Threatened gastropods under the evolutionary genetic species concept: redescription and new species of the genus *Aylacostoma* (Gastropoda: Thiaridae) from High Paraná River (Argentina–Paraguay)

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The genus *Aylacostoma* Spix, 1827, is mainly endemic to South America, and comprises about 30 nominal species, most of which were described based solely on conchological features following the typological approaches of most of the 19th and the mid-20th century authors. Here, we redescribe *Aylacostoma chloroticum* Hylton Scott, 1954, and describe ***Aylacostoma brunneum* sp. nov**. from the High Paraná River (Argentina–Paraguay) by means of morphological and molecular characters. Both are threatened species currently included into an ongoing *ex situ* conservation programme, as their habitats have disappeared because of damming and the filling up of the Yacyretá Reservoir in the early 1990s. We used DNA sequences from *cytochrome b* and *cytochrome oxidase subunit I* (*COI*) genes to estimate their genetic distances, and the *COI* sequences were also used to assess their specific status under the evolutionary genetic species concept by means of the *K*/θ method. Our results clearly demonstrate that both must be recognized as evolutionary genetic species, despite only minor differences in morphological characters other than in the shells.

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ADDITIONAL KEYWORDS: anatomy – asexually reproducing taxa – endangered freshwater snails – *ex situ* conservation programme – mitochondrial DNA.

INTRODUCTION

Thiaridae Gill, 1871, is a family of gastropods that includes species that have been intensely studied because of their reproductive and life history strategies, and their capacity for successful colonization of new habitats worldwide (Strong *et al*., 2011). Although understanding of Thiaridae has increased in recent times (e.g. Glaubrecht, 1996, 1999, 2006; Gomez, Strong & Glaubrecht, 2011; Strong *et al*., 2011), little is known about almost all South American representatives, most of which were described based solely on conchological features following the typological approaches of most of the 19th and mid-20th century authors (e.g. Reeve, 1860; Ihering, 1902, 1909).

Several genus-group names are available for Neotropical thiarids: e.g. *Aylacostoma* Spix, 1827, *Hemisinus* Swainson, 1840, *Verena* H. & A. Adams, 1854,

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Longiverena Pilsbry & Olsson, 1935, and *Basistoma* Lea, 1852 (Morrison, 1954; Gomez *et al.*, 2011). The limits amongst these taxa remain to be established; however, comparative analyses amongst them are still difficult given that the available data are very scarce and highly dispersed. An attempt to organize Neotropical thiarids was made by Morrison (1954), which included the genus *Aylacostoma* Spix, 1827, within the Thiaridae, a view that has not been changed since then; despite the fact that the concept of Thiaridae has changed radically since Morrison (Strong *et al.*, 2011 and references therein). However, the genus has never been comprehensively revised, and most of our knowledge rests on the original species descriptions. At the beginning of this century, the anatomy of three Brazilian species of *Aylacostoma* was described by Simone (2001), thus providing new data for comparison.

In Argentina and Paraguay, the occurrence of species of *Aylacostoma* was first reported by Hylton Scott (1953, 1954). She described *Aylacostoma guaraniticum* (Hylton Scott, 1953), *Aylacostoma chloroticum* Hylton Scott, 1954, and *Aylacostoma stigmaticum* Hylton Scott, 1954; which are considered to be viviparous and parthenogenetic (Quintana & Mercado Laczkó, 1997; Ostrowski de Núñez & Quintana, 2008). The three species were recorded in highly oxygenated freshwater habitats near the Yacyretá–Apipé rapids in the High Paraná River at the border area between Argentina and Paraguay (Vogler, 2012; Peso, Molina & Costigliolo Rojas, 2013a; Peso, Costigliolo Rojas & Molina, 2013b). However, these habitats have disappeared because of damming and the filling up of the Yacyretá Reservoir in the early 1990s (Quintana & Mercado Laczkó, 1997; Meichtry de Zaburlín *et al.*, 2010). Owing to this flooding, the three species, which are endemic to the area and are strictly confined to the rapids, have been threatened with extinction (Vogler, 2013). *Aylacostoma guaraniticum* and *A. stigmaticum* are listed by the International Union for Conservation of Nature (IUCN) as 'Extinct in the Wild' (Mansur, 2000a, b), but presently could be categorized as extinct as no specimens of these species have been reported to have survived in nature or captivity (Peso *et al.*, 2013a, b). It is important to mention that an ongoing *ex situ* conservation programme was developed in the 1990s. This programme, known as the '*Aylacostoma* Project', is being conducted by the Universidad Nacional de Misiones (Posadas, Argentina) jointly with the Museo Argentino de Ciencias Naturales (Buenos Aires, Argentina), and is supported by the Entidad Binacional Yacyretá (EBY, Argentina–Paraguay). Currently, only *A. chloroticum* and a recently recorded new morphotype are included in the programme (Vogler, 2013).

In the present study, we provide the taxonomic description of the two threatened species of *Aylacostoma* included in the *ex situ* conservation programme mentioned above (*A. chloroticum* has been redescribed, the new morphotype has been described as a new species) as a contribution towards future comparative studies of this poorly known genus. As both are fully asexually reproducing taxa, they have been described under the evolutionary genetic species concept (EGSC) and the description includes conchological and anatomical data.

MATERIAL AND METHODS

The study was based on the examination of specimens housed in malacological collections at the Museo de La Plata (MLP; La Plata, Argentina) and Museo Argentino de Ciencias Naturales (MACN; Buenos Aires, Argentina). Additional specimens that were collected in the High Paraná River between 1994 and 2012 came from the ongoing *ex situ* conservation programme taking place at the Universidad Nacional de Misiones (UNaM; Posadas, Argentina). Living specimens were relaxed in 0.1% Nembutal solution for 8–10 h, subsequently immersed in hot water (60–70 °C), and finally fixed in Railliet–Henry's fluid (5% formalin, 2% acetic acid, and 0.6% aqueous sodium chloride). Vouchers of muscle tissue were obtained and preserved in 96% ethanol for molecular analyses. Seven shell measurements were taken (Fig. 1): total length (TL), penultimate whorl length
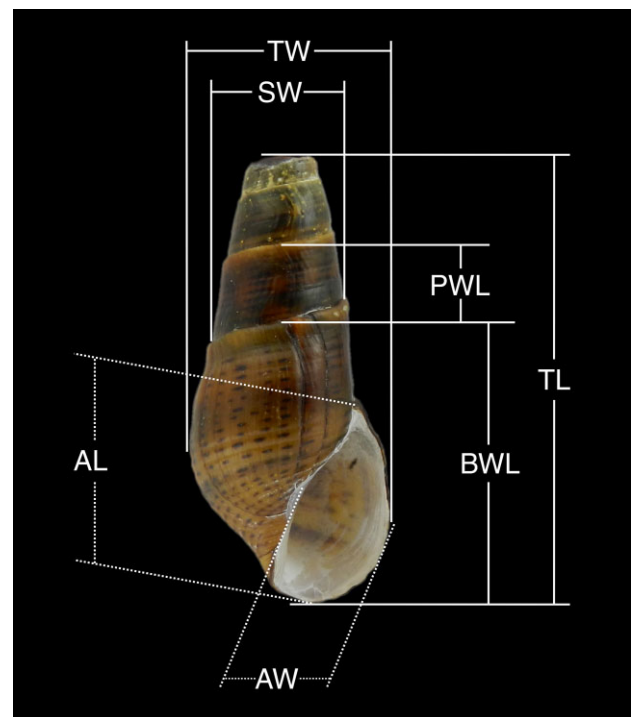

Figure 1. Shell measurements used for *Aylacostoma* species. Abbreviations: AL, aperture length; AW, aperture width; BWL, length of the body whorl; PWL, penultimate whorl length; SW, spire width; TL, total length; TW, total width.

(PWL), length of the body whorl (BWL), aperture length (AL), total width (TW), aperture width (AW), and spire width (SW). For the anatomical methodology in general we followed Simone (2001). Anatomical descriptions were based on living and preserved relaxed specimens. When necessary, a 0.1% neutral red solution was used to enhance the contrast of tissues. Internal features were examined under a Leica MZ6 stereoscopic microscope. The general descriptive format follows Simone (2001) and the terminology used follows that of Hylton Scott (1953, 1954) and Simone (2001, 2011).

Protoconchs, operculae, and radulae were also examined with a scanning electron microscope (SEM–JEOL 6360). The radulae were separated from the buccal mass and cleaned following the method described by Holznagel (1998). The terminology used for radular description follows Vogler (2013). The radular formula is described as in Gomez *et al*. (2011): rachidian (number of left side cusps/median cusp/number of right side cusps); lateral teeth (inner cusps/pronounced cusp/outer cusps); marginal teeth (number of cusps on inner marginal tooth, and number of cusps on outer marginal tooth).

A cetyltrimethylammonium bromide protocol (Gutiérrez Gregoric *et al*., 2013) was used to extract genomic DNA from foot samples of specimens belonging to *A. chloroticum*, *Aylacostoma brunneum* sp. nov., and outgroup species: *Doryssa* sp., *Pachychilus nigratus* (Poey, 1858), and *Pachychilus laevissimus* (Sowerby, 1825). Partial sequences of the mitochondrial *cytochrome oxidase subunit I* (*COI*) and *cytochrome b* (*Cyt b*) genes were amplified by means of the universal primers under the conditions detailed in Table 1. Success of PCR reactions was verified by agarose gel electrophoresis. *COI* products were purified using an AxyPrep PCR Clean-up Kit (Axygen Biosciences, Union City, California). Owing to the co-amplification of nonspecific fragments, *Cyt b* products were purified from 1.5% (w/v) agarose gels through the use of a Zymoclean Gel DNA Recovery Kit (Zymo Research, Orange, California). After purification, both DNA strands for each gene were then directly cycle sequenced (Macrogen Inc., Seoul, Korea). The resulting sequences were trimmed to remove the primers, and the consensus sequences of the individuals were compared with reference sequences in GenBank through the use of the BLASTN algorithm (Altschul *et al*., 1990) to identify similar sequences. Subsequently, a sequence alignment was performed separately for each mitochondrial region with the CLUSTAL X 2.0.12 software (Larkin *et al*., 2007), which was optimized by visual inspection and edited with a word processor.

