Freshwater gastropod diversity hotspots: three new species from the Uruguay River (South America)

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ABSTRACT

Background. The Atlantic Forest is globally one of the priority ecoregions for biodiversity conservation. In Argentina, it is represented by the Paranense Forest, which covers a vast area of Misiones Province between the Paraná and Uruguay rivers. The Uruguay River is a global hotspot of freshwater gastropod diversity, here mainly represented by Tateidae (genus *Potamolithus*) and to a lesser extent Chilinidae. The family Chilinidae (Gastropoda, Hygrophila) includes 21 species currently recorded in Argentina, and three species in the Uruguay River. The species of Chilinidae occur in quite different types of habitats, but generally in clean oxygenated water recording variable temperature ranges. Highly oxygenated freshwater environments (waterfalls and rapids) are the most vulnerable continental environments. We provide here novel information on three new species of Chilinidae from environments containing waterfalls and rapids in the Uruguay River malacological province of Argentina.

Materials & Methods. The specimens were collected in 2010. We analyzed shell, radula, and nervous and reproductive systems, and determined the molecular genetics. The genetic distance was calculated for two mitochondrial markers (cytochrome *c* oxidase subunit I (COI) and

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cytochrome b (Cyt b)) —for these three new species and the species recorded from the Misionerean, Uruguay River and Lower Paraná-Río de la Plata malacological provinces. In addition the *COI* data were analyzed phylogenetically by the neighbor-joining technique.

Results. The species described here are different in terms of shell, radula and nervous and reproductive systems. Our results also demonstrate that COI and Cyt b loci can be reliably used to distinguish species of Chilinidae. The phylogenetic analysis within Chilinidae groups the three new species together with those present in the Lower Paraná-Río de La Plata and Uruguay River Malacological provinces.

Discussion. This analysis confirms the separation between the Uruguay River and the Misioneran Malacological provinces in northeast Argentina. These new endemic species in the Uruguay River demonstrate this river to be a diversity hotspot for freshwater gastropods. These endemic species from environments with rapids and waterfalls should be taken into account by government agencies before the construction of dams that modify those ecologic niches in the Uruguay River.

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ADDITIONAL KEYWORDS: anatomy; Argentina; *Chilina luciae* sp. nov.; *Chilina nicolasi* sp. nov.; *Chilina santiagoi* sp. nov.; Conservation; Malacological provinces.

INTRODUCTION

Highly oxygenated freshwater environments (waterfalls and rapids) are the most vulnerable continental environments containing highly specific fauna with more delicate habitat requirements. Accordingly, many native snail populations are declining in numbers as a consequence of the continuous degradation and destruction of their natural ecosystems from unabated human activity (Rumi et al., 2006; Strong et al., 2008; Darrigran & Damborenea, 2011). In particular, freshwater gastropods (approximately 5% of the world's gastropod fauna) are at a disproportionately high risk of extinction (Strong et al., 2008). Of the 310 mollusc species listed as extinct in the 2015 International-Union-for-the Conservation-of-Nature (IUCN) Red List of Threatened Species (http://www.iucnredlist.org), 73 (ca. 23%) are gastropods from inland waters. The changes from fast to slow waters have caused the extinction of species—for example, those of the gastropod genus *Aylacostoma* (Mansur, 2000a, b). Despite the significance of this type of environment, the study of freshwater gastropods inhabiting them has been

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restricted to studies related to the faunas occurring upstream or beneath the rapids or waterfalls, or to descriptions of new species from those kinds of environments (Ponder, 1982; Glöer, Albrecht & Wilke, 2007; Gutiérrez Gregoric, Núñez & Rumi, 2010). Vogler et al. (2014) described a new species of *Aylacostoma* in the High Paraná River (Argentina-Paraguay), based on materials collected in 2007. In 2011, however, the locations were flooded during the last stage of filling the Yacyretá Reservoir.

The Atlantic Forest—in Argentina represented by the Paranense Forest, occupying a large part of Misiones Province—constitutes one of the global priority ecoregions for biodiversity conservation. The orography of Misiones Province is marked by a central ridge that acts as a watershed between the two great international rivers, the Paraná and the Uruguay—respectively of the Misionerean and Uruguay River malacological provinces as defined by Núñez, Gutiérrez Gregoric & Rumi (2010). The Uruguay River is among 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 (genus *Potamolithus*), Chilinidae, and Ampullariidae (genus *Felipponea*). The streams of Misiones Province contain waterfalls and rapids that have been poorly studied by malacologists.

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so I wonder how diverse this "hotspot" really is.

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In these environments several endemic freshwater gastropod entities have been recorded—*e. g.*, the genera *Acrorbis* (Planorbidae), inhabiting only waterfall environments (Hylton Scott, 1958; Ituarte, 1998; Rumi et al., 2006) and *Felipponea* spp. (Ampullariidae), recorded in the rapids of the Uruguay River and its tributaries (Castellanos & Fernandez, 1976; Rumi et al., 2006) and the species *Chilina megastoma* Hylton Scott, 1958 (Chilinidae), inhabiting the waterfalls of Iguazú National Park (Argentina and Brazil) (Hylton Scott, 1958; Ituarte, 1997), *Chilina iguazuensis* Gutiérrez Gregoric & Rumi, 2008 (Chilinidae) and *Sineancylus rosanae* (Ancylidae) (Gutiérrez Gregoric, 2012) (Planorbidae), with the last being present in the rapids of the upper Iguazú River (Argentina and Brazil) (Gutiérrez Gregoric & Rumi, 2008; Gutiérrez Gregoric, 2012, 2014).

The family Chilinidae (Gastropoda, Hygrophila) is one of the oldest families of freshwater gastropods (Duncan, 1960). Of the 21 species of *Chilina* in Argentina, 15 are endemic and nine vulnerable. Vulnerability was assessed based on or more of the following; 1, known only from the type locality (three species); 2, occurring in protected areas (four species); 3, no recent record (four species); 4, continuous restricted distribution (six species); 5, discontinuous restricted distribution (three species) (Rumi et al., 2006; Gutiérrez Gregoric & Rumi, 2010; Gutiérrez Gregoric, Ciocco & Rumi, 2014). The IUCN Red List of Threatened Species

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In this study we describe and provide information on anatomy and genetics of three new species, i.e. *Chilina nicolasi*, *Chilina santiagoi* and *Chilina luciae* from rapids and waterfalls of the Uruguay River Malacological Province. The taxonomic relationship among these Chilinidae species is estimated by phylogenetic analysis.

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MATERIALS AND METHODS

The specimens were collected in the Misiones Province (authorized by the Ministry of Ecology, Natural Renewable Resources and Tourism) and deposited in the Malacological Collection at the Museo de La Plata, Buenos Aires Province, Argentina (MLP-Ma). Additional material in MLP-Ma was also studied. Adult specimens were first relaxed in menthol for 12 hours, then immersed in hot water (70 °C), and finally stored in 96% (v/v) aqueous ethanol or fixed in modified Raillet-Henry (R-H) solution for freshwater animals—93% (v/v) distilled water, 2% (v/v) glacial acetic acid, 5% (v/v) formaldehyde, and 6 g sodium chloride per liter. Six shell measurements were taken: total length (TL), length of the last whorl (LWL), aperture length (AL), total width (TW), aperture width (AW), and aperture projection (AP). For anatomical studies of the reproductive and pallial systems, the methodology of Cuezzo (1997) was followed. Dissections were made under a Leica MZ6 stereoscopic microscope and anatomical systems drawn with the help of a *camera lucida*. Figures were drawn only for characters that showed specific differences. The terminology used for the anatomical descriptions follows that of Ovando & Gutiérrez Gregoric (2012). The length of the last whorl

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was preferred over dimensions of soft parts because that feature provided the most consistent measurement among the individuals and was not affected by the form of relaxation used. In addition, we compared these new species with *Chilina megastoma* studied by Ituarte (1997), *C. iguazuensis* described by Gutiérrez Gregoric & Rumi (2008), *C. fluminea,* studied by Gutiérrez Gregoric (2008) and *C. rushii* and *C. gallardoi* studied by Gutiérrez Gregoric (2010).

