A phylogeographical perspective on the *ex situ* conservation of *Aylacostoma* (Thiaridae, Gastropoda) from the High Paraná River (Argentina–Paraguay)

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Aylacostoma Spix, 1827, contains species that are the subject of focused conservation efforts under the auspices of the 'Aylacostoma Project', the only *ex situ* conservation programme for freshwater gastropods in South America. Two species from the High Paraná River (Argentina–Paraguay) are included in this programme (*Aylacostoma chloroticum* Hylton Scott, 1954 and *Aylacostoma brunneum* Vogler & Peso, 2014), as their habitats have disappeared as a consequence of the filling of the Yacyretá Reservoir in the 1990s. At present, *A. chloroticum* is restricted to only one known wild population in a small and fragile habitat, and wild populations of *A. brunneum* are presumed to have gone extinct. We used partial sequences of the cytochrome oxidase subunit I gene to provide the first phylogeographical perspective on these species from a limited dataset containing representatives of several wild populations that are successfully being bred in captivity. We found low genetic diversity and two haplotypes in *A. chloroticum*, and absence of variation with one haplotype in *A. brunneum*. The reservoir's entry zone was identified to be of great interest for conservation, and is where we suggest re-introductions and translocations should be targeted, to preserve the future evolutionary potential of the extant diversity.

INTRODUCTION

Non-marine molluscs represent an extremely diverse group in terms of number of described species, comprising about 24 000 terrestrial and 4000 freshwater recognized species (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). To mitigate this biodiversity loss, several conservation initiatives have been undertaken worldwide for highly imperilled molluscs such as those of Pacific islands (e.g. Pearce-Kelly *et al*., 1997; Holland & Hadfield, 2002; Hadfield, Holland & Olival, 2004).

South America is a diverse region that harbours a rich and highly endemic molluscan fauna, with two

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hotspots of freshwater gastropod diversity (Quintana, 1982; Ramírez, Paredes & Arenas, 2003; Rumi *et al.*, 2006, 2008; Simone, 2006; Strong *et al.*, 2008). Nonetheless, many of these species face increasing threats as the result of anthropogenic activities (Agudo-Padrón, 2011; Santos, Miyahira & Mansur, 2013). As for other threatened South American invertebrates, conservation strategies are still virtually non-existent for almost all imperilled molluscs in the region, which are underrepresented on the IUCN Red List of Threatened Species (Régnier, Fontaine & Bouchet, 2009).

To our knowledge, conservation strategies in South America have been adopted only for the native parthenogenetic freshwater gastropod *Aylacostoma* (Thiaridae) from the High Paraná River (Argentina–Paraguay) in the context of a conservation breeding programme, known as the '*Aylacostoma* Project' (Vogler *et al.*, 2014). This *ex situ* programme was established in the 1990s accompanying the initial filling stages of the Yacyretá Reservoir (Argentina–Paraguay) which flooded the area of the Yacyretá–Apipé rapids where three *Aylacostoma* species had been recorded: *Aylacostoma guaraniticum* (Hylton Scott, 1953), *Aylacostoma stigmaticum* Hylton Scott, 1954 and *Aylacostoma chloroticum* Hylton Scott, 1954 (Peso, Molina & Costigliolo Rojas, 2013a; Peso, Costigliolo Rojas & Molina, 2013b; Vogler, 2012, 2013; Vogler *et al.*, 2012, 2014). This conservation effort is being conducted by the Universidad Nacional de Misiones (UNaM; Posadas, Argentina), in conjunction with the Museo Argentino de Ciencias Naturales (MACN; Buenos Aires, Argentina), and is supported by the Entidad Binacional Yacyretá (EBY; Argentina–Paraguay) (Vogler, 2013). At present, only *A. chloroticum* and the recently described *Aylacostoma brunneum* Vogler & Peso 2014 are included in the programme (Fig. 1; Vogler *et al.*, 2014). The former species is restricted to only one known wild population in a small and fragile habitat under anthropogenic threat due to the construction of an artificial beach for human recreation (Fig. 2). Wild populations of *A. brunneum* are presumed to have gone extinct after their habitats were flooded in 2011 during the final filling stage of the reservoir (Vogler *et al.*, 2014). Re-introduction and translocation constitute two of the priorities of the '*Aylacostoma* Project' to preserve the existing diversity and future evolutionary potential of these species. However, the genetic composition and past history of the captive populations included in the programme remain unknown.