Phylogenetic analyses were conducted in order to identify well-supported sister clades that could be potentially different species under the EGSC, namely the K/θ method (Birky *et al*., 2010, 2011; Schön *et al*., 2012; Birky, 2013). This method, based on population genetic theory, has recently been used to successfully describe new species in a wide range of asexual organisms (e.g. Birky *et al*., 2011; Martens, Halse & Schön, 2012; Shearn *et al*., 2012). The application of the species criteria was carried out following the procedure as described in Schön *et al*. (2012) and Birky (2013): once we identified well-supported sister clades, we then estimated the mean pairwise uncorrected sequence difference d for each clade. Subsequently, we estimated the nucleotide diversity $\pi = dn/(n - 1)$, where n is the number of sequences in the clade. When $d = 0$, we calculated d as if one sequence differed from the others at one site by $d = 2/Ln$, where L is the sequence length.

Table 1. Primers and reaction conditions applied for the two mitochondrial markers used in this study

Primer	Reference	Sequence 5′–3′
COI		
LCO1490	Folmer *et al*., 1994	GGT CAA CAA ATC ATA AAG ATA TTG G
HCO2198	Folmer *et al*., 1994	TAA ACT TCA GGG TGA CCA AAA AAT CA
Master mix (final volume 50 µl): 50–100 ng template DNA, 0.1 µM of each primer, 1× PCR buffer, 50 µM dNTPs, 1.5 mM MgCl$_2$, and 1.2 U Platinum *Taq* polymerase (Invitrogen, Brazil)		
PCR conditions: 1 min at 94 °C; five cycles of 1 min at 94 °C, 40 s at 45 °C, 1 min at 72 °C; 35 cycles of 30 s at 94 °C, 40 s at 51 °C, 1 min at 72 °C; final extension for 10 min at 72 °C		
Cyt b		
UCYTB151F	Merritt *et al*., 1998	TGT GGR GCN ACY GTW ATY ACT AA
UCYTB270R	Merritt *et al*., 1998	AAN AGG AAR TAY CAY TCN GGY TG
Master mix (final volume 50 µl): 50–100 ng template DNA, 0.5 µM of each primer, 1× PCR buffer, 0.2 mM dNTPs, 1.5 mM MgCl$_2$, and 1 U Platinum *Taq* polymerase (Invitrogen, Brazil)		
PCR conditions: 4 min at 94 °C; 38 cycles of 40 s at 94 °C, 40 s at 48 °C, 1 min at 72 °C; final extension for 6 min at 72 °C		

Cyt b, cytochrome b; *COI*, cytochrome oxidase subunit I; dNTPs, deoxyribonucleotide triphosphates.

The parameter θ was estimated by $\theta = \pi/(1 - 4\pi/3)$. We then calculated the mean pairwise sequence difference between the pair of sister clades. As the pairwise sequence differences were small, we used the uncorrected sequence difference D instead of K (which is D corrected for multiple hits), and calculated the ratio D/θ as a close approximation to K/θ (Shearn *et al.*, 2012; Birky, 2013). In order to fulfil the K/θ criteria, sister clades must have $K/\theta \geq 4$ to be considered different species with probability ≥ 0.95 (Birky, 2013 and references therein).

Given that we obtained *Cyt b* sequences for only a few individuals we just calculated a pairwise distance matrix for this region, and only *COI* data were subjected to phylogenetic analyses by the methods of neighbour-joining (NJ) and Bayesian inference (BI). The NJ analysis was conducted with 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). The statistical support for the resulting phylogeny was assessed by bootstrapping with 1000 replicates (Felsenstein, 1985). The BI was carried out with the MrBayes 3.2 software (Ronquist *et al.*, 2012). Two runs were performed simultaneously with four Markov chains that went for 1 000 000 generations, sampling every 100 generations. The first 10 000 generations of each run were discarded as burn-in, and the remaining 18 000 trees were used to estimate posterior probabilities. Mean pairwise differences within and between sister clades were estimated with MEGA 5.05 using the numbers of differences (p) and 1000 bootstrap replicates.

RESULTS

Five novel sequences of 361 bp for *Cyt b* (*A. chloroticum* = 4; *A. brunneum* sp. nov. = 1) and 46 sequences of 658 bp for *COI* (*A. chloroticum* = 37; *A. brunneum* = 6; outgroup = 3) were obtained in the present study. BLAST searches identified *Cyt b* and *COI* sequences as similar to other freshwater gastropods, excluding possible contamination with DNA from other sources. Genetic distances for *Cyt b* and *COI* are shown in Table 2. For *COI*, the two different phylogenetic analyses recovered two well-supported sister clades with high posterior probabilities and bootstrap values (Fig. 2). As required for the K/θ method, genetic differences within and between the clades were estimated (Table 3). The D/θ ratio obtained was 137.97 and the EGSC criteria were clearly fulfilled. Thus, both clades were recognized as distinct evolutionary genetic species.

SYSTEMATICS

FAMILY THIARIDAE GILL, 1871

GENUS *AYLACOSTOMA* SPIX, 1827

Type species: Aylacostoma glabrum Spix, 1827, eastern Brazil.

AYLACOSTOMA CHLOROTICUM HYLTON SCOTT, 1954 (FIGS 3–6)

Aylacostoma chlorotica Hylton Scott, 1954: 45–48 (fig. 1); Castellanos, 1981: 10, 11, 17; Quintana, 1982:

Table 2. Uncorrected genetic distances for *Cyt b* and COI amongst *Aylacostoma* specimens

Cyt b	*Aylacostoma chloroticum* (KF918854)	*A. chloroticum* (KF918855)	*A. chloroticum* (KF918856)	*A. chloroticum* (KF918857)
A. chloroticum (KF918855)	0			
A. chloroticum (KF918856)	0	0		
A. chloroticum (KF918857)	0	0	0	
A. brunneum sp. nov. (KF918858)	0.0665	0.0665	0.0665	0.0665

COI	*A. chloroticum* (JF346878– JF346882)	*A. chloroticum* (JQ236692, JX203219)	***Aylacostoma brunneum* sp. nov.** (JQ236700–JQ236705)
A. chloroticum (JQ236681–JQ236691, JQ236693–JQ236699, JX203217, JX203218, JX203220–JX203225, JX244270, JX244272)	0	0.0015	0.0426
A. chloroticum (JX203226, JX244271)	0.0015	0	0.0441
A. brunneum sp. nov. (JQ236700–JQ236705)	0.0426	0.0441	–

GenBank accession numbers are indicated in parentheses.
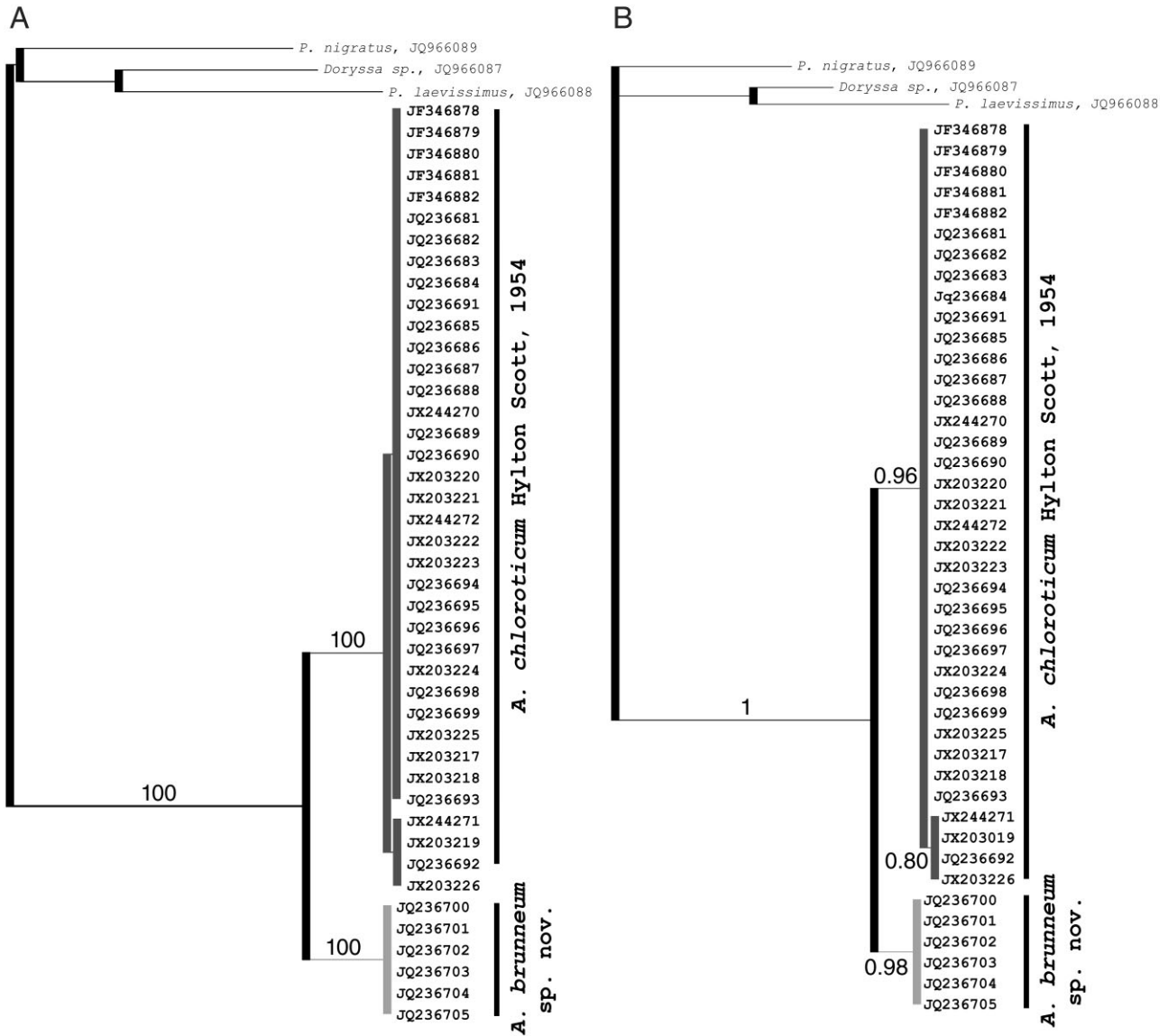*Cyt b*, cytochrome b; *COI*, cytochrome oxidase subunit I.