The radulae were separated from the buccal mass and cleaned following the method of Holznagel (1998), and mounted for scanning electron microscopy. The radular-dentition formula gives the number of teeth per row: [(number of left and right teeth)/(number of cusps) + (number of central teeth)/(number of cusps)] plus the number of transverse rows or their minimum and maximum number.

Total DNA was extracted from 2 mm^3 samples from the foot of recently collected specimens (2010) using commercial kits (Qiagen). A partial sequence of the genes encoding the mitochondrial cytochrome *c* oxidase subunit I (COI) and cytochrome b (Cyt b) were amplified by the polymerase chain reaction (PCR) with the universal primers of Folmer et al. (1994) and Merrit et al. (1998) respectively. Amplification was performed in a final volume of 50 µl, following the protocol used by Gutiérrez Gregoric et al. (2013, 2014). The PCR products were

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purified with an AxyPrep PCR Clean-up Kit (Axygen Biosciences, Union City, California) and both DNA strands for each gene were then directly cycle-sequenced (Macrogen Inc., Seoul, Korea). Sequences of *C. megastoma*, *C. iguazuensis* and *C. fluminea* (partial) were obtained from the Barcode of Life Database. The resulting sequences were trimmed to remove the primers, and the consensus sequences of the individuals were compared to reference sequences in GenBank. The sequence alignment was performed with the Clustal X 2.0.12 software (Larkin et al., 2007), optimized by visual inspection and edited with a word processor. Since we obtained Cyt b sequences for only four individuals we calculated a pairwise genetic divergence (Kimura two-parameter) for this region, and only COI data were subjected to phylogenetic analysis by the method of neighbor-joining (NJ). The NJ analysis was conducted using MEGA 5.05 software (Tamura et al., 2011) through the use of the maximum-composite-likelihood option for computing evolutionary distances (Tamura, Nei & Kumar, 2004). Statistical support for the resulting phylogeny was assessed by bootstrapping with 1000 replicates (Felsenstein, 1985).

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Ah – but now I see Table 2 and these sequences were not previously available but are new sequences. So why even refer to the project for which they were generated. They are simply additional species included in the project this paper describes. In fact it is not clear to me whether you generated the sequences first and then identified new species based on the tree you generated, or whether you identified the new species morphologically and then generated the tree, labeling the tips according to the identifications you made based on morphology.

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Code from the electronic edition alone. This published work and the nomenclatural acts it contains have been registered in ZooBank, the online registration system for the ICZN. The ZooBank LSIDs (Life Science Identifiers) can be resolved and the associated information viewed through any standard web browser by appending the LSID to the prefix http://zoobank.org/. The LSID for this publication is: urn:lsid:zoobank.org:pub:3140E36D-B1F5-4C1B-9F3C-0081CDE88B00. The online version of this work is archived and available from the following digital repositories: PeerJ, PubMed Central and CLOCKSS.

RESULTS

Four novel sequences of 388 bp for Cyt b (*C. nicolasi* sp. nov., 1; *C. luciae* sp. nov., 1; *C. fluminea*, 1; *C. gallardoi*, 1) and 15 sequences of 655 bp for COI (*C. nicolasi* sp. nov., 1; *C. santiagoi* sp. nov., 1; *C. luciae* sp. nov., 1; *C. fluminea*, 5; *C. rushii*, 1; *C. gallardoi*, 1; *C. iguazuensis*, 4; *C. megastoma*, 1) were obtained (Table 1). BLAST searches identified Cyt b and COI sequences as similar to other freshwater gastropods, excluding possible contamination with DNA from other sources.

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The COI sequences obtained here for *Chilina nicolasi* and *C. santiagoi*, differ by *ca.* 1.23%, while those of *C. luciae* differ from the other two species described in this work by 3.80% (Table 2). The phylogenetic analysis (Fig. 1) showed two groups within the Chilinidae, one belonging to the Misionerean Malacological province, and a second with representatives from the other two Malacological provinces (Uruguay River and Lower Paraná-Río de la Plata).

The Cyt b sequences obtained here for *Chilina santiagoi* and *C. luciae* differ by *ca.* 4%. Both species are at similar genetic distance from the other two species from which this gene was sequenced (Table 3).

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SYSTEMATICS

FAMILY CHILINIDAE DALL, 1870

GENUS *CHILINA* GRAY, 1828

Type species: *Auricula* (*Chilina*) *fluctuosa* Gray, 1828 (subsequent designation of Gray 1847).

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Diagnosis

Species in the genus and family have an oval (oblong to ventricose) shell with an expanded last whorl. Nervous system with vestigial chiastoneury. Pulmonary roof pigmented with kidney occupying almost entire length. Kidney inner wall with numerous transverse trabeculae of irregular contour. Rectum on right side of mantle cavity, anus near pneumostome. Incomplete division of male and female ducts; common duct opens to hermaphrodite duct, with irregular contours on both sides. Proximal portion of uterus with glandular walls. Calcareous granules in vaginal lumen and secondary bursa copulatrix or accessory seminal receptacle present. Penial terminal portion with cuticularized teeth-like structures.

Remarks

The Chilinidae include only the genus *Chilina* with 36 nominal species, 21 of which are found in Argentina (Núñez *et al*., 2010; Ovando & Gutiérrez Gregoric, 2012; Gutiérrez Gregoric *et al*., 2014) with the remainder in Chile and Brazil (Castellanos & Gaillard, 1981; Simone, 2006; Valdovinos Zarges, 2006).

CHILINA NICOLASI GUTIÉRREZ GREGORIC SP. NOV.

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(Figs 2A-C, 3, 4A-C)

Type locality and type material

Uruguay River, Alba Posse, Misiones Province, Argentina, (27°33’S; 54°40’W), coll. D.E. Gutiérrez Gregoric, V. Núñez & R.E. Vogler, March 23, 2010.

Holotype: MLP-Ma 13412-2; paratypes: MLP-Ma 13412 same data (4 specimens preserved in alcohol, R-H, and shell); MLP-Ma 14134 same data (10 specimens preserved in R-H and shell)

Etymology:

Dedicated to the first son, Nicolás, of the first author of this paper.

Diagnosis

Shell strong, oval, two columellar teeth (upper underdeveloped); radula with first lateral tooth with saw-like external side of mesocone; penis sheath twice the length of the prepuce; penis sheath inner sculpture with triangular regular pustules.

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Description

Shell (Fig. 2A-C). Strong, oval, globular, periostracum light brown with faint dark reddish zigzag bands. Spire immersed. Last whorl well developed. Aperture 90% of LWL, slightly expanded, with white callus of terminal portion slightly widened and flattened. Width 73% of LWL. Aperture projected 35% of TW. Two columellar teeth, lower tooth more prominent and developed than upper. Dimensions: see Table 4.

