresponds to 10.67% of the pleuro-suprasophageal connective. RPG ratio 0.51. Cerebral ganglion 0.32 mm; cerebral commissure 0.16 mm; right pleural ganglion 0.16 mm, left pleural ganglion 0.18 mm; suprasophageal ganglion 0.37 mm; osphradium-mantle nerve 0.32 mm; subesophageal ganglion 0.25 mm; pedal ganglion 0.38 mm; commissure pedal 0.05 mm.

3.6.4.6. Genetic information. Four COI sequences of 658 bp were obtained and registered in the GenBank under the identification numbers MT299933 to MT299936.

3.6.4.7. Remarks. The limit of protoconch is not observed because it is eroded. In *Potamolithus grossus* sp. nov. there are no differences between males and females in terms of body coloration, number of gill filaments or shell. This species is the second largest described, after *Potamolithus peristomatus misionum* (original drawing by Parodiz, 1966), with a maximum total length of 9 mm, recorded for the town of San Javier (Misiones) (Fig. 4; Taxonomic key). The type material of the last subspecies has not been found in the Carnegie Museum of Natural History. *Potamolithus peristomatus misionum* differs from *P. grossus* sp. nov. in the shape of the shell (longer than wider), the coloration of the shell (black), and by the presence of a protuberant hump in the dorsal view. *Potamolithus peristomatus* (d'Orbigny, 1835) (original drawing by d'Orbigny 1840, NHMUK 1854.12.4.338 Lectotype, NHMUK 1854.4.12.4.338, Paralectotypes) has a trochoid-globose shell, high spire and a fine face. While the shell of *P. grossus* sp. nov. is globose, like that of *P. doeringi* Pilsbry, 1911 (ANSP 103074, Cotype), it differs from this in the form of the opening (pyriform vs oval), outer lip (deformed vs. not deformed)