Figure 2. Phylogenetic trees of *Aylacostoma* (*A.*) specimens based on 658 nucleotides of the partial *cytochrome oxidase subunit I* gene. A, neighbour-joining (NJ) tree. B, Bayesian consensus tree. The support values: bootstrap values (NJ) and posterior probabilities (Bayesian inference) are shown above and below the branches. The trees show two well-supported clades (each in a different grey), corresponding to the species described in this study. Numbers within clades are GenBank accession numbers. Abbreviation: *P.*, *Pachychilus*.

Table 3. D/θ ratio calculations for *Aylacostoma cytochrome oxidase subunit I* sequences

	θ	D	D/θ ratio	n1, n2
Aylacostoma chloroticum – **Aylacostoma brunneum** sp. nov.	0.0003096*	0.0427174	137.97	37, 6

*Birky (2013) suggested using the larger value of θ. However, we used the smaller value because it was based on real data; the larger value was estimated from a clade of six identical individuals by supposing that one sequence differed from each of the others at one site.

θ, mean pairwise sequence difference within a clade; D, mean pairwise sequence difference between sister clades; n1, number of sequences within the *Aylacostoma chloroticum* clade; n2, number of sequences within the *Aylacostoma brunneum* sp. nov. clade.
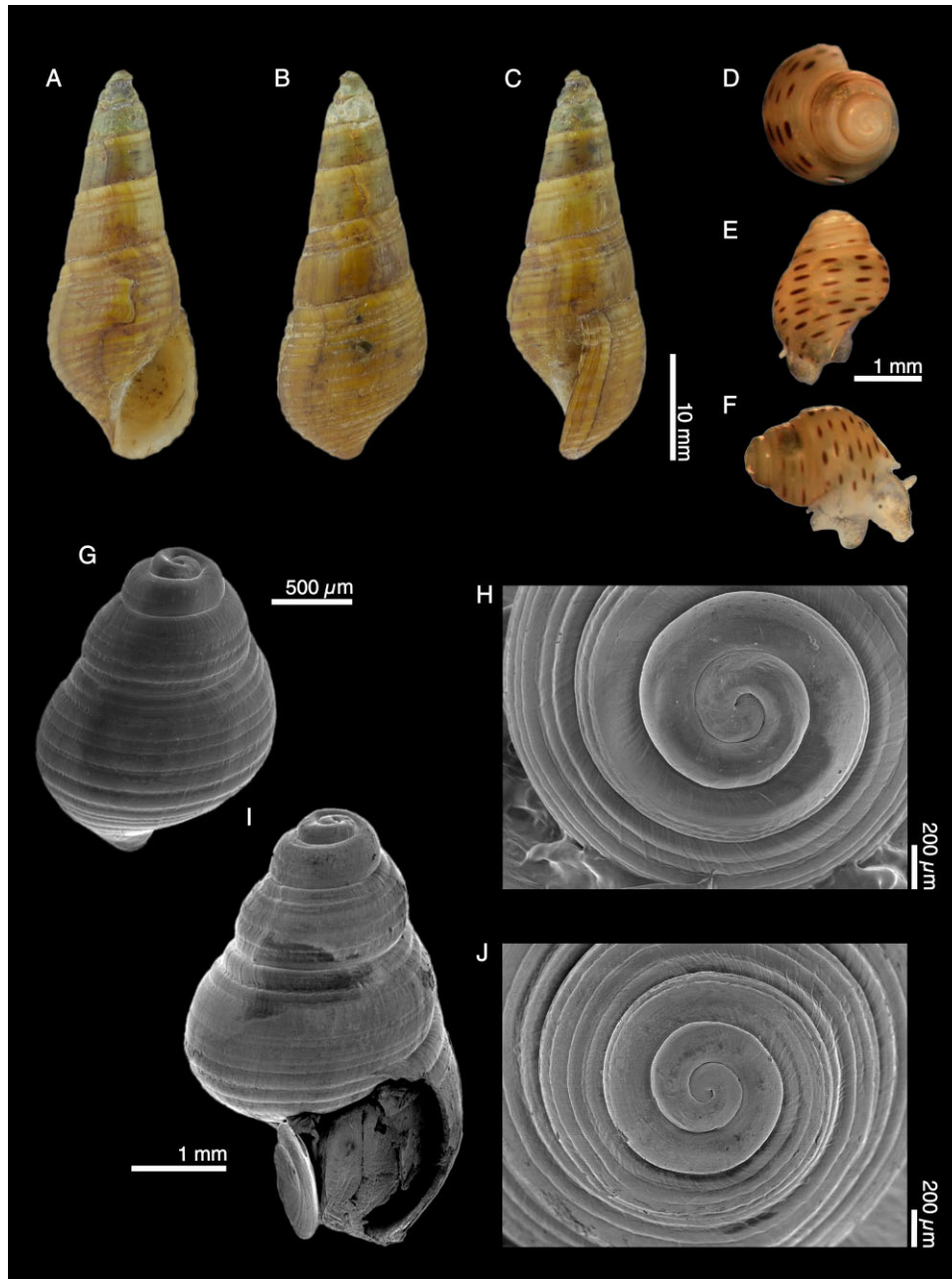
Figure 3. Shell of *Aylacostoma chloroticum*. A–C, holotype (MLP 10958): A, frontal, B, dorsal, and C, lateral view. D–J, juveniles from brood pouch and protoconch views. D–F, stereoscopic microscope: D, apical–lateral, E, dorsal, and F, lateral view. G–J, scanning electron micrographs: G, dorsal, I, frontal, and H, J, apical view.

118; Castellanos & Landoni, 1995: 774, 796 (fig. 34); Rumi *et al*., 2004: 214; Simone, 2006: 12, 79 (fig. 185).

Aylacostoma chloroticum – Cazzaniga, 1992: 303; Quintana & Mercado Laczkó, 1997: 26 (fig. 4); Mansur, 2000c; Quintana, Peso & Pérez, 2001: 110; Gutiérrez Gregoric *et al*., 2006: 54, 57; Rumi *et al*., 2006: 199, 204; Gutiérrez Gregoric *et al*., 2007: 109; Ostrowski de Núñez & Quintana, 2008: 647, 648, 652–654 (fig. 1);

Rumi *et al*., 2008: 80; Núñez, Gutiérrez Gregoric & Rumi, 2010: 50, 58; Cazzaniga, 2011: 178; Vogler, 2012: 43–45 (fig. 1); Vogler *et al*., 2012: *passim* (fig. 1b).

Hemisinus chloroticus – Martín & César, 2004: 15.

Type locality
'Isla Ibicuy, Alto Paraná, Paraguay'. At present, the Isla Ibicuy (27°16′–27°19′S, 56°04′–56°14′W), as well
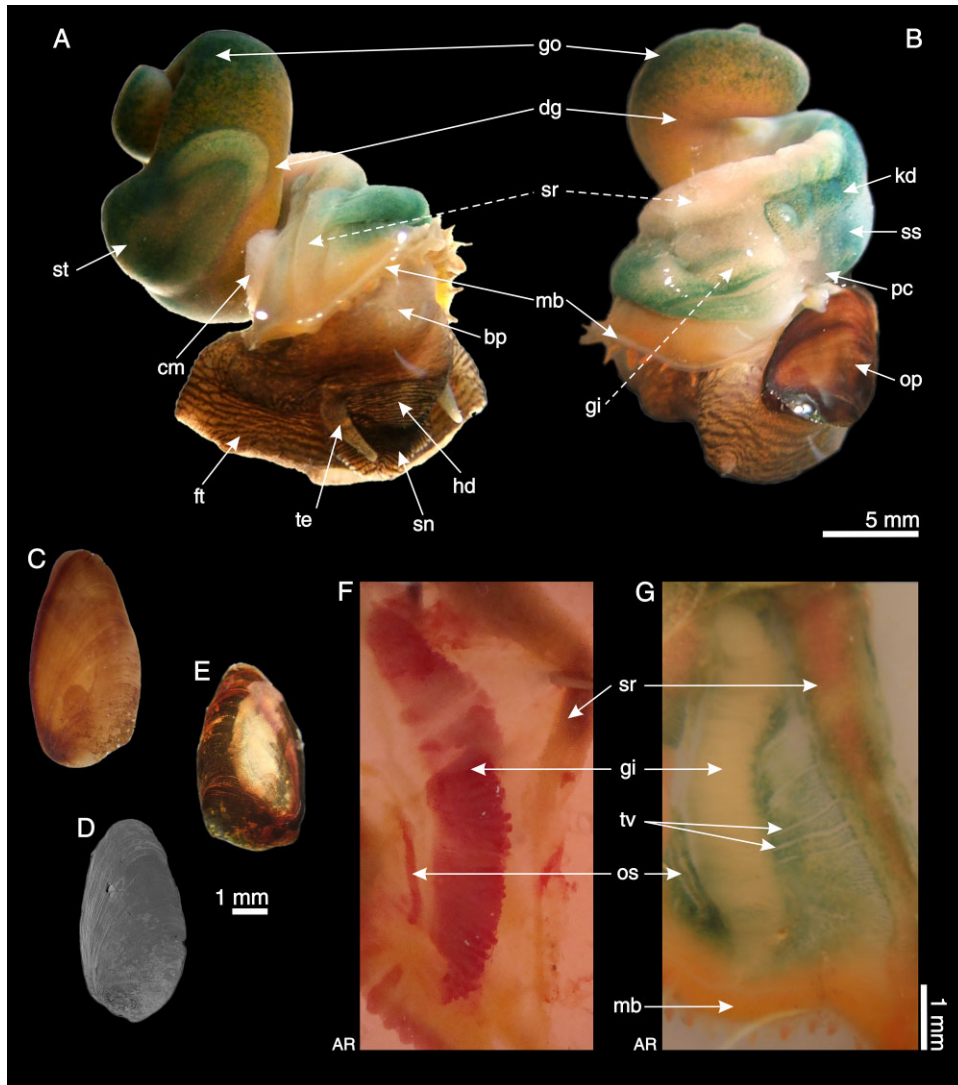
Figure 4. Anatomy of *Aylacostoma chloroticum*. A, B, specimen extracted from the shell: A, dorsolateral right view; B, ventrolateral left view. C–E, operculum: C, D, external view, stereoscopic microscope and scanning electron microscope, respectively; E, internal view, stereoscopic microscope. F, G, detail of mantle organs, inner-ventral view: F, fixed and stained with neutral red; G, live. Dashed arrows indicate internal structures. Abbreviations: AR, anterior region; bp, brood pouch; cm, columellar muscle; dg, digestive gland; ft, foot; gi, gill; go, gonad; hd, head; kd, kidney; mb, mantle border; op, operculum; os, osphradium; pc, pericardial chamber; sn, snout; sr, adrectal sinus; ss, style sac; st, stomach; te, cephalic tentacle; tv, transverse vessels between gill and adrectal sinus.

as many other islands of the High Paraná River between Argentina and Paraguay have disappeared because of damming and the filling up of the Yacyretá Reservoir in the early 1990s (Vogler, 2013).