Reproductive System (Fig. 3). (i) Female reproductive system. Bursa copulatrix duct long (average 7.17 mm; SD 0.23, N 2), five times bursa sac diameter. Bursa copulatrix sac spherical, located on left side of cephalopedal haemocoel between pericardial cavity and columellar base. Secondary bursa copulatrix short, emerging from base of uterus, cylindrical (8% the length of bursa copulatrix duct). Vagina cylindrical, longer than wide, folded over free oviduct and entering female atrium. (ii) Male reproductive system. Prostate gland extending to lower half of uterus and consisting of variable size and with cylindrical acini. Vas deferens coiled twice, overlapping vagina. At level of penis complex, vas deferens bent back on itself. Penis sheath muscular, twice the length of the prepuce, with slight convexity on right side. Penis-sheath inner

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sculpture with triangular pustules over entire surface. Penis elongated (as long as the penis sheath), robust, with outer surface crossed by transverse lamellae, triangular in cross section. Prepuce cylindrical, thin, with constriction marked by oblique lines arranged in a V making connection with penis sheath.

Radula (Fig. 4 A-C): Average number of rows 55 (n= 3; SD= 3.79; range= 52 to 59). Average number of teeth per half row 41 (n= 3; SD=0.58; range= 40 to 41). Central tooth asymmetrical, bicuspid, elongated base higher than wide, left cusp more developed. Both cusps with slight sawlike edges. Presence of longitudinal groove between cusps. First lateral tooth tricuspid or tetracuspid, with mesocone (central cusp) more developed, saw-like external side of mesocone, base of tooth narrower than the apical part (cusp area). Second lateral tooth tetracuspid with the outermost second cusp more developed and saw-like. In a radula, notable that the second lateral tooth presented three cusps. Last teeth with thin base and five to seven cusps similarly developed. Radular formula: [41/(3-7) + 1/2] 55.

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Nervous system (Fig. 3C; Table 5). All connectives between ganglia relatively thin compared to size of both ganglia and central nervous system in general. Left connective joining the cerebral ganglion with the pleural one is longer than the right one (10.10% vs. 8.97% of LWL). Right pleuroparietal connective passes over the penis complex. Length of left pleuroparietal connective shorter than right (3.79% vs 9.22% of LWL). Parietal-subesophageal connective shorter than parietal-visceral connective (15.08% vs 23.26% of LWL). One very short connective (5.72% of LWL) linking subesophageal ganglion to visceral ganglion and closing posterior nerve ring. Pleurovisceral connectives with incomplete torsion characteristic of the genus.

Distribution (Fig. 5)

Only known from the type locality. A hydroelectric dam is going to be built in the area where the new species described here were collected. This hydroelectric dam will raise the level the Uruguay River, causing the disappearance of the environment inhabited by the species.

DNA barcoding

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The data from the analysis of the cytochrome *c* oxidase subunit I (COI) of 655 bp from paratype material (MLP-Ma 14134) was deposited in GenBank under the number KT830419.

Remarks: Of the Chilinidae species described so far through characters of the radula, *C. nicolasi* is the only one with the first and second lateral tooth of the outer edge of the mesocone serrated. *Chilina nicolasi* has been recorded in the Uruguay River rapids as has *C. gallardoi*. Both species have two columellar teeth in the aperture, but in *C. gallardoi* both teeth are strong. The Aperture Length /Last Whorl Length ratio in *C. gallardoi* is lower than in *C. nicolasi* (78% vs 89%) (Gutiérrez Gregoric, 2010). In addition, *C. gallardoi* has a keel (or sub-keel) along the whorls (so does *C. rushii*), a character absent in *C. nicolasi*. The radula of both species has a similar number of rows and teeth per row, but marginal teeth in *C. nicolasi* can have up to seven cusps, while *C. gallardoi* has only five. *Chilina nicolasi* differs in the length of the aperture from *C. iguazuensis*, which feature in the latter is of the same total length; moreover, the last whorl is more globose in *C. iguazuensis*. The radula of *Chilina iguazuensis* also has more rows and teeth per row (Gutiérrez Gregoric & Rumi, 2008).

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Chilina santiagoi Gutiérrez Gregoric **sp. nov.**

(Figs 2D-F, 4D-F, 6)

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Type locality and type material

Horacio Foerster Falls, Misiones Province, Argentina (27°08'S 53°55'W), coll. D. E. Gutiérrez Gregoric, V. Núñez & R.E. Vogler, March 24, 2010, Holotype**,** MLP-Ma 14135. Paratypes**:** MLP-Ma, 13417 same data (five specimens preserved in alcohol), MLP-Ma, 14136 same data (six specimens preserved in R-H and shell).

Other material examined

MLP-Ma 14137: Horacio Foerster Falls, Misiones Province, Argentina (27°08'S 53°55'W), coll. C. Galliari, May 2009, (four dry shells); MLP-Ma 14138: Moconá Falls, Misiones Province, Argentina (27°08'S 53°53'W), coll. C. Galliari, May 2009 (12 specimens preserved in alcohol); MLP-Ma 14139: Moconá Falls, Misiones Province, Argentina (27°08'S 53°53'W), coll. A. Rumi, S.M. Martín & I. César, October 20, 2011 (10 specimens preserved in R-H and shell);

MLP-Ma 14140: Yerba Falls, Paraíso Stream, El Soberbio, Misiones Province, Argentina (27°14'S 54°02'W), without coll. and date (two specimens preserved in alcohol)

Etymology

Dedicated to my second son, Santiago.

Diagnosis

Shell small, thin, one columellar tooth; radula with asymmetrical bicuspid central tooth; penis sheath twice the length of prepuce; penis sheath inner sculpture with regular conical pustules and longitudinal folds.

Description

Shell (Fig. 2D-F): Small, thin, oval, of 3¼ whorls. Spire low and conical. Last whorl large (97% of the total length). Width 79.7% of LWL Aperture expanded, of 94.5% of LWL, with strong white callus. One columellar tooth. Aperture projection 50% of TW. Light brown periostracum with stronger thin longitudinal reddish bands. Dimensions: see Table 4.

Reproductive System (Fig. 6): Female genital system: Bursa-copulatrix duct large (average= 4.69 mm; DS= 0.88; N= 4). Bursa-copulatrix sac oval. Secondary bursa copulatrix long (18% bursa copulatrix duct length), comprised of a long duct and expanded at the distal end. Male genital system: Muscular penis sheath, nearly twice as long as prepuce. Penis-sheath inner sculpture with pustules of conical aspect and longitudinal folds. Penis slightly longer than penis sheath, robust, with outer surface cut by transverse lamellae, triangular in cross-section. Prepuce inner sculpture with numerous smooth, very tight longitudinal folds.

Radula (Fig. 4D-F): Average number of rows 44 (n= 3; SD= 0.58; range= 43 to 44). Average number of teeth per half row 32 (n= 3; SD= 0.58; range= 32 to 33). Central tooth asymmetrical, bicuspid, elongated base higher than wide, mesocone more developed and serrated, with longitudinal groove between the two cusps. First lateral tooth tricuspid with mesocone more developed, base of tooth same width as apical part (cusp area). Second lateral tooth tricuspid (mainly) or tetracuspid, with mesocone (of the tricuspid) or the outermost second cusp (in the tetracuspid) more developed, base of tooth narrower than apical part of tooth. Last teeth with thin base, having five similarly developed cusps. Radular formula: [32(3-5) + 1/2] 44.