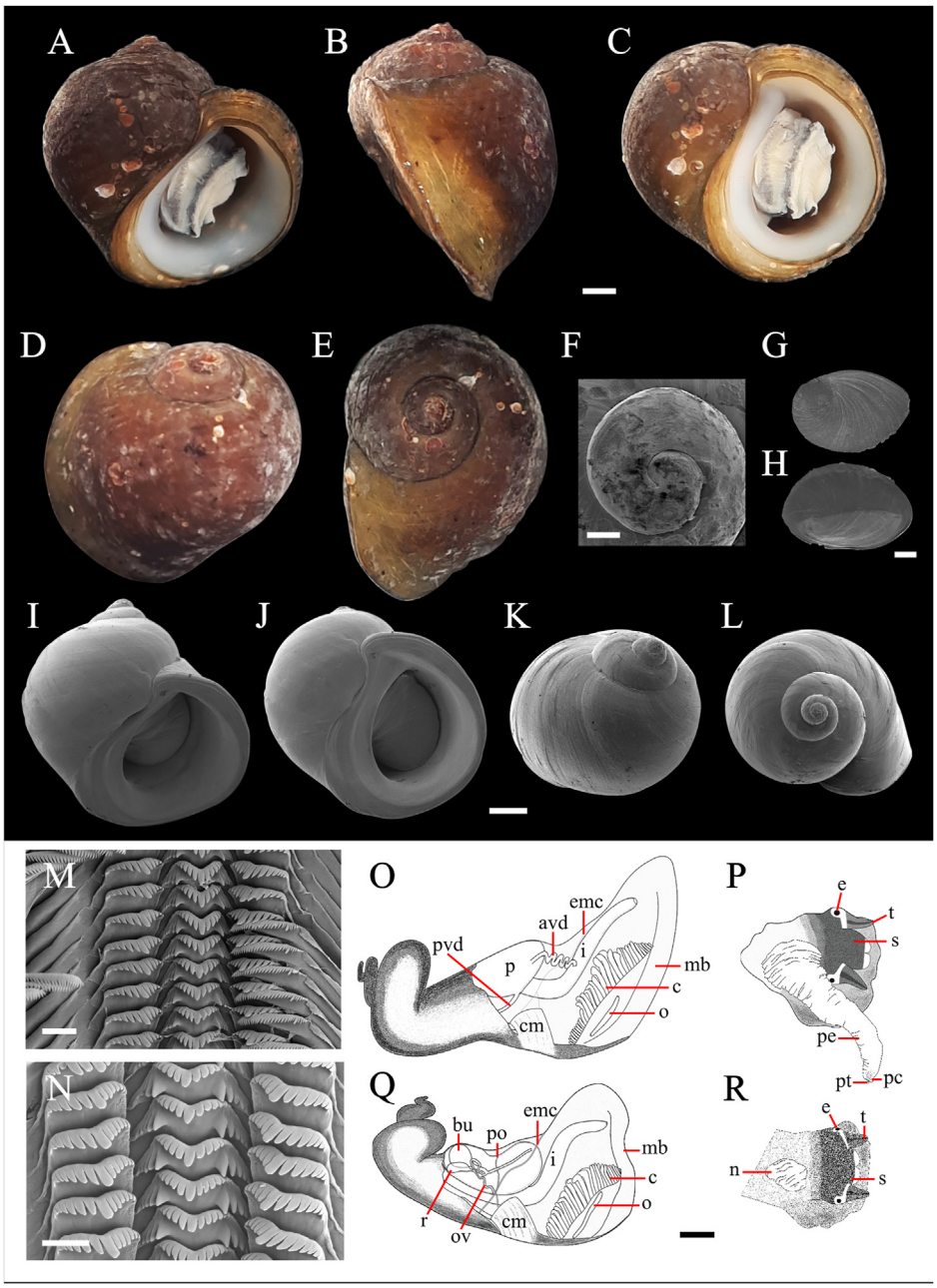

Fig. 8. *Potamolithus grossus* sp. nov. (A–E) Holotype (MLP-Ma 15066) shell; (F–N) Paratypes (MLP-Ma 15069, MLP-Ma 15073) and additional material (MLP-Ma 15068); (F, I-L) Shell (images of the SEM-SE-); (G–H) Operculum: external view, internal view (images of the SEM-SE-); (M–N) Radula (images of the SEM-BSE-); (O–P) Male; (Q–R) Female. Abbreviations: avd, anterior vas deferens; bu, bursa; c, ctenidium; cm, columellar muscle; e, eye; i, intestine; mb, mantle border; n, nuchal node; o, osphadium; ov, oviduct; p, prostate; pc, prepucial collar; pe, penis; po, pallial oviduct; pt, terminal papilla; pvd, posterior vas deferens; r, receptacle; s, snout; t, tentacle. Scale bars: A-E, I-L, O-R = 1 mm; F = 100 μm, G-H = 500 μm; M-N = 20 μm.

and the callus in the outer lip (presence vs. absence). In addition, *P. doeringi* inhabits the Iguazú river, which belongs to a different basin not related to the basin in which *P. grossus* sp. nov. inhabits. Only the shell is known for *P. doeringi*.

3.7. *Potamolithus nelidae de Lucía & Gutiérrez Gregoric sp. nov*

(Fig. 9) lsid:zoobank.org:act:B2829E67-514F-4AF6-B742-FC3C4F00DEFB

3.7.1. *Etymology*

Dedicated to the mother of the first author of this paper called Nélida.

3.7.2. *Type-material*

Holotype: MLP-Ma 15075. Pepirí Miní Stream (27°08′42″S; 53°55′41″W), Misiones Province, Argentina. *Coll.* F. Brusa, L. Negrete and A. Zivano, 2016.