Type material
Holotype: MLP 10958 (examined; dry shell from type locality; Fig. 3A–C); paratypes (all from type locality): MACN 488–1 (236 dry shells, examined); MLP 11596 (12 juvenile shells and two operculae, examined).

Other material examined
PARAGUAY. Itapúa; Río Beach (27°24′29.83′′S, 55°49′32.94′′W): MLP 13521 (one soft part in Railliet–Henry's fluid), 13522 (one soft part in Railliet–Henry's fluid), 13523 (six unbroken juvenile shells with soft parts in 96% ethanol), 13762 (three soft parts in Railliet–Henry's fluid); conservation programme at UNaM (seven living specimens). ARGENTINA. Corrientes; Apipé Rapids (27°28′57.41′′S, 56°44′0.82′′W): conservation programme at UNaM (five living specimens). Misiones; Posadas Port (27°21′43.10′′S,
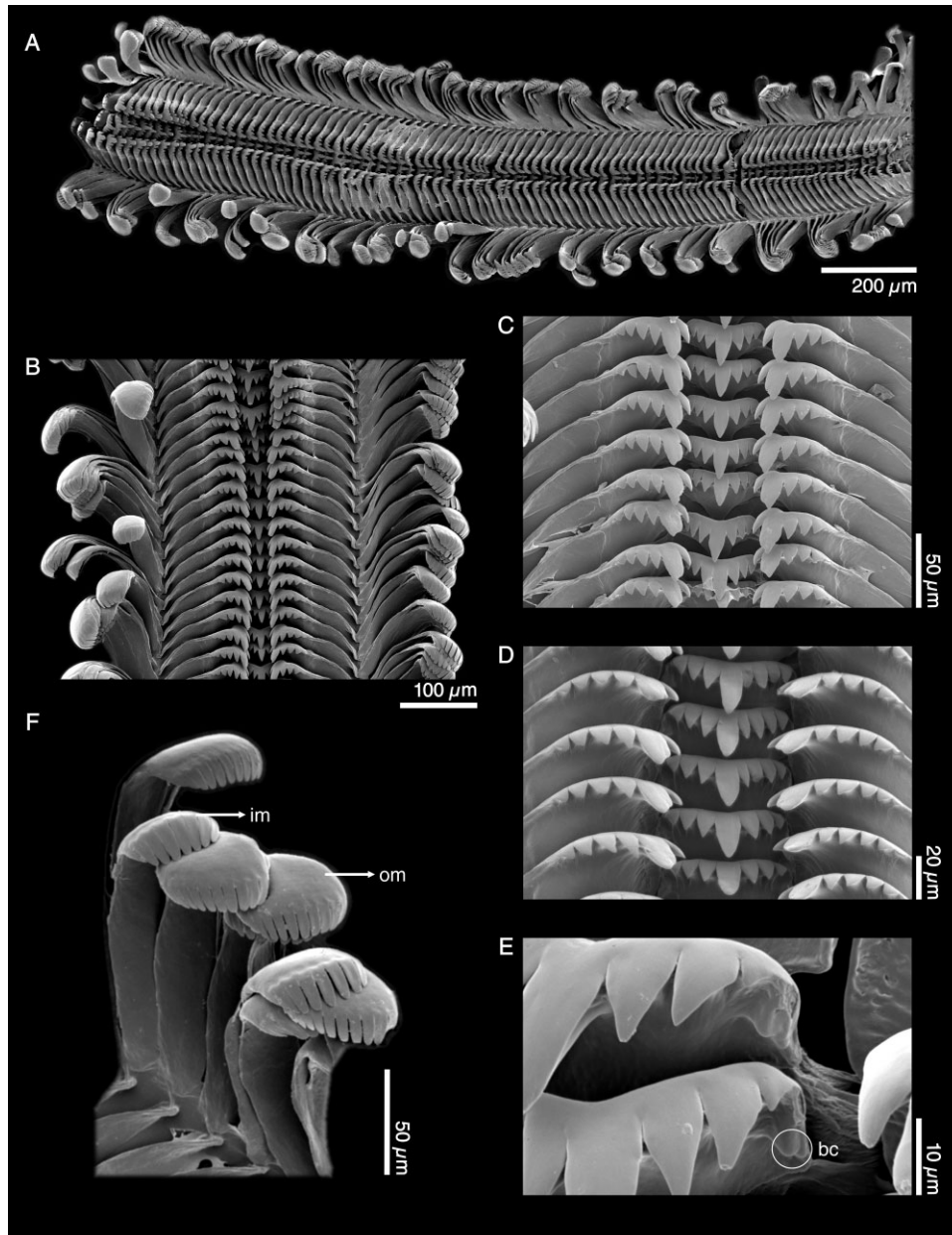
Figure 5. Scanning electron micrographs of radula of *Aylacostoma chloroticum*. A, radular ribbon. B, radular segment. C, D, detail showing central and lateral teeth. E, rachidian with basal cusps. F, marginal teeth. Abbreviations: bc, basal cusps; im, inner marginal; om, outer marginal.

55°53′5.20″W): conservation programme at UNaM (three specimens); San José Lagoon (27°22′11.40″S, 55°52′49.64″W): conservation programme at UNaM (ten living specimens); Candelaria (27°26′50.96″S, 55°45′0.84″W): conservation programme at UNaM (ten living specimens); mouth of San Juan Stream (27°25′0.95″S, 55°42′46.88″W): conservation programme at UNaM (two living specimens). *Additional material*: four lots from three localities, 36 shell speci-

mens at MLP; anatomical material from various individuals from Candelaria, Misiones, Argentina and Río Beach, Itapúa, Paraguay.

Taxonomic history

According to Hylton Scott's (1954) original description, the holotype and paratypes were deposited at the MACN under no. 488–1. Tablado & Mantinian (2004) reported that the holotype was not found amongst the
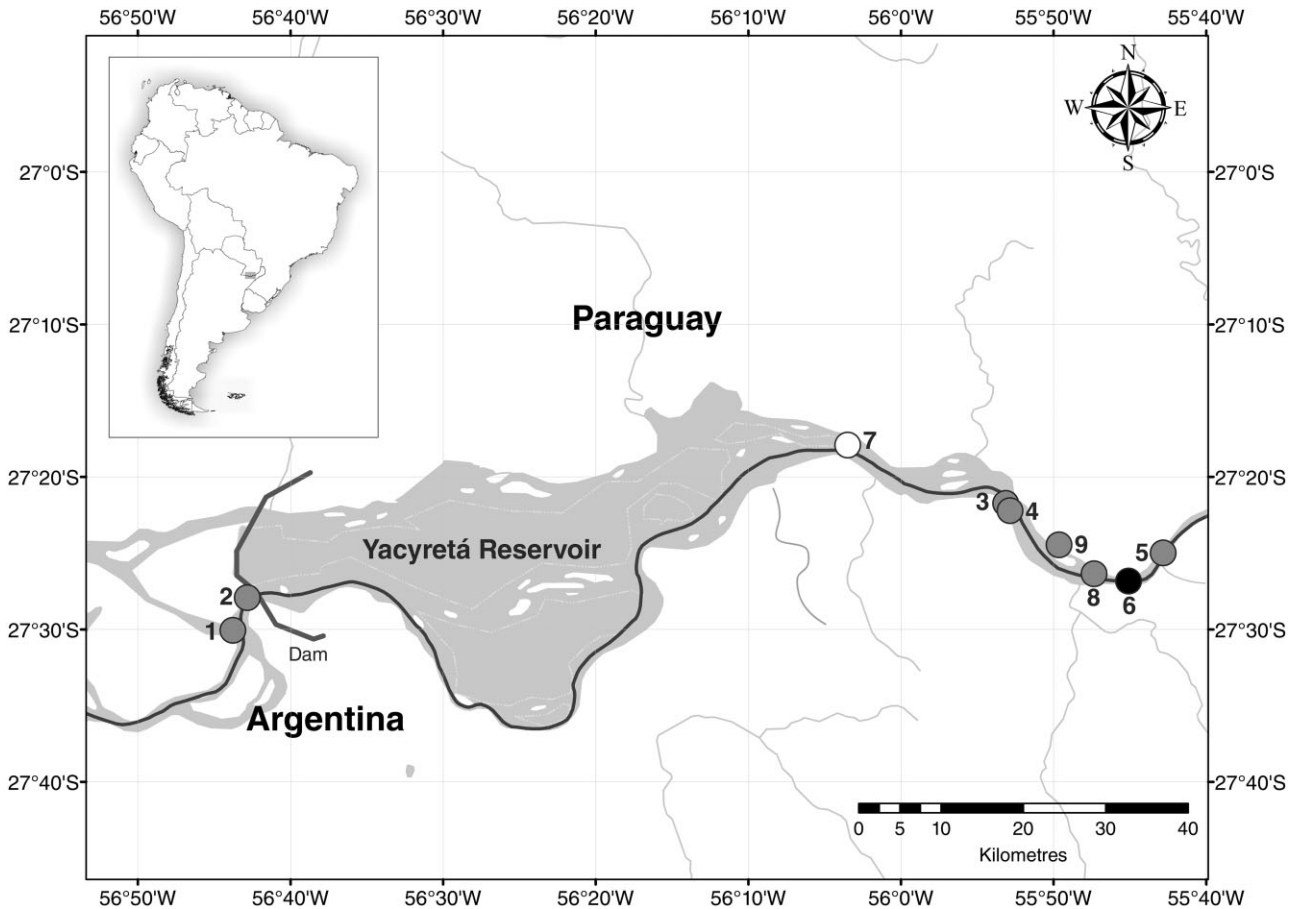
Figure 6. Distribution of *Aylacostoma chloroticum* in the High Paraná River. Type locality (no. 7) shown in white. Location numbers correspond to the numbers in Table 5. Localities 1, 2, 7, and 8: historical records prior to the damming and filling up of the Yacyretá Reservoir. Localities 3, 4, 5, 6, and 9: records obtained during the successive stages of the filling up of the reservoir. Locality 6 (shown in black): only known wild population after completion of the filling up process in 2011.

type specimens housed in the National Invertebrate Collection at the MACN. For unknown reasons the holotype is deposited in the Type Specimens Collection at the MLP under no. 10958. This specimen is the same as that measured and shown in Hylton Scott (1954), which was also illustrated in Simone (2006), and photographed in this study.