Nervous system (Fig. 6 Table 5): Length of the left connective joining the cerebral ganglion with the pleural one slightly greater than the right one (12.20 vs. 11.24% of LWL). There are differences in the lengths of the pleuroparietal connectives with the left one smaller than the right one (4.50 vs 12.80% of LWL). Long connective (ratio= 19.35% of LWL) linking left parietal ganglion to subesophageal ganglion, located above posterior half of columellar muscle. Long connective (ratio= 20.11% of LWL) linking right parietal ganglion to visceral ganglion. One very short connective (ratio= 3.46% of LWL) linking subesophageal ganglion to visceral ganglion and closing posterior nerve ring.

Distribution (Fig. 5)

Horacio Foerster Falls is located within the Yabotí Biosphere Reserve. It is a small waterfall coming from the Oveja Negra Stream, which flows into the Uruguay River. Water quality parameters of the Horacio Foerster Falls, were taken on 3-24-2010, and are the following: water temperature: 23.2C°; pH: 7.62; dissolved oxygen: 6.3 mg/l; conductivity: 0.015 mS. Moconá Falls is located in the Moconá Provincial Park, which is within the Yabotí Biosphere Reserve. This waterfall is peculiar in the sense that it spills along a ridge parallel to the river course. Its height varies with the level of the river and it is the second largest waterfall in Misiones Province after the Iguazú Falls.

DNA barcoding

The data from the analysis of the *cytochrome c oxidase subunit I* (*COI*) of 655 bp and *Cytochrome b* (*Cyt b*) of 388 bp from Paratype MLP-Ma 14136 material were deposited in the GenBank under the numbers KT820416 and KT820424 respectively.

Remarks: The spire is not preserved in all specimens. This failure occurs in several species of Chilinidae, especially in those that inhabiting fast-running water such as *C. iguazuensis* (Gutiérrez Gregoric & Rumi, 2008). Regarding to *C. megastoma*, which inhabits waterfalls environments, it mainly differs in size from the others because *C. santiagoi* reaches a maximum last whorl length of 9.60mm, whereas *C. megastoma* is of 17.33mm (Gutiérrez Gregoric, 2008).

Chilina megastoma has a striated shell (but not in *C. santiagoi*) and two columellar teeth (with one in *C. santiagoi*). Both species have thin shells. In *C. megastoma* there is a slight swelling not forming a true ganglion between the left pleural and the subesophageal ganglia (Ituarte, 1997; Gutiérrez Gregoric, 2010), but was not detected in *C. santiagoi*.

CHILINA LUCIAE GUTIÉRREZ GREGORIC **SP. NOV.**

(Figs. 2G-I; 4F-I; 7)

Type locality and type material

Pesiguero stream, Misiones Province, Argentina (27°58'S 55°26'W), coll. D. E. Gutiérrez Gregoric, March 21, 2010, Holotype**,** MLP-Ma 14141. Paratypes**:** MLP-Ma, 13413 same data (5 specimens preserved in alcohol, R-H, and shell), MLP-Ma 14142 same data (4 specimens preserved in alcohol).

Etymology

Dedicated to my daughter, Lucía.

Diagnosis

Shell strong, two strong columellar teeth; radula, central tooth bicuspid and with saw-like external side; prepuce 37% of length of penis sheath; penis sheath inner sculpture with two regions, one with polygonal pustules and the other with longitudinal folds in zigzag.

Description

Shell (Fig. 2G-I): Strong, slightly elongated. Spire eroded. Width 73.7% of LWL. Aperture somewhat expanded, of 82.8% of LWL, with strong white callus. Two strong columellar teeth. Aperture projection 32.6% of TW. Light reddish periostracum with some dark brown spots. Dimensions: see Table 4.

Reproductive System (Fig. 7): Female genital system: bursa-copulatrix duct large (average= 4.67 mm; DS= 0.14; N: 3). Bursa-copulatrix sac spherical. Secondary bursa copulatrix short (11% of the length of bursa copulatrix duct), cylindrical, expanded at its distal portion. Male genital system: muscular penis sheath, a little more than twice the length of prepuce (2.08mm vs

0.78mm). Penis-sheath inner sculpture with polygonal pustules and longitudinal zigzag folds. Penis 92% the length of penis sheath, robust, with outer surface cut by transverse lamellae, triangular in cross section. Inner sculpture of prepuce with numerous smooth, very tight longitudinal folds.

Radula (Fig. 4G-I): Average number of rows 50 (n= 2). Average number of teeth per half row 41 (n= 2; SD= 0.58; range= 40 to 41). Central tooth asymmetrical, bicuspid, elongated base higher than wide, both cusps with serrated edges. First lateral tooth tretracuspid with innermost second cusp more developed, base of tooth same width as apical part (cusp area). Second lateral tooth tetracuspid, with innermost second cusp more developed, base of tooth narrower than apical part of tooth. Last teeth with thin base, having five similarly developed cusps. Radular formula: [41(4-5) + 1/2] 50.

Nervous System (Fig. 7; Table 5): Length of the left connective joining the cerebral ganglion with the pleural one greater than the right one (10.30 vs. 8.83% of LWL). There are differences in the length of the pleuroparietal connectives with the left one smaller than the right one (5.89

vs 17.66% of LWL). Long connective (ratio= 22.07% of LWL) linking left parietal ganglion to subesophageal ganglion, located above posterior half of columellar muscle. Long connective (ratio= 16.18% of LWL) linking right parietal ganglion to visceral ganglion. One very short connective (ratio= 3.46% of LWL) linking subesophageal ganglion to visceral ganglion and closing posterior nerve ring.

Distribution (Fig. 5)

Only cited for the type locality. Pesiguero Stream drains into Uruguay River and is located in the Concepción de la Sierra District in Misiones Province. The Uruguay River is located 10 km away from the collection site.

DNA barcoding

The data from the analysis of the *cytochrome c oxidase subunit I* (*COI*) of 655 bp and *Cytochrome b* (*Cyt b*) of 388 bp from paratype material (MLP-Ma 14142) were deposited in the GenBank under the numbers KT820420 and KT820425 respectively.

Remarks: *Chilina luciae,* like *C. gallardoi and C. rushii,* was recorded in the rapids of a stream that flows into the Uruguay River. *Chilina luciae* differs from both of those species by not having a keel in the shell. *Chilina luciae* has two strong columellar teeth as in *C. gallardoi* and *C. rushii*; while *C. nicolasi* also has two columellar teeth, but weak ones. The Aperture Length / Last Whorl Length ratio in *C. luciae* is lower than in *C. nicolasi* (83% vs 89%), but higher than in *C. gallardoi* (78%) (Gutiérrez Gregoric, 2010). In the radula of *C. luciae* carries the same number of teeth per row as in *C. nicolasi* (41), and a closer number of rows (50 vs 55). In the lateral teeth, *C. luciae* has between four and five cusps, while in *C. nicolasi* has between three and seven. Compared with *C. iguazuensis,* and like *C. nicolasi,* it differs in the length of the aperture; in *C. iguazuensis* is of the same total length. In addition, the last whorl is more globose in *C. iguazuensis*. *Chilina iguazuensis* has a radula with more rows and teeth per row (Gutiérrez Gregoric & Rumi, 2008).