In the present study, we provide the first phylogeographical perspective on *A. chloroticum* and *A. brunneum* from a limited dataset containing representatives of several wild populations that are successfully being bred in captivity, to shed light on the genetic composition of captive populations and their past history. This information is crucial for making in-

Figure 1. Living specimens of *Aylacostoma* born in captivity within the *ex situ* conservation programme. A, *Aylacostoma chloroticum* (note the juvenile on the adult specimen). B, *Aylacostoma brunneum*.

formed management decisions regarding re-introduction of captive-bred individuals and field translocations.

MATERIALS AND METHODS

The study was based on partial sequences of the cytochrome *c* oxidase subunit I (COI) gene of *Aylacostoma* specimens from the ongoing *ex situ* conservation programme at UNaM collected in the High Paraná River between 1994 and 2011. This dataset was made available by Vogler *et al.* (2014) when re-describing *A. chloroticum* and describing *A. brunneum* under the evolutionary genetic species concept. Ingroup taxa included 37 *A. chloroticum* from six populations spanning its known range in the High Paraná River, and six *A. brunneum* from the only two known geographical records for the species. Outgroups included *Doryssa* sp., *Pachychilus laevissimus* (Sowerby, 1825) and *Pachychilus nigratus* (Poey, 1858). An overview of the samples analysed is presented in Table 1.

Sequence alignment was performed with Clustal X 2.0.12 (Larkin *et al.*, 2007) with manual corrections as

Figure 2. Evolution of the old port of Candelaria city (Misiones, Argentina; 27°26′50.96″S, 55°45′0.84″W). This is the only location along the High Paraná River where a wild population of *Aylacostoma chloroticum* is still known to occur after completing the filling of the Yacyretá Reservoir in 2011. Note the drastic modification of the environment between 2003 and November 2011, with the construction of an artificial beach for recreational use. The white arrow highlights the increase in water level between August and December 2010.

necessary. The sequences were translated into amino acids to check for stop codons and frameshift mutations in ORF Finder (http://www.ncbi.nlm.nih.gov/projects/gorf/). Several methods were used to quantify and understand the distribution of polymorphisms in *Aylacostoma* species. The number of haplotypes (h), number of segregating sites (S), as well as nucleotide (π) and haplotype (H_d) diversities (Nei, 1987) were estimated using DnaSP 5.10 (Librado & Rozas, 2009). Nucleotide composition of haplotypes was analysed in BioEdit 7.0.9 (Hall, 1999). Genetic distances were explored in DAMBE 5.3.8 (Xia, 2013) by means of the Jukes–Cantor (JC) and Kimura's two-parameter (K2P) substitution models. Tajima's D (Tajima, 1989), Fu's F_S (Fu, 1997) and R_2 (Ramos-Onsins & Rozas, 2002) tests were computed in DnaSP 5.10 to evaluate deviation of sequence variation from predictions of evolutionary neutrality, and to explore possible demographic forces that may have shaped existing genetic diversity. Statistical significance of these tests was computed through the number of segregating sites (S) and 5000 coalescent simulations. To assess the amount of population genetic structure, an analysis of molecular variance (AMOVA) was carried out in Arlequin 3.5.1.3 (Excoffier & Lischer, 2010). Pairwise Φ_{ST} statistics were estimated to test for genetic differentiation between populations. An AMOVA considering three hierarchical levels (amongst regions Φ_{CT}, amongst populations within regions Φ_{SC}, and within populations Φ_{ST}) was also performed using the main channel of the High Paraná River as the regionalization criterion, pooling the Argentinean and Paraguayan populations separately. In all cases, significance of the obtained values was evaluated using 16 000 random permutations (Guo & Thompson, 1992). In addition, the null hypothesis of no association between genetic and geographical distances was assessed by conducting a Mantel test (Mantel, 1967) with 1000 random permutations in Alleles in Space (Miller, 2005).