Paratypes: MLP-Ma 15076, MLP-Ma 15078, MLP-Ma 15080 and MACN-In 43339. Pepirí Miní Stream (27°08′42″S; 53°55′41″O),

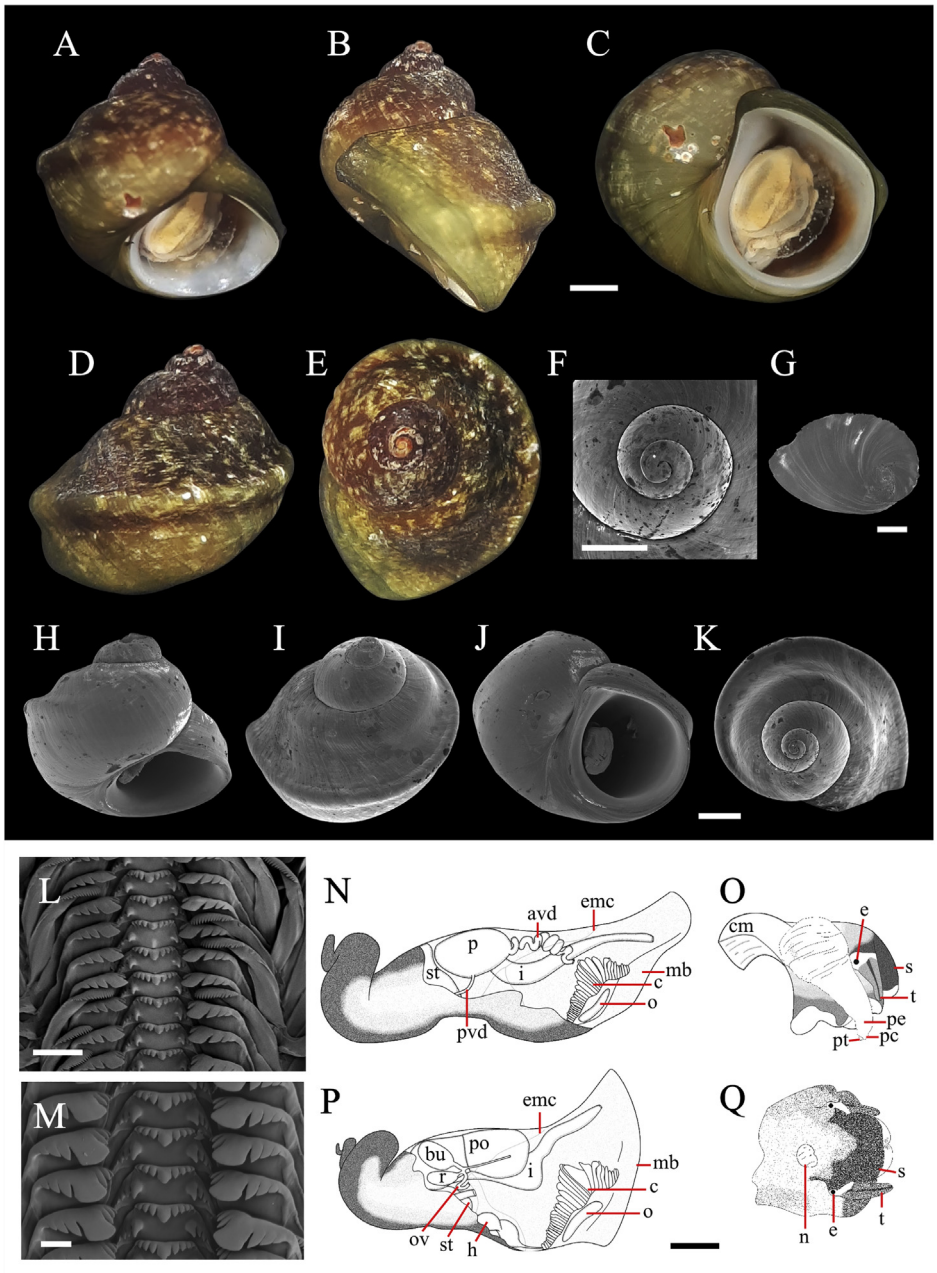

Fig. 9. *Potamolithus nelidae* sp. nov. (A–E) Holotype (MLP-Ma 15075) shell; (F–M) Paratypes (MLP-Ma 15076); (F, H–K) Shell (images of the SEM-SE-); (G) Operculum external view (image of the SEM-SE-); (L–M) Radula (images of the SEM-BSE-); (N–O) Male; (P–Q) Female. Abbreviations: avd, anterior vas deferens; bu, bursa; c, ctenidium; cm, columellar muscle; emc, posterior end of mantle cavity; e, eye; h, heart; i, intestine; mb, mantle border; n, nuchal node; o, osphadium; ov, oviduct; p, prostate; pc, prepucial collar; pe, penis; po, pallial oviduct; pt, terminal papilla; pvd, posterior vas deferens; r, receptacle; s, snout; st, stomach; t, tentacle. Scale bars: A-E, H–K, N-Q = 1 mm; F-G = 500 μm; L = 50 μm; M = 20 μm.