Description
Shell (Fig. 3): Shape conical to fusiform; strong; apex worn, sometimes completely eroded; large size, reaching up to at least 42 mm (Table 4). Colour: greenish-yellow to mid greenish-brown; some specimens bearing reddish dark brown, minute, spiral spots. Protoconch present only in juveniles (from brood pouch) with two whorls smooth, convex, rounded; spiral sculpture beginning gradually from third whorl, becoming sharp in successive whorls (Fig. 3G–J). From

embryonic stages juveniles always with delicate red wine colour spots, except for subsutural band, which has no decoration (Fig. 3D–F). Teleoconch with up to eight whorls, in most cases with three to five persistent whorls. Spire high with flat-convex whorls; body whorl moderately convex. Surface sculptured with spiral nearly flat cords somewhat irregular in each whorl, about six in penultimate whorl; narrow furrow amongst spiral cords. Some specimens with sculpture attenuated, up to whorls entirely smooth. Subsutural cord a little wider than other cords, sometimes lighter. Suture shallow, not excavated. Aperture elliptical, long, internally white, angled posteriorly, with shallow anterior canal.

Head–foot (Fig. 4A, B): Head, snout, tentacles, and dorsal region of foot light to mid-brown, with dark brown transverse striae. Snout large, dorsoventrally

Table 4. Shell measurements of *Aylacostoma chloroticum* from the holotype, and 17 living specimens included in the conservation programme taking place at Universidad Nacional de Misiones (UNaM; Posadas, Argentina)

	TL	TW	BWL	PWL	SW	AL	AW	NW	Location
Holotype, MLP 10958	34.78	13.43	19.38	5.74	9.46	14.35	6.10	6	Isla Ibicuy, PY
Specimen no. 1	41.59	16.13	20.95	6.22	10.43	16.02	7.07	8	San Juan St., AR
Specimen no. 2	40.49	14.5	19.91	5.82	10.18	15.32	7.12	7	San Juan St., AR
Specimen no. 3	33.59	11.20	17.08	5.19	8.11	12.86	5.66	7	Candelaria, AR
Specimen no. 4	32.07	12.12	17.90	4.60	8.35	13.17	6.20	6	Candelaria, AR
Specimen no. 5	30.11	14.05	19.29	5.25	9.33	14.88	6.55	4	Candelaria, AR
Specimen no. 6	29.54	10.20	15.92	3.45	7.26	12.17	5.26	6	Candelaria, AR
Specimen no. 7	29.29	10.40	16.63	4.04	7.14	13.01	5.10	6	Candelaria, AR
Specimen no. 8	29.20	11.47	17.64	4.56	8.34	12.52	5.29	5	Candelaria, AR
Specimen no. 9	26.72	9.82	15.89	4.02	6.97	11.76	5.01	5	Candelaria, AR
Specimen no. 10	24.96	10.19	15.38	4.04	7.20	11.30	5.68	5	Candelaria, AR
Specimen no. 11	24.68	8.65	14.02	3.86	6.49	9.81	4.67	5	Candelaria, AR
Specimen no. 12	23.70	8.72	13.80	3.58	6.33	10.06	4.72	5	Candelaria, AR
Specimen no. 13	22.84	10.28	15.27	4.08	6.87	11.42	5.80	4	Río Beach, PY
Specimen no. 14	22.20	10.14	14.88	3.81	6.82	10.74	5.80	3	Río Beach, PY
Specimen no. 15	21.45	9.60	14.08	4.09	6.50	10.45	5.43	4	Río Beach, PY
Specimen no. 16	21.01	9.14	14.40	3.60	6.22	10.99	5.27	3	Río Beach, PY
Specimen no. 17	20.90	8.95	13.67	3.73	6.33	10.64	5.13	4	Río Beach, PY

Measurements in mm.
TL, total length; TW, total width; BWL, length of body whorl; PWL, penultimate whorl length; SW, spire width; AL, aperture length; AW, aperture width; NW, number of whorls; AR, Argentina; PY, Paraguay.
References to localities are provided in the main text in the Other material examined section for the species.

flattened, anterior margin somewhat bilobed. Tentacles long, thick, eyes on outer side of ocular peduncles. Foot of medium size, without divisions; anterior pedal gland a furrow on anterior edge of foot. Brood pouch situated on posterior region of head, extending into neck region; several specimens with developing embryos inside. Aperture of brood pouch ventrally situated near right fusion of mantle to head–foot. Columellar muscle of about one and a half whorls.

Operculum (Fig. 4C–E): Ovate, corneous, brown, paucispiral with nucleus subterminal, externally lamellate. Muscle scar elliptical, near inner margin.

Mantle organs: Mantle border with small papillae (Fig. 4A), more developed at both extremities. Mantle cavity of about two whorls. Osphradium conspicuous, ridge-like, lying on left side of mantle cavity, less than half of gill length; anterior extremity posterior to that of gill (Fig. 4F, G). Gill very long, almost same length as pallial cavity; filaments triangular; ctenidial vein well developed. Area between gill and rectum without distinctive vascularization in all fixed specimens; but some living specimens with distinct transverse vessels; hypobranchial gland located in same area. Adrectal sinus proportionally broad with rectum running free inside.

Pallial oviduct running through right side of pallial cavity.

Digestive system: Mouth longitudinal, ventral, at anterior extremity of the snout. Buccal mass situated within snout, just behind mouth; oesophagus dorsal, odontophore ventral. Radula taenioglossate, short, S-shaped in side view, with about 93 rows of teeth ($N = 6$; Fig. 5A). Rachidian teeth pentagonal, broader than longer, with v-shaped base. All of rachidian bearing pointed apical cusps (3–4/1/4–3), central cusp noticeably longer, lanceolate (Fig. 5B–D); rachidian with two basal cusps at each outer corner (Fig. 5E). Lateral teeth long, in general with six to seven pointed cusps, second one larger, somewhat rectangular (1/1/4–5; Fig. 5B–D). Marginal teeth similar to one another, long, spatulate, narrow on base, with slender, fringe-like cusps. Inner marginal teeth with about eight cusps; outer marginals with about 11 thinner cusps (Fig. 5F). Stomach large, complex, about half whorl, separating almost completely digestive gland–gonad from pericardial and pallial structures. Style sac large, about half of stomach size. Style elliptical, transparent. Digestive gland of about two whorls, posterior to stomach. Intestine long, narrow, with dorsal loop on style sac. Rectum broad, with several inner folds. Anus siphoned, in right extremity of mantle border, posterior to anterior end of pallial oviduct.

Table 5. Historical records of *Aylacostoma chloroticum* in Argentina and Paraguay

Country	No.	Locality	Year	Latitude	Longitude	Reference
Argentina	1	Isla Apipé Grande	1981	27°30′5.4″S	56°43′45.48″W	Castellanos, 1981
	2	Apipé Rapids	1994	27°28′57.41″S	56°44′0.82″W	Quintana & Mercado Laczkó, 1997
	3	Posadas Port	2006	27°21′43.10″S	55°53′5.20″W	This work
	4	San José Lagoon	2008	27°22′11.40″S	55°52′49.64″W	This work
	5	San Juan Stream (mouth)	2009	27°25′0.95″S	55°42′46.88″W	This work
	6	Candelaria	2006–2013	27°26′50.96″S	55°45′0.84″W	Vogler, 2012; this work
Paraguay	7	Isla Ibicuy*	1954	27°17′56.76″S	56°3′28.44″W	Hylton Scott, 1954
			1982			Quintana, 1982
	8	Campichuelo	1982	27°26′20.04″S	55°47′18.24″W	Quintana, 1982
	9	Río Beach	2007	27°24′29.83″S	55°49′32.94″W	This work

*Type locality. The coordinates of localities 1, 2, 7, and 8 are approximations; only the localities' names were reported in the consulted sources and the precise locations are uncertain.

Circulatory and excretory systems: Heart situated posteriorly in pallial cavity behind gill; auricle somewhat triangular, large, anterior; ventricle spherical, small, posterior. Kidney proportionally large, situated posterior to pallial cavity, limited behind by digestive gland and style sac, anterior end extending into mantle roof.

Genital system: Only females found. Ovary developed in largest specimens, occupying the superior regions of each whorl on digestive gland. Pallial oviduct complex, anterior extremity posterior to anus, close to aperture of brood pouch. Brood pouch a bulging sac situated behind head (Fig. 4A). Surface internally smooth, without conspicuous folds. Up to 13 juveniles within brood pouch occurring at different growth stages, from one to three whorls; mucus not visible around juveniles, but with transparent membrane present in one- and two-whorled specimens. Aperture of brood pouch sphincter-like.

Distribution
Neotropical species endemic to the High Paraná River, between Argentina and Paraguay. Its historical distribution was within the Argentinean Misionerean Gastropod Province (Núñez, Gutiérrez Gregoric & Rumi, 2010), and comprised a narrow geographical range between 56°50′W and 55°40′W longitude (< 150 km; Table 5, Fig. 6). The area was characterized by numerous small islands, shallow waters, and rapids (Quintana & Mercado Laczkó, 1997). The damming and filling up of the Yacyretá Reservoir (Argentina–Paraguay) from 1993 to 2011 had a negative impact on the distribution of the species, which is currently restricted to only one known wild population in Candelaria city (Misiones, Argentina). The relict population occurs in a small and fragile habitat under several anthropogenic threats because of the construction of an artificial beach for human recreation in the area.

DNA barcoding
Partial sequences of mitochondrial *COI* (*N* = 37) and *Cyt b* (*N* = 4) genes for specimens from several locations have been deposited in GenBank under the following accession numbers: *COI*: JF346878–JF346882; JQ236681–JQ236699; JX203217–JX203226; JX244270–JX244272. *Cyt b*: KF918854–KF918857.

Parasitism
Stephanoprora aylacostoma, a digenean species was recently described from infected specimens (Ostrowski de Núñez & Quintana, 2008). This parasite is frequently found in the gonads; but in heavy infections cercaria may also be numerous in the pallial cavity.