Maximum-likelihood (ML) was used for phylogenetic inference in PhyML (Guindon & Gascuel, 2003) available via the Phylemon2 webserver (http://phylemon.bioinfo.cipf.es; Sánchez *et al.*, 2011). The optimal model of nucleotide substitution (TIM3+I) was

Table 1. Origin and year of sampling of *Aylacostoma* specimens included in the conservation programme taking place at Universidad Nacional de Misiones from where the COI dataset studied was obtained; information on the three outgroup species is also provided

Species	Locality	Coordinates	Year	N	GenBank accession nos.
Aylacostoma chloroticum	Apipé Rapids, AR	27°28′57.41″S 56°44′0.82″W	1994	5	JQ236687–JQ236690; JX244270
	Posadas Port, AR	27°21′43.10″S 55°53′5.20″W	2006	3	JQ236698; JQ236699; JX203225
	San José Lagoon, AR	27°22′11.40″S 55°52′49.64″W	2008	10	JQ236694–JQ236697; JX203220–JX203224; JX244272
	San Juan St. (mouth), AR	27°25′0.95″S 55°42′46.88″W	2009	2	JQ236685–JQ236686
	Candelaria, AR	27°26′50.96″S 55°45′0.84″W	2008	5	JF346878–JF346882
			2011	5	JQ236681–JQ236684; JQ236691
	Río Beach, PY	27°24′29.83″S 55°49′32.94″W	2007	7	JQ236692; JQ236693; JX203217–JX203219; JX203226; JX244271
Aylacostoma brunneum	Ita Cuá, PY	27°24′42.13″S 55°48′45.69″W	2007	5	JQ236700–JQ236704
	Río Beach, PY	27°24′29.83″S 55°49′32.94″W	2007	1	JQ236705
Doryssa sp.*	Venezuela	–	2010	1	JQ966087
*Pachychilus laevissimus**	Venezuela	–	2010	1	JQ966088
*Pachychilus nigratus**	Cuba	–	1997	1	JQ966089

COI, cytochrome oxidase subunit I; AR, Argentina; PY, Paraguay; N, number of specimens analysed from each locality. *Outgroup species.

evaluated with the likelihood-ratio test and selected by means of the corrected Akaike information criterion (AICc) in Jmodeltest 0.1.1 (Posada, 2008). Nodal support values were calculated with 100 bootstrap replicates (Felsenstein, 1985). A median joining network was also constructed based on the number of nucleotide substitutions between haplotypes using Network 4.6 (Bandelt, Forster & Röhl, 1999).

To estimate relative divergence times, molecular clock analyses were conducted. The molecular clock hypothesis was tested using Tajima's non-parametric relative rate test (Tajima, 1993) in MEGA 5.05 (Tamura *et al.*, 2011), and by means of a likelihood-ratio test on ML trees with and without enforcing a molecular clock in PAUP*4.0b10 (Swofford, 2002), with the parameters from the best model for molecular evolution as identified by Jmodeltest 0.1.1. Divergence time (T) between phylogeographical groups was estimated following Brunhoff *et al.* (2003) and Vega *et al.* (2010) as $T = Da/2\mu$, where Da is the net nucleotide divergence (Nei, 1987) and 2μ indicates the divergence rate. Net divergence and standard errors (SE) were estimated in MEGA 5.05 using the JC and K2P models and 1000 bootstrap replicates. The 95% confidence intervals (CI) for the divergence times were calculated as ± 1.96 SE of the net distances (Brunhoff *et al.*, 2003). The mitochondrial invertebrate divergence rates of 1.22 ± 0.27% (JC) and 1.23 ± 0.26% (K2P) per Myr (Wilke, Schultheiß & Albrecht, 2009) were used to obtain age estimates. Additionally, estimates of divergence times were carried out by means of Bayesian inference in MrBayes 3.2 (Ronquist *et al.*, 2012). Two runs were performed simultaneously with four Markov chains for 2000 000 generations, with the first 20 000 generations discarded as burn-in, and assuming a divergence rate of 1.76 ± 0.66% per Myr under the General Time Reversible model with gamma-distributed rate heterogeneity and invariant sites (GTR+I+G).

RESULTS

Partial COI sequences consisted of 658 bp for 43 *Aylacostoma* individuals from the High Paraná River (*A. chloroticum* = 37; *A. brunneum* = 6). Two haplotypes were identified in *A. chloroticum* (H_1 and H_2), which differed from each other by one nucleotide. A single haplotype was identified in *A. brunneum* (H_3), differing in 28 and 29 nucleotides from H_1 and H_2, respectively (Table 2). Base frequencies as well as AT and GC content are shown in Table 3. Stop codons were

Table 2. Haplotype structure in *Aylacostoma* specimens from the High Paraná River based on 658 nucleotides of the partial cytochrome oxidase subunit I gene