Moconá Falls (Uruguay River) (27°8′43″S, 53°53′23″W), Misiones Province, Argentina.

Additional material: MLP-Ma 15077, MLP-Ma 15079 and MLP-Ma 15081. Same as paratypes.

Distribution: Same as paratypes.

3.7.3. Diagnosis

Trochoid shell with a medium keel, TL 4.19-6.07 mm and 3.84–4.84 whorls. Gill filaments: 33–42. Females with nuchal node.

3.7.4. Description

3.7.4.1. Shell and operculum (supplementary material SM.3 and SM.4) (n = 34). Trochoid, thick, TL 4.19-6.07 mm (holotype: 5.87 mm); TW 4.00-5.95 mm (holotype: 5.57 mm); 3.84–4.84 whorls. Dark brown in first whorls and with lighter spots (no defined pattern). In last whorl, it acquires a greenish-brown hue, while peristome remains dark green. With medium keel. Short spire (13% of TL). Aperture piriform (65% of TL). Slightly concave columella.

3.7.4.2. Radula (supplementary material SM.5) (n = 10). Radular ribbon: large size (41% of TL shell), length 2.06 mm, width 0.24 mm. Total rows 84 (in development30). Central tooth: 7–11 anterior cusps and 3 pairs of basal cusps on each side, trapezoidal shape (width of the anterior surface 38.63 μm; the width of the basal surface is 70.86 μm), the dorsal surface with slight concavity, and without excavation (24.10%) at the base of the tooth; Lateral tooth: 6–9 cusps, more developed mesocone with parallel edges taking a square shape; Internal marginal tooth: 12–18 cusps; External marginal tooth: 20–28 cusps.

3.7.4.3. Head-foot and non-genital anatomy (supplementary material SM.6) (n = 13). Black head except for lips (in few individuals lips in upper area presented a slight black pigmentation). Dark grey tentacles with black middle band. Grey foot that turns black towards the base. Mantle dorsally black/dark grey, lighter on sides, ventrally unpigmented. Female with nuchal node (0.51 mm) rises a little from right side, with stretch marks. Distance from node to eyes 0.34 mm. Ctenidium occupies 33% of VML, with 33–42 filaments. Osphradium occupies 45% of CL.

3.7.4.4. Reproductive system (supplementary material SM.6) (n = 13). Male: White penis (occupies 34% of VML). Distance from penis to eyes 0.27 mm. Anterior vas deferens in some specimens is spiral and in others, it is undulated, with 2 or 3 spiral turns, thicker than posterior vas deferens. *Female*: Seminal receptacle oval, duct very short, ventral to bursa copulatrix, in some specimens it makes contact with the wall of the same. Bursa copulatrix oval, positioned over middle part of albumen gland, about half length of albumen gland, bursal duct enters bursa anteriorly. Albumen gland not present in mantle cavity, slightly smaller than capsule gland.

3.7.4.5. Nervous system (n = 3). Pleuro-subsophageal connective corresponds to 19% of the pleuro-supraesophageal connective. RPG ratio 0.42. Cerebral ganglion 0.39 mm; cerebral commissure 0.14 mm; right pleural ganglion 0.20 mm, left pleural ganglion 0.15 mm; supraesophageal ganglion 0.18 mm; osphradium-mantle nerve 0.17 mm; subesophageal ganglion 0.17 mm; pedal ganglion 0.39; commissure pedal 0.04 mm.

3.7.4.6. Genetic information. Two sequence of the COI gene of 658 bp were obtained and registered in GenBank under the identification numbers MT299937 and MT299938.