DISCUSSION

This report provides anatomical, molecular-genetic, and distributional information on the species of *Chilina* of lotic environments from the Uruguay River Malacological Province, increasing the number of known freshwater gastropod species in this province from 51 to 54. This province exhibits the highest gastropod richness in Argentina, and also contains the highest number of vulnerable (14) and endemic species (seven of the province and another five of Argentina), most belonging to *Potamolithus* spp. (Tateidae) and to *Felliponea* spp. (Ampullariidae; Núñez, Gutiérrez Gregoric & Rumi, 2010). The Chilinidae species richness of this province now increases from three to six species. These new endemic species from the Uruguay River have confirmed this river as a diversity hotspot of freshwater gastropods as originally proposed by Strong et al. (2008). The family Chilinidae is represented by 24 species in Argentina, of which 17 are endemic.

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The interspecific genetic distances found in the present study for *COI* were above 1.2%, and the intraspecific lower than 0.5%. Studies in Lymnaeidae (Gastropoda, Hygrophila) have also suggested a similar interspecific genetic distance for COI among neotropical species (Correa et al., 2011). For land molluscs, Davison et al. (2009) estimated interspecific genetic distances of 12% and intraspecific of 3%, but the authors clarified that the interspecific genetic distance can actually be quite low, around 1%. For this reason, we suggest that an integrative vision is

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necessary—one that complements molecular-genetics information with conchological, anatomical, and ecological data.

The phylogenetic analysis of Chilinidae corroborated segregation of the freshwater gastropod fauna of Misiones Province from those of other provinces, as suggested by Núñez, Gutiérrez Gregoric & Rumi (2010). The species described here for the Uruguay River Malacological Province are distinct from those of the Misionerean Malacological Province—*e. g. C. megastoma* and *C. iguazuensis*. As mentioned in the Introduction, the Misiones Province contains a central ridge that acts as a watershed between the two great border rivers, the Paraná and the Uruguay. Nevertheless, the species from the Río de la Plata River (*C. fluminea* and *C. rushii*) are more closely associated with those of the Uruguay River. The species in the Cuyo Malacological Province (*C. mendozana* and *C. sanjuanina*) are distinct from those from the Del Plata basin. Likewise, species of *Aylacostoma* (Thiaridae) and *Acrorbis* (Planorbidae) in the Misioneran Malacological Province have not been recorded in the Uruguay River Province (Núñez, Gutiérrez Gregoric & Rumi, 2010). Despite malacological differences, ichthyologic classifications (Ringuelet, 1975; López, Morgan & Montenegro, 2002) suggest that Misiones Province (as a political division) is considered an ecoregion or province.

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With the examples described here, the number of endemic species found in waterfall environments increases. Thus, species living in Misiones Province are *Chilina megastoma*, endemic to Iguazú National Park; *Acrorbis petricola*, from the waterfalls of Iguazú National Park and the Encantado Falls (Aristólubo del Valle); and *Chilina santiagoi* in the Uruguay River. In addition, *C. nicolasi* and *C. luciae* have been added to the species recorded in rapids along rivers in Misiones Province, which include *Chilina iguazuensis*, *Sineancylus rosanae*, *Felipponea* spp. and *Aylacostoma* spp. These endemic species should be taken into account by government agencies before the construction of dams that modify these types of environments within the Uruguay River.

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ACKNOWLEGMENTS

We thank the curator and technical staff of the malacological collection of La Plata Museum (MLP), G. Darrigran and M. Tassara for their generosity in lending the material under study, and R. Vogler, V. Núñez, and A. Rumi for support during field work. We are especially grateful to Dr. M. Griffin for her helpful comments. Dr. D.F. Haggerty, a retired academic career investigator and native English speaker, edited the final version of the manuscript.

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Table 1. Information on the specimens of *Chilina* used in DNA sequence analysis.

Species	Site / Malacological Province	GenBank	
		COI	*Cyt b*
Chilina nicolasi	Alba Posse / III	KT830419*	--
Chilina santiagoi	H. Foerster Fall / III	KT820418*	KT820424*
Chilina luciae	Pesiguero Stream / III	KT820420*	KT820425*
Chilina gallardoi	Monte Caseros / III	KT820421*	KT820427*
Chilina rushii	Gualeguaychú river / III	KT820423*	--
Chilina fluminea	Punta Lara / IV	KT807833*#	--
Chilina fluminea	Punta Lara / IV	KT807832*#	--
Chilina fluminea	Punta Lara / IV	KT807831*#	--
Chilina fluminea	Punta Lara / IV	KT807834*#	--
Chilina fluminea	Punta Lara / IV	KT820422*	KT820426*
Chilina iguazuensis	Iguazú National Park / I	KT807837*#	--
Chilina iguazuensis	Iguazú National Park / I	KT807838*#	--
Chilina iguazuensis	Iguazú National Park / I	KT807836*#	--
Chilina iguazuensis	Iguazú National Park / I	KT807835*#	--
Chilina megastoma	Iguazú National Park / I	KT807839*#	--
Chilina sanjuanina	Aguas Negras / VI	KC347574	--
Chilina mendozana	Uspallata / VI	KC347575	--
Lymnaea diaphana		JF909501	--

* new sequences. # sequences generated by the BOLD program. Numerals corresponding to the Malacological provinces: I. Misionerean; III. Uruguay River; IV. Lower Paraná - Río de la Plata; VI. Cuyo.

Table 2. Pairwise genetic divergence (Kimura two-parameter, %) among species of the genus *Chilina* assessed by means of *cytochrome c oxidase subunit I* (*COI*) gene sequences. GenBank accession numbers are indicated in parentheses.

		1	2	3	4	5	**6**	**7**	**8**	9	10	11	12	13
1	*C. fluminea* (KT807831/33; KT820422)													
2	*C. fluminea* (KT807832)	0.15												
3	*C. fluminea* (KT807834)	0.31	0.46											
4	*C. rushii* (KT820423)	1.24	1.39	1.55										
5	*C. gallardoi* (KT820421)	2.98	3.14	3.3	2.66									
6	***C. santiagoi* sp. nov. (KT820418)**	2.97	3.13	3.29	3.14	2.66								
7	***C. nicolasi* sp. nov (KT820419)**	2.97	3.13	3.29	3.14	2.34	1.24							
8	***C. luciae* sp. nov. (KT820420)**	5.27	5.44	5.61	5.45	4.29	3.79	3.79						
9	*C. iguazuensis* (KT807838)	8.01	8.19	7.66	8.75	7.68	6.67	7.35	8.58					
10	*C. iguazuensis* (KT807833/37)	7.84	8.01	7.48	8.57	7.5	6.49	7.17	8.4	0.15				
11	*C. iguazuensis* (KT807836)	7.49	7.67	7.14	8.22	7.16	6.48	6.83	8.05	0.46	0.31			
12	*C. megastoma* (KT807839)	8.52	8.7	8.17	8.9	9.63	8.72	8.9	9.62	7.83	7.65	7.66		
13	*C. sanjuanina* (KC347575)	11.8	12	11.4	12	11.6	11.3	11.8	12	10.7	10.5	10.1	13.8	
14	*C. mendozana* (KC347574)	10.8	11	10.5	10.3	11.4	10.7	11.2	10.7	11.2	11	10.7	12.8	3.64

Table 3. Pairwise genetic divergence (Kimura two-parameter, %) among species of the genus *Chilina* assessed by means of *cytochrome b* (*Cyt b*) gene sequences. GenBank accession numbers are indicated in parentheses.