Conservation status
Representatives of several wild populations that were extinct are successfully being reproduced in captivity within the '*Aylacostoma* Project', the *ex situ* conservation programme developed in the 1990s being conducted by the UNaM and the MACN, and supported by EBY. *Aylacostoma chloroticum* is listed by IUCN as 'Extinct in the Wild' (Mansur, 2000c).

AYLACOSTOMA BRUNNEUM VOGLER & PESO **SP. NOV.**
(FIGS 7–11)

Type material
Holotype (Fig. 7A–E; Table 6): MLP 13757, iii.2007, Peso, J.G. col., female, 24.98 mm, dissected, voucher pedal tissue preserved in the tissue collection at the Museo de La Plata. Paratypes (Fig. 7F–Q; Table 6): MLP 13758 (one dry shell from type locality), 13613 (15 dry shells and two unbroken shells with soft parts in 96% ethanol
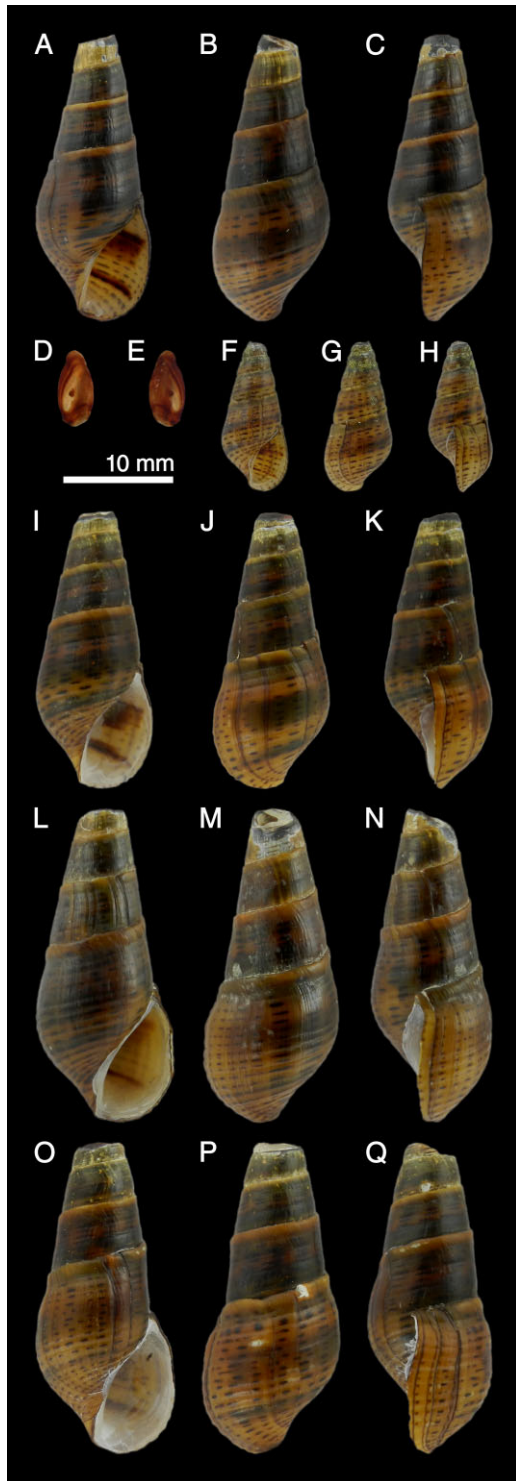
Figure 7. *Aylacostoma brunneum* **sp. nov.** A–E, holotype (MLP 13757). F–H, paratype no. 16, juvenile (MLP 13613). I–K, paratype no. 12 (MLP 13613). L–N, paratype no. 1 (MLP 13613). O–Q, paratype no. 7 (MLP 13613). A, F, I, L, O, frontal; B, G, J, M, P, dorsal; C, H, K, N, Q, lateral view. Measurements in Table 6. Operculum: D, external view; E, internal view.

from Ita Cuá, High Paraná River; Itapúa; Paraguay; 27°24′42.13″S, 55°48′45.69″W).

Type locality
High Paraná River; Paraguay, Itapúa, Río Beach, 27°24′29.83″S, 55°49′32.94″W.

Other material examined
PARAGUAY, Itapúa; Río Beach (27°24′29.83″S, 55°49′32.94″W): MLP 13759 (one juvenile shell from brood pouch of a specimen from the type locality), conservation programme at UNaM (four living specimens); Ita Cuá (27°24′42.13″S, 55°48′45.69″W): MLP 13761 (two soft parts in Railliet–Henry's fluid), conservation programme at UNaM (four living specimens born in captivity from wild progenitors). *Additional material:* Three lots from two localities containing five shell specimens and two soft parts at MLP; descriptive anatomy from several individuals from type locality and Ita Cuá.

Etymology
Allusion to the shell colour. From the Latin word '*brunneum*', meaning 'brown'. Grammatical gender: neuter.

Diagnosis
Shell dark brown with alternating lighter brown bands, decorated by reddish dark brown, minute, spiral spots; surface with opaque sheen. Osphradium conspicuous, almost half of gill length; area between gill and adrectal sinus with distinctive transverse vessels. Rachidian with tip of central cusp constricted, dagger-like. *COI*: GenBank JQ236705. *Cyt b*: GenBank KF918858.

Description
Shell (Figs 7, 8): Shape conical to ovate; strong; apex worn, sometimes completely eroded; medium size, reaching up to at least 28 mm (Table 6). Periostracum persistent. Colour: dark brown with alternating lighter bands, delicately decorated by reddish dark brown, minute, spiral spots regularly spaced in all specimens (Fig. 7). Protoconch present only in juveniles (from brood pouch) with two whorls almost smooth, convex, rounded; spiral sculpture beginning gradually from third whorl, with dark spiral spots, quite regularly spaced (Fig. 8). Teleoconch with up to five whorls, in most cases with three persistent whorls. Spire high, profile of whorls flattened, body whorl moderately convex. Surface with opaque sheen; spiral sculpture weak, almost absent on spire, body whorl sculptured by broad spiral cords, uniform in size with narrow and shallow furrow between; suture narrow and shallow, not excavated; small furrow running parallel to suture clearly limiting lighter subsutural cord, somewhat broad.
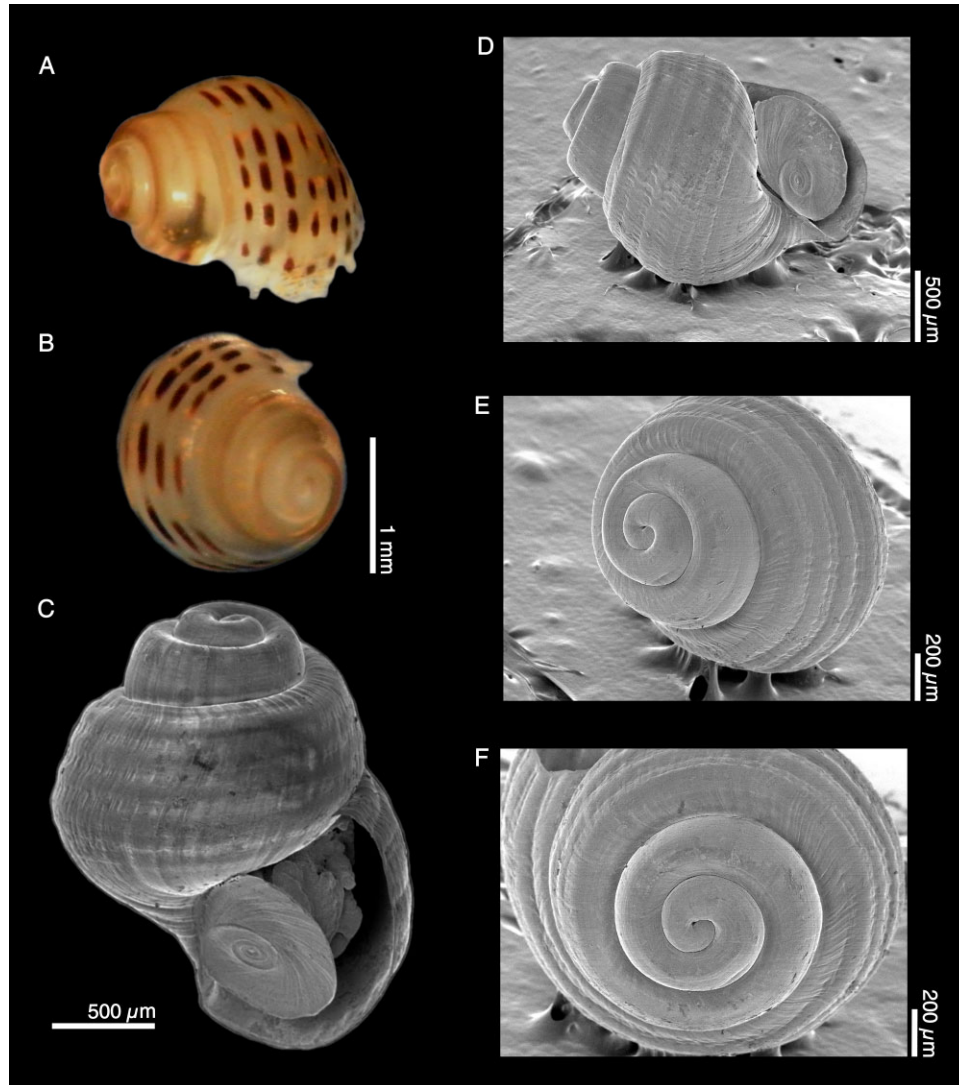
Figure 8. Juvenile of *Aylacostoma brunneum* **sp. nov.** from brood pouch and protoconch views (MLP 13759); A, B, stereoscopic microscope: A, lateral–dorsal, B, apical–lateral view. C–F, scanning electron micrographs: C, D, frontal, E, apical–lateral, F, apical view.

Aperture vertically elongated, angled posteriorly, with anterior canal defined. Outer lip D-shaped, curvature projecting towards base. When not calcified, aperture glossy with dark, linear spiral spots showing through; some specimens with two darker bands similar to slats, ending just before the edge.

Head–foot: Similar to that of preceding species. Transverse striae in cephalic region comparatively darker.

Operculum (Figs 7D, E; 8C, D): Similar to that of preceding species.

Mantle organs: Similar to those of preceding species. Comparatively, more transparent mantle. Osphradium length approximately half of gill length. Area between gill and adrectal sinus also with distinctive transverse vessels (Fig. 9).