3.7.4.7. Remarks. Seven specimens of the Moconá Falls are more rounded, and their keel is less evident. However, the internal anatomy and their radula identify them as *P. nelidae* sp. nov. The limit of protoconch is not observed because it is eroded. *Potamolithus hidalgoi* (Pilsbry, 1896) (ANSP 69687, Lectotype) has a light brown trochoid shell with a basal keel on the penultimate and last whorl; due to these characteristics, it differs from *P. nelidae* sp. nov. (Fig. 4; Taxonomic key). *Potamolithus peristomatus* (original drawing by d'Orbigny 1840, NHMUK 1854.12.4.338, Lectotype, NHMUK 1854.4.12.4.338, Paralectotypes) has a fine keel (as a cord) in a basal position, which does not correspond to the medium keel that owns the *P. nelidae* sp. nov. *Potamolithus peristomatus misionum* (original drawing by Parodiz, 1966) is larger than *P. nelidae* sp. nov. (9 mm vs 5 mm) and does not have a keel, but it shows a protruding hump when in dorsal view.

3.8. Taxonomic key

Taxonomic key of the six new species of *Potamolithus* and those are similar:

1	Globose or trochoid shell, with or without keel	2
1′	Conic shell, without keel	3
2	Shell with three keels	4
2′	Shell with one keel	6
2″	Shell without keel	7
3	Shell 1.74 to 2.16 mm in total length; black mantle roof; black tentacles, up to 17 gill filaments	*P. nezibrus*
3′	Shell from 3.20 to 4.25 mm in total length; mantle roof black with white spots and patches; white tentacles with a dorsal dark grey line, up to 24 gill filaments	*P. simplex*
4	Keels with little development	*P. tricostatus*
4′	Middle keel slightly more pronounced than the others and forming two furrows (one above and one below)	5
5	Dark grey tentacles with thin white line in the middle; female without nuchal node.	*P. tricarinatus*
5′	White tentacles with a medium black band; female with nuchal node.	*P. recurvatus*
5″	Keel on the penultimate whorl	*P. microthauma*
6	Keel weak bordering in upper position; total length of shell does not exceed 1.20 mm	*P. elenae*
6′	Mid-position keel, total shell length up to 6.07 mm	*P. nelidae*
6″	Basal keel on the penultimate and last whorl, total length up to 5.2 mm	*P. hidalgoi*
6‴	Thick keel with hump-like appearance protruding dorsally, total length of shell up to 9 mm	*P. peristomatus misionum*
7	Total length of shell less than 5 mm	8
7′	Total length of shell greater than 5 mm	11
8	Vas deferens enters prostate; expansion in preputial collar; penis with terminal papilla; females may or may not present a nuchal node	9
8′	Vas deferens does not enter prostate; without expansion in preputial collar; penis without terminal papilla; females without nuchal node; total length up to 2.5 mm	10
9	Total length of shell up to 2.63 mm; female without nuchal node; up to 18 gill filaments	*P. rauli*

(*continued*)

9′	Total length of shell up to 5 mm; female with nuchal node; up to 32 gill filaments	*P. ribeirensis*
10	Presence of eyes	*P. karsticus*
10′	Absence of eyes	*P. troglobius*
11	Globose shell; total length of shell up to 8.22 mm; piriform opening; callus on external lip; female with nuchal node	*P. grossus*
11′	Globose shell; total length up to 7 mm; oval opening; no callus on external lip	*P. doeringi*
11″	Trochoid-globose shell; total length up to 6.5 mm; oval or piriform opening; no callus on external lip; with pronounced chord above the suture of last whorl	*P. peristomatus peristomatus*

3.9. Statistical analyses

The descriptive statistics of the six species are provided in Supplementary material SM.3 and SM.4.