	1	5	31	74	160	166	181	202	211	212	217	223	271	280	316	319	343	346	358	466	475	478	481	535	544	550	631	637	640	N
Aylacostoma chloroticum																														
H_1	G	T	C	T	G	G	G	C	A	T	T	A	G	G	A	C	A	C	T	G	G	G	A	T	A	A	C	G	T	33
H_2	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	4
Aylacostoma brunneum																														
H_3	A	C	T	C	A	A	A	G	G	C	C	G	T	A	G	T	C	T	C	A	A	A	G	C	G	G	A	C	C	6

H, haplotype; N, number of individuals with that particular haplotype. Numbers heading the columns indicate the position of polymorphic sites. Haplotype 1 is shown as a reference sequence; dots indicate nucleotides matching the reference sequence.

absent, and an ORF = +2 was identified, representing 219 codons. The amino acid sequence was conserved amongst haplotypes, indicating that the polymorphic sites represent synonymous mutations; third codon position substitutions were most common (89.28%). Corrected sequence divergence amongst haplotypes is presented in Table 4.

The results of the estimated molecular diversity and neutrality tests are shown in Table 5. For *A. chloroticum*, mean haplotype diversity (H_d) was 0.198 ± 0.08 and mean nucleotide diversity (π) was 0.00030 ± 0.00012, with most of the geographical populations lacking variation. No genetic diversity was detected in *A. brunneum* ($H_d = 0$; $\pi = 0$). No statistically significant deviations from neutrality were observed.

Owing to the absence of genetic variation in *A. brunneum*, only *A. chloroticum* was considered further. For this species, the metrics confirm what is intuitively clear given the low levels of genetic variability, and the geographical distribution of the two haplotypes. The Φ_{ST} value across all populations showed a significant amount of genetic variation amongst the six populations ($\Phi_{ST} = 0.49368$, $P < 0.01$) indicating that at least one of the populations presented significant heterogeneity. The results of Φ_{ST} pairwise comparisons and hierarchical AMOVA are presented in Tables 6 and 7, respectively. Genetic differentiation amongst populations was not found to be correlated with distance between populations ($r = -0.1039$, $P = 0.922$).

The haplotype network illustrated that H_1 was the dominant haplotype in *A. chloroticum*, and that H_2, exclusive to Río Beach, is separated by a single mutation from H_1. The unique haplotype of *A. brunneum* (H_3) is separated by 28 mutations from H_1 (Fig. 3). The phylogenetic analysis confirmed the relationships obtained in Vogler *et al.* (2014) and recovered *A. chloroticum* and *A. brunneum* as sister groups; two subgroups were recognized within *A. chloroticum*, one containing individuals from all six geographical populations, and the other containing individuals exclusively from Río Beach (Fig. 4).

Divergence between *A. chloroticum* and *A. brunneum* is inferred to have occurred around 3.6 Mya (JC, K2P) to 2 Mya (GTR+I + G), between the mid-Pliocene and early Pleistocene. When taking into account the standard deviation of the substitution rates under the JC, K2P and GTR+I+G models, the maximum time interval for this divergence is estimated to be 6.34–0.61 Mya (Fig. 5, Supporting Information, Tables S1 and S2). Within *A. chloroticum*, haplotypes H_1 and H_2 are estimated to have split between 0.38 Mya (GTR+I+G) and 0.12 Mya (JC, K2P), during the late Pleistocene. The maximum time interval inferred for this split is estimated to be 0.84–0.0008 Mya (Fig. 5, Supporting Information, Tables S1 and S3).

Table 3. Sequence composition for the COI haplotypes found in *Aylacostoma* specimens

	A	C	G	T	AT content	GC content
Aylacostoma chloroticum						
H_1	172 (26.14%)	119 (18.08%)	126 (19.15%)	241 (36.63%)	62.77%	37.23%
H_2	173 (26.29%)	119 (18.08%)	125 (19%)	241 (36.63%)	62.92%	37.08%
Aylacostoma brunneum						
H_3	173 (26.29%)	124 (18.85%)	124 (18.85%)	237 (36.02%)	62.31%	37.69%

COI, cytochrome oxidase subunit I; *H*, haplotype.

Table 4. Corrected genetic distances amongst *Aylacostoma* haplotypes under the Jukes–Cantor (below the diagonal) and Kimura's two-parameter (above the diagonal) substitution models

	Aylacostoma chloroticum		*Aylacostoma brunneum*
	H_1	H_2	H_3
Aylacostoma chloroticum			
H_1	–	0.001522	0.044278
H_2	0.001521	–	0.045934
Aylacostoma brunneum			
H_3	0.043808	0.045421	–

H, haplotype.