		1	**2**	**3**
1	*C. fluminea* (KT820426)			
2	***C. santiagoi* sp. nov. (KT820424)**	2.91		
3	***C. luciae* sp. nov. (KT820425)**	4.26	3.99	
4	*C. gallardoi* (KT820427)	4.00	3.46	4.82

Table 4. Average and range of six measurements for *Chilina nicolasi* sp. nov., *C. santiagoi* sp. nov., and *C. luciae* sp. nov. LWL: last whorl length; AL: aperture length; TW: total width; AW: aperture width; AP: aperture projection.

		LWL	AL	TW	AW	AP
	Holotype	13.50	11.99	9.76	7.96	3.39
Chilina nicolasi (n=15)	Mean	13.16	11.74	9.63	7.45	3.41
	SD	1.4	1.16	1.11	0.88	0.56
	Max	16.46	14.26	12.2	9.49	4.93
	Min	10.84	9.69	8.08	6.34	2.46
Chilina santiagoi	Holotype	8.47	7.96	6.37	5.15	2.9
(n=40)	Mean	7.17	6.77	5.68	4.56	2.83
	SD	1.33	1.29	0.96	0.79	0.55
	Max	9.6	9.04	7.76	6.08	4.00
	Min	4.55	4.3	3.75	3.00	1.70
Chilina luciae	Holotype	10.62	8.91	7.84	5.91	2.53
(n=10)	Mean	11.54	9.56	8.51	6.15	2.78
	SD	0.86	0.68	0.71	0.46	0.25
	Max	12.91	10.56	9.82	7.24	3.09
	Min	10.54	8.77	7.65	5.57	2.38

Table 5. Ratio between the lengths of ganglia and last whorl in *Chilina nicolasi* (n = 5), *C. santiagoi* (n = 5) and *C. luciae* (n = 4).

	Chilina nicolasi			*Chilina santiagoi*			*Chilina luciae*		
	Ratio	Mean	SD	Ratio	Mean	SD	Ratio	Mean	SD
lc - rc	14.39	1.83	0.19	19.46	1.41	0.11	16.88	1.90	0.14
lpe – rpe	6.46	0.82	0.05	6.16	0.45	0.03	5.26	0.59	0.19
lc – lpl	10.10	1.28	0.07	12.20	0.88	0.17	7.11	0.80	0.26
rc – rpl	8.97	1.14	0.07	11.24	0.81	0.14	8.40	0.95	0.01
c – p	12.32	1.56	0.22	17.30	1.25	0.56	13.05	1.47	0.62
rpl – rp	9.22	1.17	0.29	12.80	0.93	0.13	14.01	1.58	0.31
lpl – lp	3.79	0.48	0.11	4.50	0.33	0.06	5.14	0.58	0.09
lp – so	15.08	1.91	0.18	19.35	1.40	0.20	17.25	1.94	0.38
rp – v	23.26	2.95	0.37	20.11	1.45	0.24	17.51	1.97	0.14
so – v	5.72	0.73	0.14	3.46	0.25	---	4.21	0.47	0.14

Abbreviations for each ganglion: c, cerebral; lc, left cerebral; lp, left parietal; lpe, left pedal; lpl, left pleural; p, pedal; rc, right cerebral; rp, right parietal; rpe, right pedal; rpl, right pleural; so, subesophageal; v, visceral. Measurements in mm.

Figure 1. Phylogenetic tree (neighbor-joining [NJ] method) of Chilinidae from the Del Plata basin based on a 655-bp fragment of *COI* gene. The support value—i. e., NJ bootstrap values—is shown above and below the branches. The tree contains two well supported clades corresponding to the species of Misioneran (light-grey bar) and Uruguay River and Lower Paraná – Río de la Plata (dark-grey bar) Malacological provinces. The numbers within the clades are the corresponding GenBank-accession numbers.

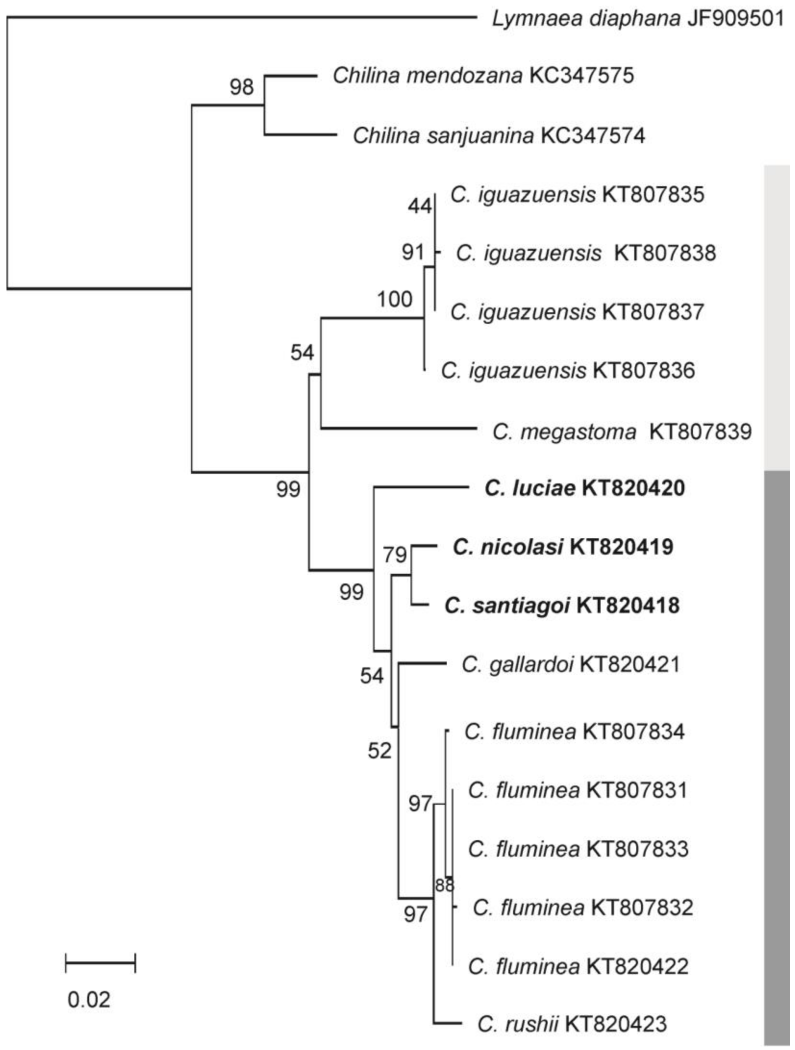

Figure 2. Shells of new species (Holotypes). A-C. *Chilina nicolasi*. D-F. *Chilina santiagoi*. G-I. *Chilina luciae*. Scale bars: 1.0 mm.