The correlation matrix indicated a high value for variables TL, TW, LA and WA, being removed from the DA. The *P. recurvatus* sp. nov. population has not been included in this analysis because the number of individuals sampled is insufficient. Fig. 10 represents the variables distributed in two axes, which together explain 94.21% of the analysis. The axis 1 and axis 2 received the greater influence of the LS/TL and LBW/TL proportions, respectively. The DA classification matrix has indicated 100% morphometric identity in the species *P. rauli* sp. nov., *P. grossus* sp. nov. and *P. nezibrus* sp. nov. *Potamolithus tricarinatus* sp. nov. and *P. nelidae* sp. nov. show low identity with 74% and 71%, respectively. The result has been correctly classified at 90.17% by the Jackknifed calculation.

3.10. Molecular analyses

The greatest genetic distances in the COI gene among the new species are established between *P. rauli* sp. nov. and *P. nelidae* sp. nov. with 12% in terms of p distances and 13.48% in terms of corrected distances. The lowest distances occur between *P. nelidae* sp. nov. and *P. tricarinatus* sp. nov. with 4.40% for p distances and 4.58% for corrected distances. *Potamolithus nelidae* sp. nov. shows a genetic variability of 0.59%, while *P. grossus* sp. nov. ranges from 0.39% to 2.20%. Given these results, we can establish that the limit to separate the *Potamolithus* species would be between 3 and 4% (supplementary material SM.8). The genetic distance between the genus *Potamolithus* and *Tatea* is 13–16% in terms of p distances and 14-18% in terms of corrected distances.

The phylogenetic analyses performed using BI (Fig. 11) and ML (not shown) recovered four main clades. Clade I includes *P. rauli* sp. nov., *P. grossus* sp. nov., *P. nezibrus* sp. nov., *P. elenae*, and *P. ribeirensis*. This clade presents species from the Northeast of Argentina, Brazil, and one from the Argentine Patagonia. Clade II was formed only by sequences of *P. simplex* (Misiones Province). Clade III was composed of species from Chile together with "*Heleobia*" *hatcheri* and "*Heleobia*" sp. from Western Argentina. Clade IV was constituted by *P. nelidae* sp. nov., *P. tricarinatus* sp. nov., *P. supersulcatus* Pilsbry, 1896 (Entre Ríos Province), *P. buschii* (Frauenfeld, 1865) and *P. agapetus* Pilsbry, 1911 (Buenos Aires Province). The phylogenetic analyses recovered *P. nelidae* sp. nov. and *P. tricarinatus* sp. nov. as sister species. Although not well supported, the phylogeny also shows that *P. rauli* sp. nov. and *P. grossus* sp. nov are closely related species. The systematic position of *P. nezibrus* sp. nov. was not resolved, but it is more closely related to these two species than the first two. BI analyses and ML do not resolve the relationships between the four clades.

4. Discussion

This work provides information (shell, anatomy, molecular-genetic (COI) and distribution) on six new species of *Potamolithus*

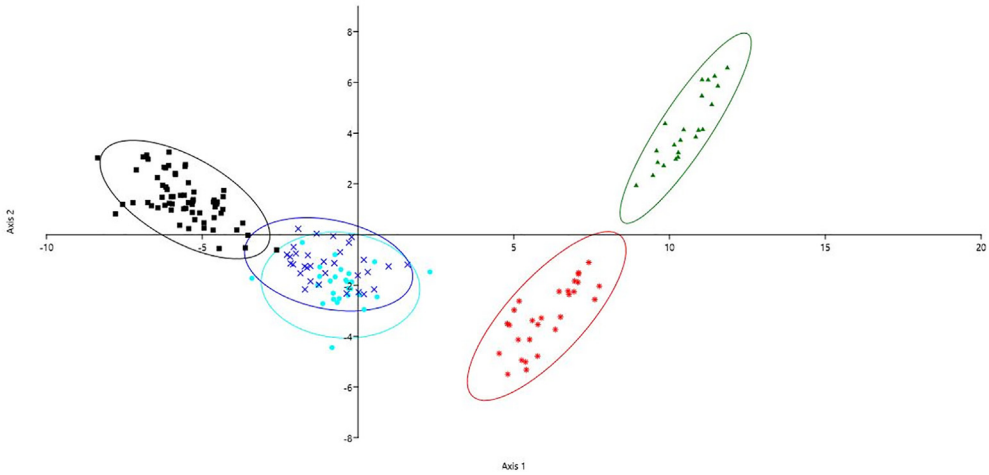

Fig. 10. Discriminant Analysis of five species of *Potamolithus* from Misiones Province, Argentina. ✳*P. rauli* sp. nov; ▲ *P. nezibrus* sp. nov.; ● *P. tricarinatus* sp. nov.; ■ *P. grossus* sp. nov; ✖ *P. nelidae* sp. nov.