Digestive system: Characters similar to previous species. Radula short, with about 94 rows of teeth ($N = 3$; Fig. 10A). Rachidian teeth pentagonal, broader than longer, with v-shaped base. All of rachidian bearing pointed apical cusps (3–4/1/4–3); central cusp noticeably longer, lanceolate, with tip constricted, dagger-like (Fig. 10B–E); rachidian with two basal cusps at each outer corner (Fig. 10E). Lateral teeth long, in general with six pointed cusps, main cusp larger, somewhat rectangular, with asymmetrically lobate margin and small posterior projection (1/1/4; Fig. 10B–D); teeth
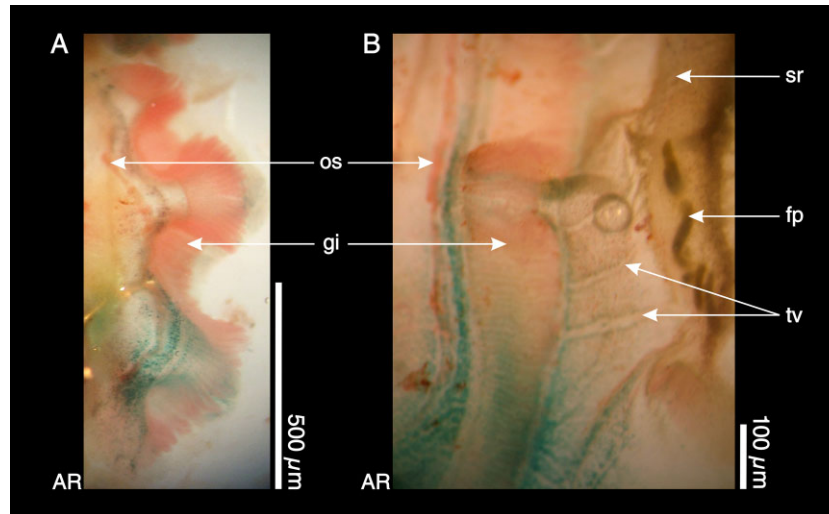
Figure 9. Mantle organs of *Aylacostoma brunneum* **sp. nov.**, inner–ventral view. A, gill and osphradium. B, detail of transverse vessels between the gill and adrectal sinus. Abbreviations: AR, anterior region; fp, faecal pellet; gi, gill; os, osphradium; sr, adrectal sinus; tv, transverse vessels between gill and adrectal sinus.

Table 6. Shell measurements of the type series of *Aylacostoma brunneum* **sp. nov.**

	Collection	TL	TW	BWL	PWL	SW	AL	AW	NW
Holotype	MLP13757	24.98	9.99	15.78	3.98	7.25	11.58	4.86	4
Paratype no. 1	MLP13613	26.95	11.28	17.41	4.70	8.09	12.61	5.80	4
Paratype no. 2	MLP13613	27.89	12.38	18.41	4.39	9.24	13.72	6.51	4
Paratype no. 3*	MLP13613	13.47	6.32	9.52	2.15	4.66	6.80	3.61	3
Paratype no. 4	MLP13613	23.63	11.47	17.33	4.57	7.90	11.99	5.56	3
Paratype no. 5	MLP13613	23.56	11.80	17.25	4.47	8.47	11.56	6.50	3
Paratype no. 6	MLP13613	18.03	9.29	13.99	3.99	6.66	10.03	5.10	3
Paratype no. 7	MLP13613	26.49	11.41	17.05	4.86	8.07	12.18	5.95	4
Paratype no. 8	MLP13613	24.96	10.65	16.27	4.31	7.54	12.13	5.34	4
Paratype no. 9	MLP13613	25.09	10.86	16.94	4.06	7.60	11.84	5.57	4
Paratype no. 10	MLP13613	25.60	9.98	16.04	4.01	7.21	11.74	4.94	5
Paratype no. 11	MLP13613	24.84	9.86	15.96	3.89	7.12	11.24	5.12	5
Paratype no. 12	MLP13613	24.20	9.77	15.82	4.13	7.03	11.42	5.18	4
Paratype no. 13	MLP13613	21.21	8.56	13.56	3.35	6.41	9.80	4.70	5
Paratype no. 14	MLP13758	21.94	9.01	14.80	3.57	6.52	10.75	4.90	4
Paratype no. 15	MLP13613	20.00	9.26	14.03	4.03	6.72	10.25	4.83	3
Paratype no. 16*	MLP13613	13.30	6.06	8.68	2.45	4.18	6.13	3.05	4
Paratype no. 17*	MLP13613	13.53	6.04	9.32	2.37	4.46	6.61	3.38	3
Paratype no. 18*	MLP13613	14.74	7.01	10.08	2.82	4.90	7.31	3.60	3

Measurements in mm.
TL, total length; TW, total width; BWL, length of body whorl; PWL, penultimate whorl length; SW, spire width; AL, aperture length; AW, aperture width; NW, number of whorls.
*juvenile specimens.

of some rows with cusps showing different levels of fusion, occasionally developing bifid or trifid points. Marginal teeth similar to one another, long, spatulate, narrow on base, with slender, fringe-like cusps. Inner marginal teeth with about eight cusps; outer marginals with about 11 thinner cusps (Fig. 10F). Rectum lacking transverse inner folds. Several faecal pellets inside rectum oblique compacted (Fig. 9B).

Circulatory and excretory systems: Heart and kidney characters and location similar to those of preceding species.
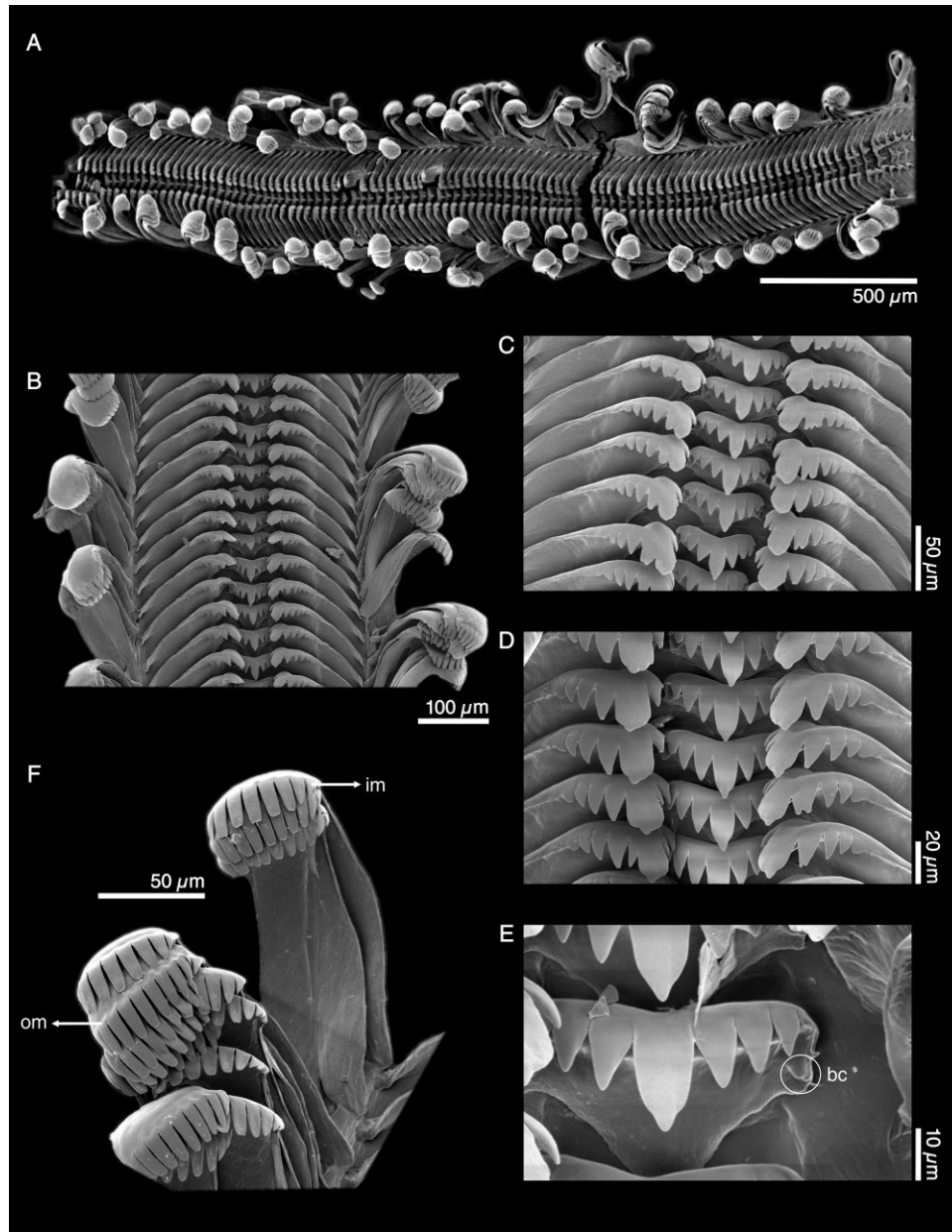
Figure 10. Scanning electron micrographs of radula of ***Aylacostoma brunneum* sp. nov.** A, radular ribbon. B, radular segment. C, D, detail showing central and lateral teeth. E, rachidian with basal cusps. F, marginal teeth. Abbreviations: bc, basal cusps; im, inner marginal; om, outer marginal.

Genital system: Similar to preceding species. Only females found, with up to four specimens within brood pouch.

Distribution (Fig. 11)
Endemic to High Paraná River at the border area between Argentina and Paraguay. This new species was recorded in 2007 from only two localities. As the main channel of the river is the boundary between the two countries, both localities territorially correspond to Para-

guay: Río Beach, Itapúa, 27°24′29.83″S, 55°49′32.94″W (type locality) and Ita Cuá, Itapúa, 27°24′42.13″S, 55°48′45.69″W. In 2011, these localities were flooded in the last filling stage of the Yacyretá Reservoir.