DISCUSSION

GENETIC DIVERSITY IN *AYLACOSTOMA*

The data obtained for the *Aylacostoma* species from seven populations within their narrow ranges in the High Paraná River demonstrated the existence of low genetic diversity in *A. chloroticum* ($H_d = 0.198$, $\pi = 0.00003$), and the absence of variation in *A. brunneum*. As this is the first such study for the genus *Aylacostoma*, comparisons with other congeners are presently not possible. However, comparably low levels of genetic diversity have been reported for other members of three families of Caenogastropoda where apomictic parthenogenesis is present as a reproductive strategy: Hydrobiidae Troschel, 1857 [e.g. *Tryonia protea* (Gould, 1855); Hershler, Liu & Mulvey, 1999], Thiaridae Gill, 1871 [e.g. *Melanoides tuberculata* (Müller, 1774); Stoddart, 1983; Samadi *et al.*, 1999] and Viviparidae Gray, 1847 [e.g. *Campeloma decisum* (Say, 1817); Johnson, 1992].

The analysis of structure and spatial distribution of genetic diversity in *A. chloroticum* showed uniformity in the gene pools of five of the populations, with the exclusive presence of H_1. The exception was represented by the Paraguayan population of Río Beach, with two haplotypes (H_1, H_2) and the greatest genetic diversity ($H_d = 0.571$, $\pi = 0.00087$) among the sampled populations. This pattern could be due to a range expansion of H_1. Alternatively, the broad spatial distribution of H_1 could be the result of its parthenogenetic reproductive strategy (Quintana & Mercado Laczkó, 1997). As stated by Wares & Turner (2003), mating system has a profound effect on the distribution of genetic diversity, and for parthenogenetic species is expected to result in genetic homogeneity resulting from the persistence of a few clonal lineages. In the case of *A. brunneum*, knowledge of the natural history is even scarcer than in *A. chloroticum*, although parthenogenesis is the most likely explanation.

By analysing the spatial distribution observed and with support in differentiation values obtained in the AMOVA, we suggest that the greatest genetic diversity of the genus *Aylacostoma* in the High Paraná River would fit to a longitudinal zonation model. Meichtry de Zaburlín *et al*. (2010) recognize four zones for the Yacyretá Reservoir, referred to as: 1, the entry zone, river or lotic; 2, the transition zone, intermediate or river–reservoir; 3, the reservoir zone or lacustrine; and 4, the subreservoirs or lateral arms. It was observed that the greatest genetic diversity is allocated within the entry zone, which also constitutes the eastern boundary of the historical distribution of *Aylacostoma* in the High Paraná River. This suggests that the genetic diversity observed in this area is not accidental, but as the area furthest from the Yacyretá dam, the entry zone most resembles the natural conditions of the river and has suffered only indirect changes resulting from increases in water level (Peso, 2012). Several studies on the spatial and temporal variation of planktonic and benthic communities of the Yacyretá influence area support this idea, and have shown that the attributes of the communities in the entry zone (e.g. species richness, diversity, density) have remained more or less stable since before the filling of the reservoir (Meichtry de Zaburlín, 1999, 2012; Peso & Bechara, 1999; Meichtry de Zaburlín *et al*., 2010; Peso, 2012).

PHYLOGEOGRAPHICAL PATTERNS AND HISTORY OF THE HIGH PARANÁ RIVER

According to the divergence time estimates, the split between *A. chloroticum* and *A. brunneum* probably

Table 5. Summary of genetic diversity and neutrality tests in *Aylacostoma*

	N	h	$H_d \pm$ SD	$\pi \pm$ SD	S	D_T	F_S	R_2
Aylacostoma chloroticum	37	2	0.198 ± 0.08	0.00030 ± 0.00012	1	−0.25209*	0.2*	0.0991*
San Juan St., AR	2	1	0	0	0	NA	NA	NA
Candelaria, AR	10	1	0	0	0	NA	NA	NA
San José Lagoon, AR	10	1	0	0	0	NA	NA	NA
Posadas Port, AR	3	1	0	0	0	NA	NA	NA
Apipé Rapids, AR	5	1	0	0	0	NA	NA	NA
Río Beach, PY	7	2	0.571 ± 0.119	0.00087 ± 0.00018	1	1.34164*	0.856*	0.2857*
Aylacostoma brunneum	6	1	0	0	0	NA	NA	NA
Ita Cuá, PY	5	1	0	0	0	NA	NA	NA
Río Beach, PY	1	1	NA	NA	NA	NA	NA	NA

AR, Argentina; PY, Paraguay; N, number of individuals; h, number of haplotypes; H_d, haplotype diversity; π, nucleotide diversity; S, number of segregating sites; D_T, Tajima's D; F_S, Fu's F_S; SD, standard deviation; NA, not applicable. *Non-significant value.