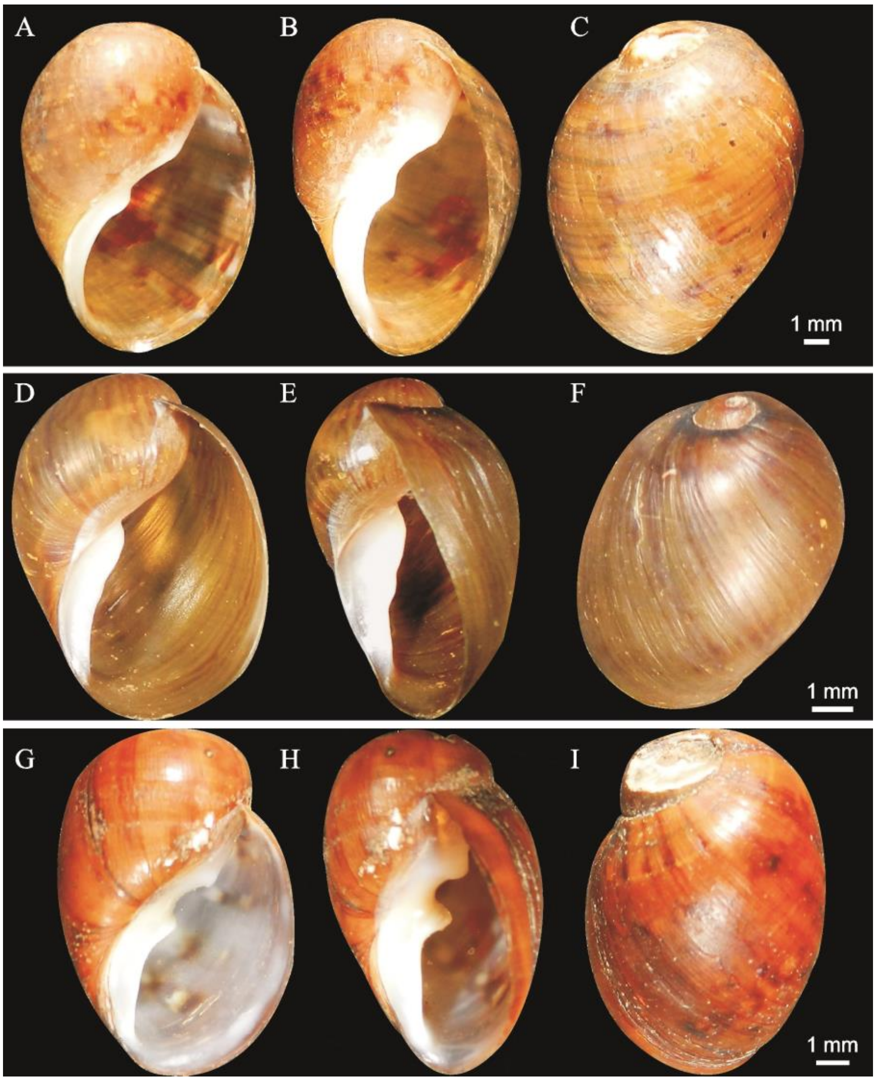

Figure 3. *Chilina nicolasi* sp. nov. A. Dorsal view of the reproductive system. B. Penis inner wall. Abbreviations: ag, albumen gland; bc, bursa copulatrix; bcd, bursa copulatrix duct; pe, penis; pr, prostate; pp, preputium; prm, penis retractor muscle; ps, penis sheath; pu, pustules; sbc, secondary bursa copulatrix; va, vagina; vd, vas deferens. C. Diagram of nervous system. Abbreviations: lc, left cerebral; lpe, left pedal; lp, left parietal; lpl, left pleural; rc, right cerebral; rpe, right pedal; rp, right parietal; rpl, right pleural; so, subesphageal; v, visceral. Scale bar: 1.0 mm.

Commented [ANON74]: Why do you not show the ovotestis and hermaphrodite duct?

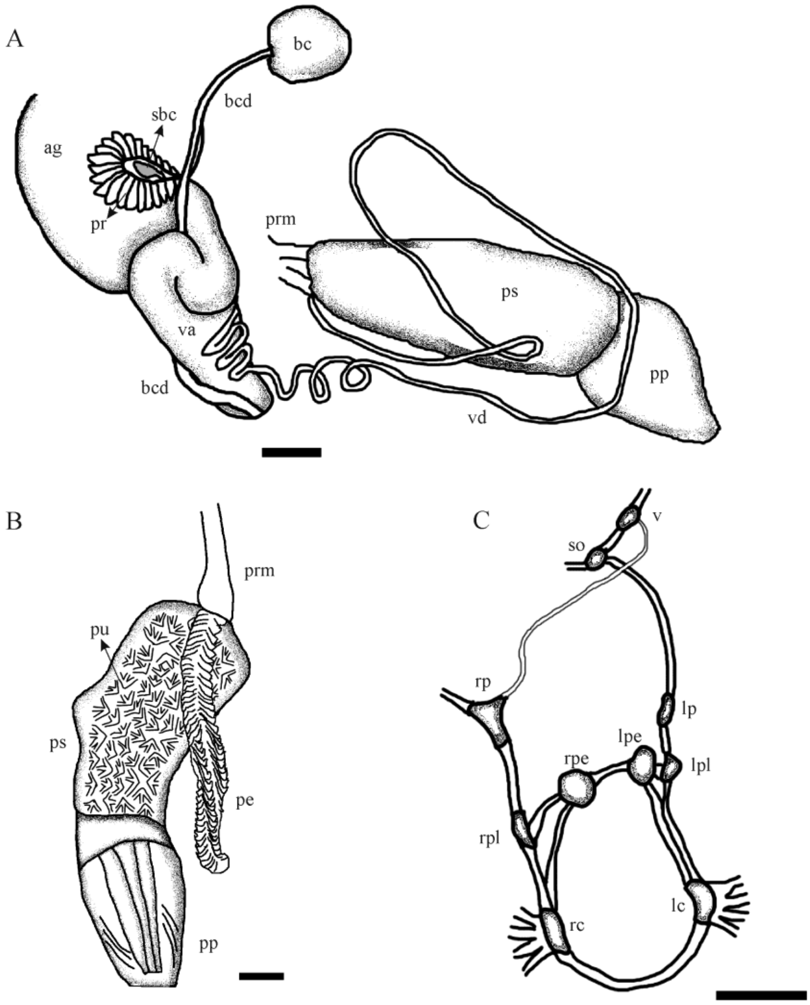

Figure 4. Radulae: A-C. *Chilina nicolasi* sp. nov. from Alba Posse, Misiones province, Argentina. D-F. *Chilina santiagoi* sp. nov. from Horacio Foerster Fall, Misiones Province, Argentina. **G-I**. *Chilina luciae* sp. nov. from Persiguero Stream, Misiones Province, Argentina. A, D, G. Central tooth and first lateral teeth. B, E, H. Central tooth. C, F, I. Lateral teeth.