DNA barcoding
Partial sequences of mitochondrial *COI* and *Cyt b* genes of the holotype have been deposited in GenBank with accession numbers JQ236705 and KF918858, respectively. Additional partial *COI* sequences from other living
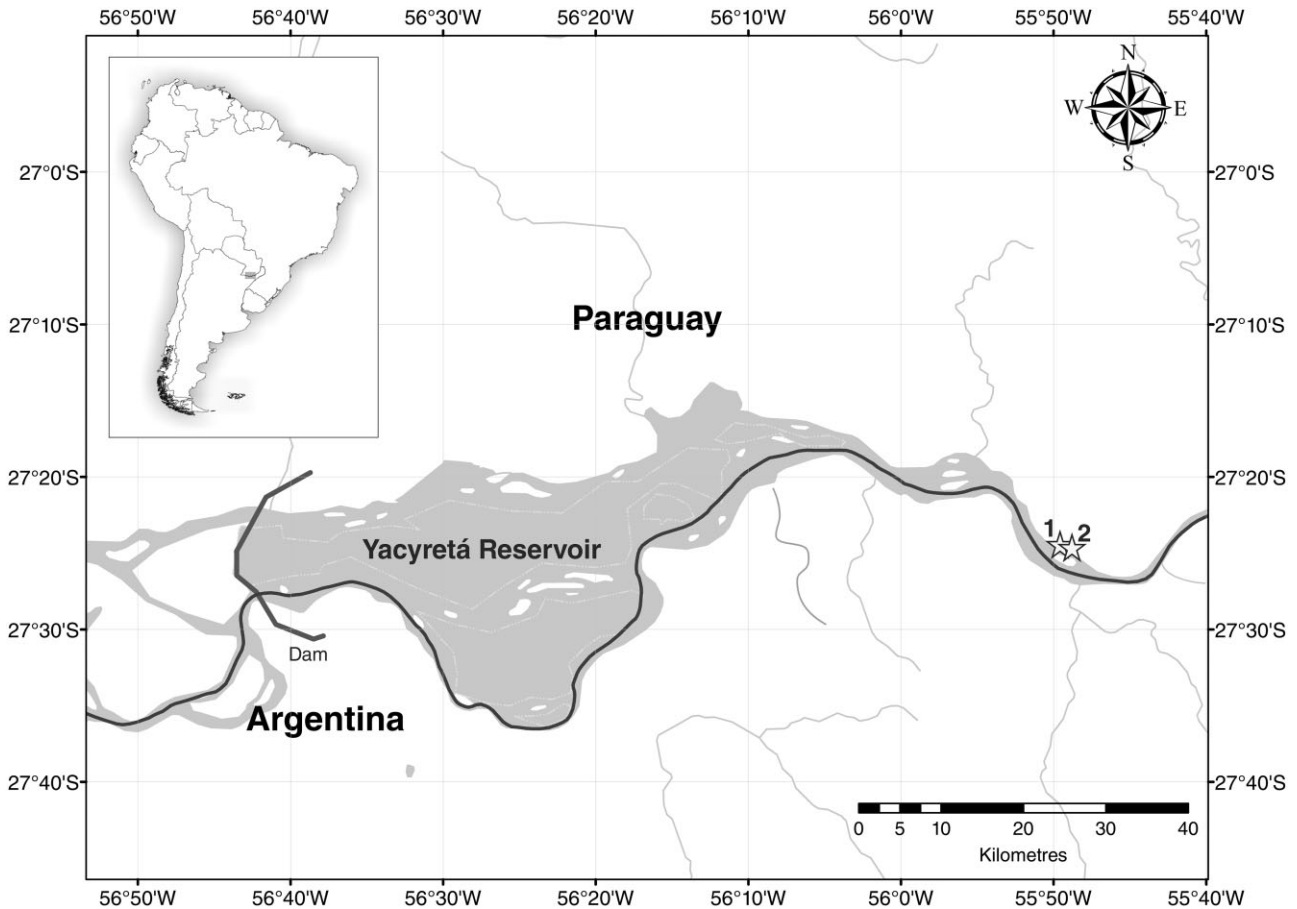
Figure 11. Known distribution of ***Aylacostoma brunneum* sp. nov.** in the High Paraná River. 1, Río Beach, Itapúa, Paraguay (type locality). 2, Ita Cuá, Itapúa, Paraguay.

specimens born in captivity at UNaM have been deposited in GenBank under accession numbers JQ236700–JQ236704.

Conservation status
Representatives of two known geographical populations are reproducing successfully in captivity within the '*Aylacostoma* Project' at UNaM. The status of the species in the wild remains uncertain, but the populations are assumed to have become extinct after the completion of the filling up process of the Yacyretá Reservoir in 2011. The inclusion of *A. brunneum* in the IUCN and Convention on International Trade in Endangered Species of Wild Fauna and Flora lists is recommended.

DISCUSSION

Nonmarine molluscs belong to the second most diverse animal phylum in terms of number of described species (Lydeard *et al*., 2004). The estimated number of nonmarine molluscs comprises about 24 000 terres-trial and 4000 freshwater valid described species (Lydeard *et al*., 2004; Strong *et al*., 2008). However, molluscs have the highest number of documented extinctions of any major taxonomic group (Lydeard *et al*., 2004; Strong *et al*., 2008). In particular, freshwater gastropods (approximately 5% of the world's gastropod fauna) are at disproportionately high risk of extinction (Strong *et al*., 2008). Of the 310 species listed as extinct in the 2013 IUCN Red List of Threatened Species (http://www.redlist.org), 73 (~23%) are gastropod species from inland waters. Moreover, when corrected for knowledge bias, the number of known extinctions is markedly increased (Régnier, Fontaine & Bouchet, 2009).

Argentina has a total of 101 recorded species of freshwater gastropods distributed in ten families, 40 of which have been recognized as endemic and 45 considered vulnerable (Rumi *et al*., 2006, 2008; Núñez *et al*., 2010). Of the latter, conservation strategies have been adopted only for the native thiarids from the High Paraná River in the context of the '*Aylacostoma* Project'. To our knowledge, this conservation breeding programme is the only one being developed for

freshwater gastropods in South America and constitutes an example of conservation success, similar to those programmes being developed for other seriously threatened snails of the Pacific islands, such as *Achatinella* and *Partula* (Pearce-Kelly *et al.*, 1997; Hadfield, Holland & Olival, 2004).

Within the framework of the aforementioned project, this study has described for the first time the morphological and genetic characters of two threatened representatives of *Aylacostoma* from the High Paraná River, *A. chloroticum* and the new species *A. brunneum*. The most notable differences between them are concentrated on their shells, which are mainly distinguished by their distinctive coloration patterns. The new species also differs from *A. chloroticum* and the extinct *A. guaraniticum* in having a lower number of whorls. Owing to its relatively low spire and the swelling of the body whorl, *A. brunneum* resembles the extinct *A. stigmaticum* although it differs from it by lacking the yellow horn colour and by the absence of its typical black spots (Hylton Scott, 1954; Peso *et al.*, 2013b).

Anatomically, the general scheme of the internal organization of the species described here fits well with that reported for *A. guaraniticum* by Castellanos (1981). The new species differs from *A. chloroticum* by the constant presence of the distinctive vessels in the area between the gills and the adrectal sinus. The lack of conspicuous vascularization in that area found in some of the specimens of *A. chloroticum* resembles the Brazilian *Aylacostoma ci* Simone, 2001, described and illustrated in Simone (2001). Nonetheless, both species from the High Paraná River differ from their Brazilian congeners by their shells and the presence of two basal cusps on the rachidian teeth, both of which are lacking in the three Brazilian species characterized by Simone (2001, 2011). These cusps also discriminate the species studied here from *A. guaraniticum*, for which only one basal cusp has been reported (Hylton Scott, 1953), but not from *A. stigmaticum*, whose radula (Vogler, 2013) is similar to those reported in this study. As noted by Vogler (2013), the basal cusps are not visible at first sight under SEM, and the presence and number of basal cusps as a useful character for differentiating species, at least amongst those from Argentina–Paraguay and Brazil, needs to be confirmed by more evidence from further species.

Although it was possible to discriminate the two species from High Paraná River studied here from those for which anatomical data are available, remarkably no major differences were found to allow an accurate diagnosis based on anatomical characters alone, at least at the level of detail of this study. Some evidence for this situation can be found in the recent study of comparative anatomy published by Simone (2011), where 305 species of Caenogastropoda were analysed by means of 676 morphological characters with 2291 states, including three *Aylacostoma* representatives from Brazil, *A. ci*, *Aylacostoma exoplicata* Simone, 2001, and *Aylacostoma tenuilabris* (Reeve, 1860). Despite the goal of Simone's study being to reconstruct higher-order relationships, by analysing the characters matrix presented, we found that only six of the 676 characters were coded with different states for the Brazilian species (Simone, 2011).

In order to make sound decisions regarding the specific status of members of the genus *Aylacostoma*, we suggest that an integrative vision is necessary, complementing the conchological and anatomical data with evidence from other approaches. In this sense, ecology and life history data could be helpful for this decision process; however, as noted in the Introduction, this information is still scarce and constitutes a generalization for all the species currently included in *Aylacostoma*. The molecular data presented here, the first for any species of the genus according to our knowledge, have shown that the mitochondrial regions employed constitute useful markers for discriminating between the species. Taking into account that *A. chloroticum* and *A. brunneum* are considered to be fully asexually reproducing taxa, they seem to be highly divergent in terms of genetic distance (as evidenced by values of *c*. 4.26 and 4.41% for *COI* and 6.65% for *Cyt b*), especially when compared with the smaller interspecific distances of other endemic freshwater gastropods (e.g. Hershler, Liu & Thompson, 2003; Köhler, Panha & Glaubrecht, 2010). With these percentages in hand, and in view of the fact that they are reciprocally monophyletic, the High Paraná representatives could be delineated as species by applying the 10% sequence divergence rule (10X rule) proposed by Hebert *et al.* (2004) as their divergence clearly exceeds this recommended threshold. However, the use of divergence thresholds has been strongly discouraged (Frézal & Leblois, 2008). On the contrary, the EGSC has been proposed and applied to delineate parthenogenetic species of mites, rotifers, and ostracods (Birky & Barraclough, 2009; Birky *et al.*, 2010, 2011; Martens *et al.*, 2012; Shearn *et al.*, 2012). This concept is based on evolutionary and population genetics, as well as data from DNA sequences, and has been developed with special emphasis on the speciation of asexual organisms (Birky *et al.*, 2005, 2010; Birky & Barraclough, 2009).

By using the K/θ method with our *COI* data, we have clearly recognized *A. chloroticum* and *A. brunneum* as distinct evolutionary genetic species, despite the lack of major distinguishable morphological features other than shells. Thus, we suggest that the EGSC could be a useful framework with which to comprehensively review the specific status of the gastropods of this genus. This would probably open the way to a greater understanding of the species included in *Aylacostoma* and their evolutionary relationships.

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