Table 6. Φ_{ST} values amongst the six geographical populations of *Aylacostoma chloroticum*

	San Juan St.	Candelaria	San José Lagoon	Posadas Port	Apipé Rapids
San Juan St., AR	–	–	–	–	–
Candelaria, AR	0	–	–	–	–
San José Lagoon, AR	0	0	–	–	–
Posadas Port, AR	0	0	0	–	–
Apipé Rapids, AR	0	0	0	0	–
Río Beach, PY	0.25664	0.56656*	0.56656*	0.34375	0.43850

*$P < 0.05$; AR, Argentina; PY, Paraguay.

Table 7. Hierarchical analysis of molecular variance in *Aylacostoma chloroticum* using the main channel of the High Paraná River as regionalization criterion

Source of variation	% of variation	Φ statistics	P
Amongst regions	78.44	$\Phi_{CT} = 0.7844$	0.166
Amongst populations within regions	−4.77*	$\Phi_{SC} = -0.2214$*	1.000
Within populations	26.33	$\Phi_{ST} = 0.78441$	0.006

Regions defined: Argentina (grouping together the San Juan St., Candelaria, San José Lagoon, Posadas Port and Apipé Rapids populations), and Paraguay (Río Beach population).
*Negative values should be interpreted as zero (Tzeng, Yeh & Hui, 2004).

occurred roughly 3.6–2 Mya, placing the origin of the divergence between the mid-Pliocene and early Pleistocene. If we consider the maximum estimate for this divergence, this origin would not be older than the late Miocene. Similarly, the divergence between *A. chloroticum* haplotypes H_1 and H_2 probably occurred roughly 0.38–0.12 Mya in the late Pleistocene, and not older than mid-Pleistocene, according to the maximum estimate. Given that fossils of *Aylacostoma* are known in South America since the Palaeocene (Parodiz, 1969; Camacho & Del Río, 2008), both species can be understood to be of relatively recent

origin. The mid-Pliocene to early Pleistocene divergence between *A. chloroticum* and *A. brunneum* seems consistent with the known geomorphological history of the Paraná River during this time interval. The sediments of the Ituzaingó Formation, a unit that has been dated as mid-Pliocene in age (Herbst, 2000; Popolizio, 2006), can be recognized from a few kilometres to the east of the Argentinean city of Ituzaingó (27°36′S, 56°40′W), from where the Paraná River runs towards the east and north encased in a basalt bed (known as Alto Paraná Encajonado *sensu* Matteucci *et al.*, 2004). According to Herbst (2000), where the basalts are no
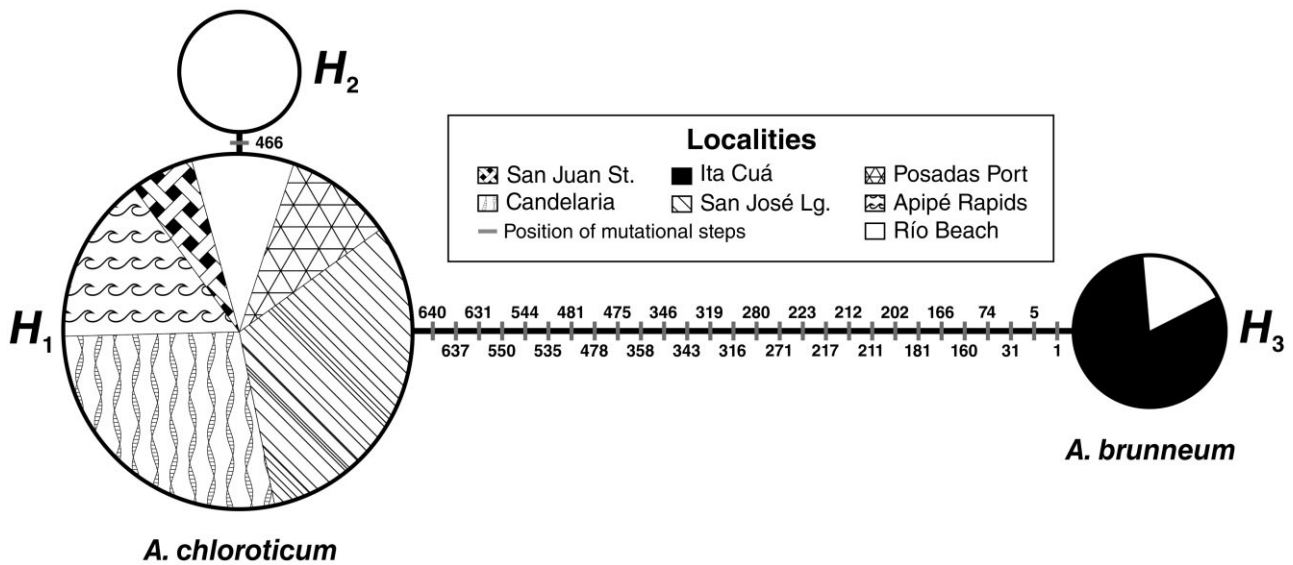
Figure 3. Median-joining network depicting all haplotypes found for the *Aylacostoma* specimens from the High Paraná River included in the *ex situ* conservation programme. The size of circles is proportional to haplotype frequency. Mutational steps separating haplotypes are indicated. For localities, see Table 1.