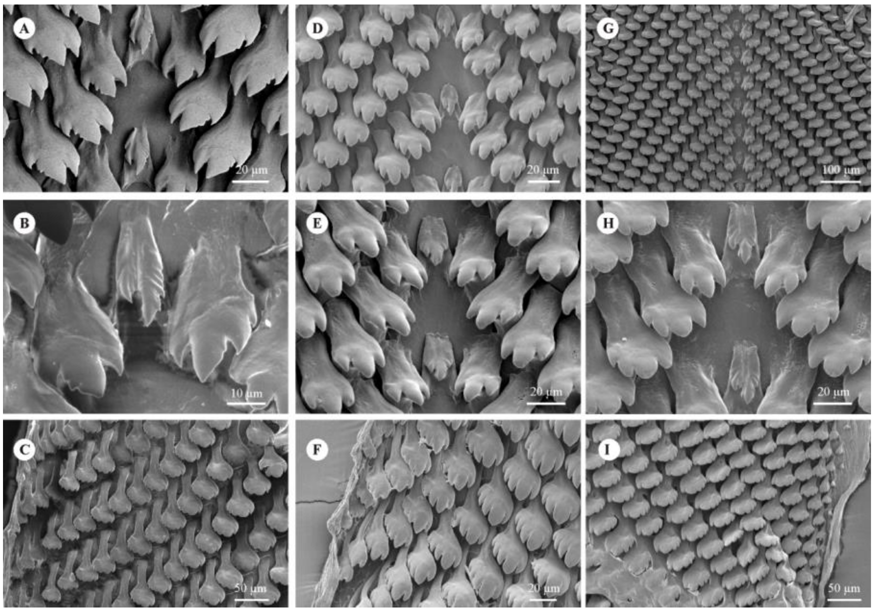

Figure 5. A. Malacological provinces of Argentina, I. Misionerean; II. Middle Paraná; III. Uruguay River; IV. Lower Paraná - Río de la Plata; V. Central; VI. Cuyo; VII. Northern Patagonia; VIII. Southern Patagonia. Diagonal pattern: Transitional Zone. B. Species distribution of Chilinidae in the Misiones province, Argentina: light grey; Misionerean Malacological Province; dark grey: Uruguay River Malacological Province: ●: *Chilina santiagoi* sp. nov.; ★: *Chilina nicolasi* sp. nov.; ⬟**:** *Chilina luciae* sp. nov.; ▲: *Chilina megastoma*; ■: *Chilina iguazuensis*; ▼: *Chilina gallardoi*; ○: *Chilina rushii*; □: *Chilina guaraniana*.

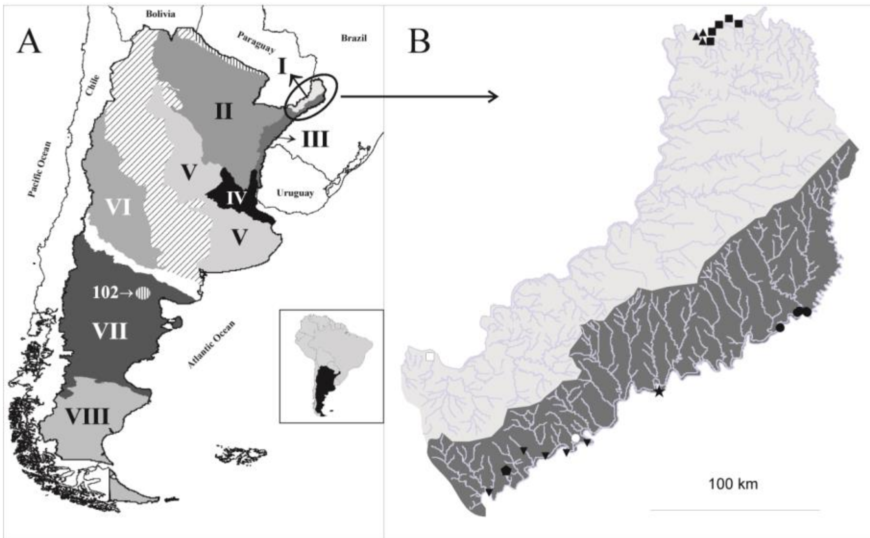

Figure 6. ***Chilina santiagoi*** **sp. nov**. **A**. Dorsal view of the reproductive system. **B.** Penis inner wall. Abbreviations: ag, albumen gland; bc, bursa copulatrix; bcd, bursa copulatrix duct; pe, penis; pr, prostate; pp, preputium; prm, penis retractor muscle; ps, penis sheath; pu, pustules; va, vagina; vd, vas deferens. **C**. Diagram of nervous system: Abbreviations: lc, left cerebral; lpe, left pedal; lp, left parietal; lpl, left pleural; rc, right cerebral; rpe, right pedal; rp, right parietal; rpl, right pleural; so, subesphageal; v, visceral. Scale bar: 1.0 mm.

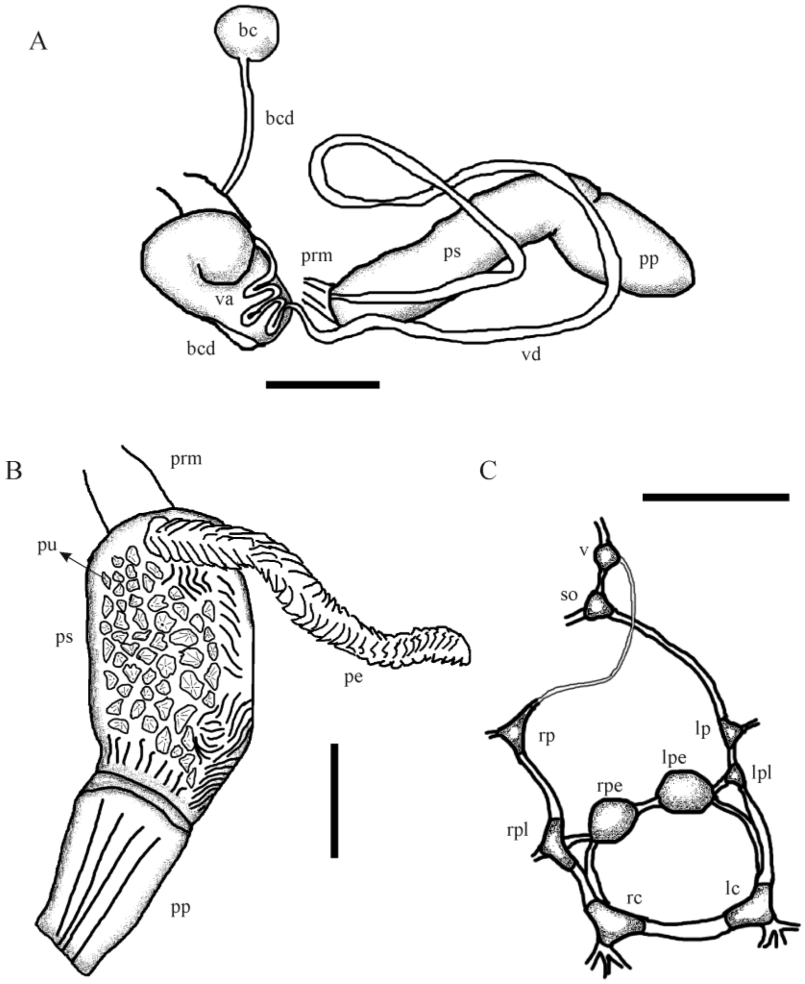

Figure 7. ***Chilina luciae*** **sp. nov**. **A**. Dorsal view of the reproductive system. **B:** Penis inner wall. Abbreviations: ag, albumen gland; bc, bursa copulatrix; bcd, bursa copulatrix duct; lf: longitudinal folds; pe, penis; pr, prostate; pp, preputium; ps, penis sheath; pu, pustules; sbc, secondary bursa copulatrix; va, vagina; vd vas deferens. **C**. Diagram of nervous system: Abbreviations: lc, left cerebral; lpe, left pedal; lp, left parietal; lpl, left pleural; rc, right cerebral; rpe, right pedal; rp, right parietal; rpl, right pleural; so, subesphageal; v, visceral. Scale bar: 1.0 mm.