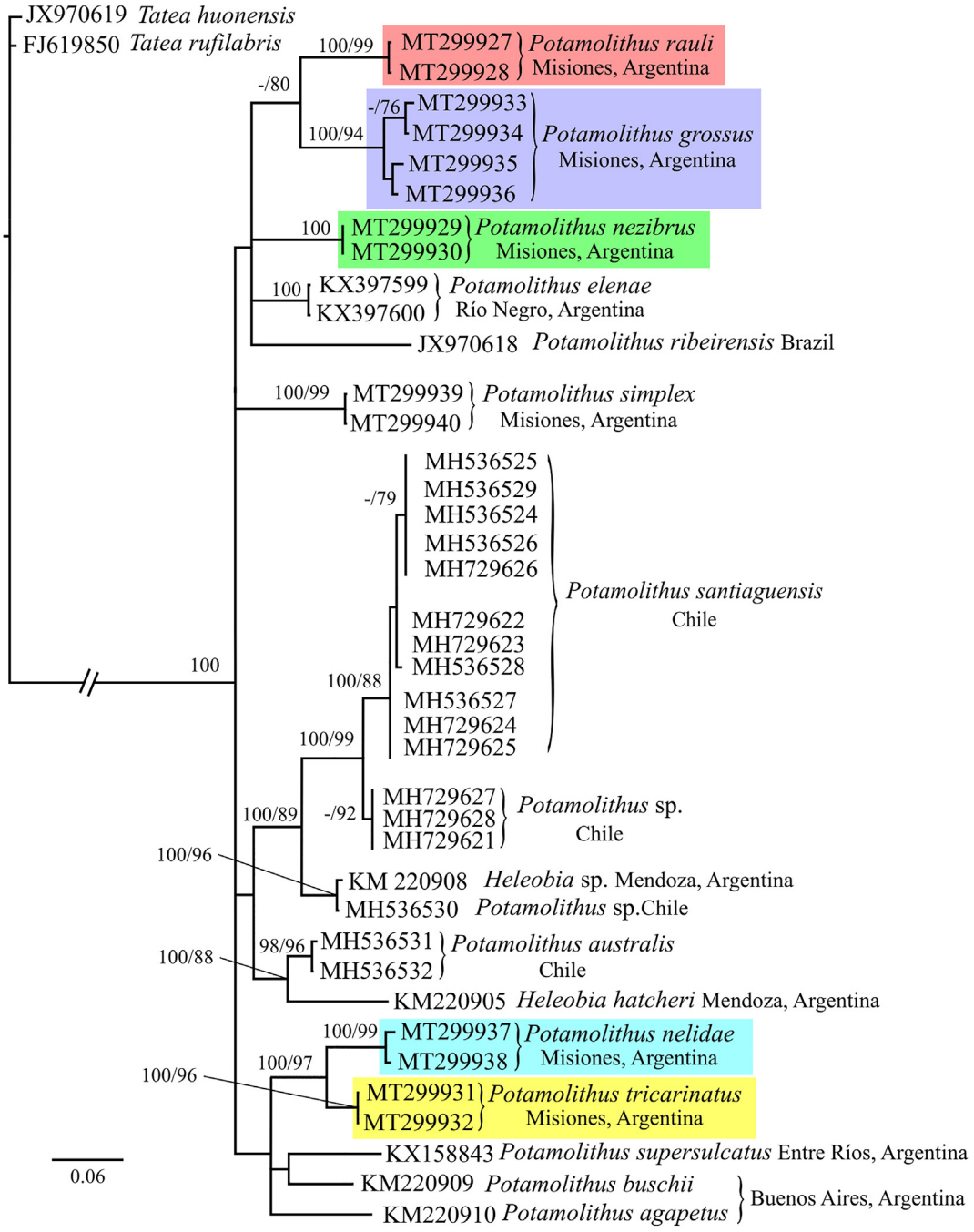

Fig. 11. Phylogenetic relationships amongst COI lineages in *Potamolithus* spp. Topology and branch lengths are from BI analyses, with significant support values given as posterior probability and bootstrap percentage from ML analyses (post bar values). When BI and ML values are equal, only one value is expressed. New species are found in coloured boxes.

of lotic environments from the Uruguay River Malacological Province. *Potamolithus grossus* sp. nov., is the largest species of the six described here, while *P. rauli* sp. nov. and *P. nezibrus* sp. nov. are the smallest. *Potamolithus recurvatus* sp. nov., *P. tricarinatus* sp. nov., and the *P. nelidae* sp. nov. are similar in shell shape (trochoid) and size. Due to these similarities in the shells, *P. nelidae* sp. nov. and *P. tricarinatus* sp. nov. are close regarding the DA. However, they all have anatomical and genetic differences that distinguish them from each other.

The new species increase the number of known freshwater gastropod species in Uruguay River Malacological Province from 58 to 64 (Núñez et al., 2010; Peso et al., 2010; Ovando et al., 2014; Gutiérrez Gregoric and de Lucía 2016; de Lucía and Gutiérrez Gregoric 2017a, 2017b; Vogler et al., 2019), confirmed by the map in the Fig. 1. It is possible to see through the map, that most of the locations sampled in this work had not yet registered the genus, especially in the preserved area Yabotí Biosphere Reserve. Most of the records of *Potamolithus* species are concentrated in the regions San Javier (Uruguay basin) and Iguazú River (Paraná basin). This may be due to the fact that these areas have long been tourist areas with paved roads, which allowed easier access to the sampled sites. In contrast, regions that had not yet been investigated proved to be of great importance for the genus. The Uruguay River Malacological Province has the highest richness, the highest number of endemic

(21), and vulnerable (21) species (Núñez et al., 2010; Gutiérrez Gregoric and de Lucía, 2016; Vogler et al., 2019). These new species increase the number of the *Potamolithus* species to 47 and add further support to the suggestion that the Uruguay River is a biodiversity hotspot for freshwater gastropods in South America (Strong et al., 2008).

The province of Misiones has one of the largest systems of protected natural areas in Argentina, comprising different categories ranging from the strictest protected areas, to those available for sustainable use. Three of the species described here are found within the protected "Yabotí Biosphere Reserve", while three are found outside protected areas. However, two of these species (*P. rauli* sp. nov. and *P. tricarinatus* sp. nov.) have been described within the CIAR (Centro de Investigaciones Antonia Ramos) which is not a protected area, but several studies on biodiversity have been carried out there in recent years. These include several new species and new records of both vertebrates and invertebrates (arthropods, flatworms) (Negrete and Brusa 2017; Avigliano et al., 2019) indicating that conservation studies should not only be carried out in protected areas.

Two invasive molluscs, *Limnoperna fortunei* and *Melanoides tuberculata* have been recorded for the Uruguay River in recent years (Peso et al., 2010; Darrigran et al., 2012; Oliveira et al., 2015). The presence of these invasive species represents a threat to native gastropod species (as well as other invertebrates), as they compete for space and resources, causing the displacement of native fauna. Furthermore, a hydroelectric dam is going to be built in the area where *Potamolithus recurvatus* sp. nov. has been collected. *Chilina nicolasi* Gutiérrez Gregoric and de Lucía, 2016 has also been recently described from this site. The presence of endemic species should be considered by government agencies before the construction of dams, since they will cause the disappearance of the environment inhabited by those species just as it happened with the *Aylacostoma guaraniticum* (Hylton Scott, 1953) from Apipé falls (Vogler et al., 2014). We hope that the information provided in the present work will contribute to conservation strategies aimed at preserving endemic molluscs.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jcz.2021.03.003.

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