longer exposed at surface, in the area of the now flooded Yacyretá–Apipé rapids, the old Paraná River (Paleoparaná) opened into numerous arms in a large alluvial fan. Its waters would have been more or less clear with a sandy bottom, and with emergent sandbanks during drought, and with a diversity of environments in the area (Herbst, 2000). In this scenario, and bearing in mind that five *Aylacostoma* species have been recorded from the Yacyretá–Apipé rapids (Hylton Scott, 1953, 1954; Quintana & Mercado Laczkó, 1997), two of which have never been formally described, it is possible to hypothesize that speciation of the common ancestor of *A. chloroticum* and *A. brunneum* may have occurred through the colonization of transitional environments, as predicated by the model described above. Additionally, a recent panbiogeographical analysis by Arzamendia & Giraudo (2012) highlights that the convergence of the High Paraná River and the Alto Paraná Encajonado is located along a transition zone between two biogeographical provinces (the Atlantic Forest and Chaco), characterized by high species richness and taxonomic elements of different origins. Thus, this area represents a biogeographical crossroads that reflects the spatial and temporal interrelationships of different biotic and geological components (Arzamendia & Giraudo, 2012). However, further palaeontological studies are required to investigate the geological context of the diversification of *Aylacostoma* in this region.

The recent Pleistocene divergence between the haplotypes of *A. chloroticum* would have occurred in an ecological context very similar to that prior to the damming of the Yacyretá Reservoir, characterized by

broad meanders enclosing several islands on a broad floodplain (Neiff, 1986; Álvarez *et al.*, 1995; Neiff *et al.*, 2000; Arzamendia & Giraudo, 2012).

A CONTRIBUTION TO THE CONSERVATION OF THE *AYLACOSTOMA* SNAILS

One of the primary goals of conservation biology is to ensure the maintenance of biodiversity, of which genetic diversity is a fundamental component (DeSalle & Amato, 2004). An efficient conservation programme should include unequivocal identification of management units representing different evolutionary lineages, for which genetic information provides an objective means to determine conservation priorities (King & Burke, 1999; DeSalle & Amato, 2004). In the present study we have identified three evolutionary lineages among *Aylacostoma* representatives from the High Paraná River. While *A. brunneum* was only recently described (Vogler *et al.*, 2014), we suggest the two haplotypes found in *A. chloroticum* deserve special attention as independent lineages. Although the geographical populations where these haplotypes occur do not qualify under the strictest definition of evolutionarily significant units (ESUs; Ryder, 1986; Moritz, 1994; Ladle & Whittaker, 2011), we suggest that the two haplotypes of *A. chloroticum* (H_1 and H_2) should be recognized as ESUs in a broad sense. This proposition is based on the fact that captive populations derived from single individuals containing these haplotypes represent independent evolutionary histories reflecting the apomictic parthenogenetic reproductive mode of the species. Al-
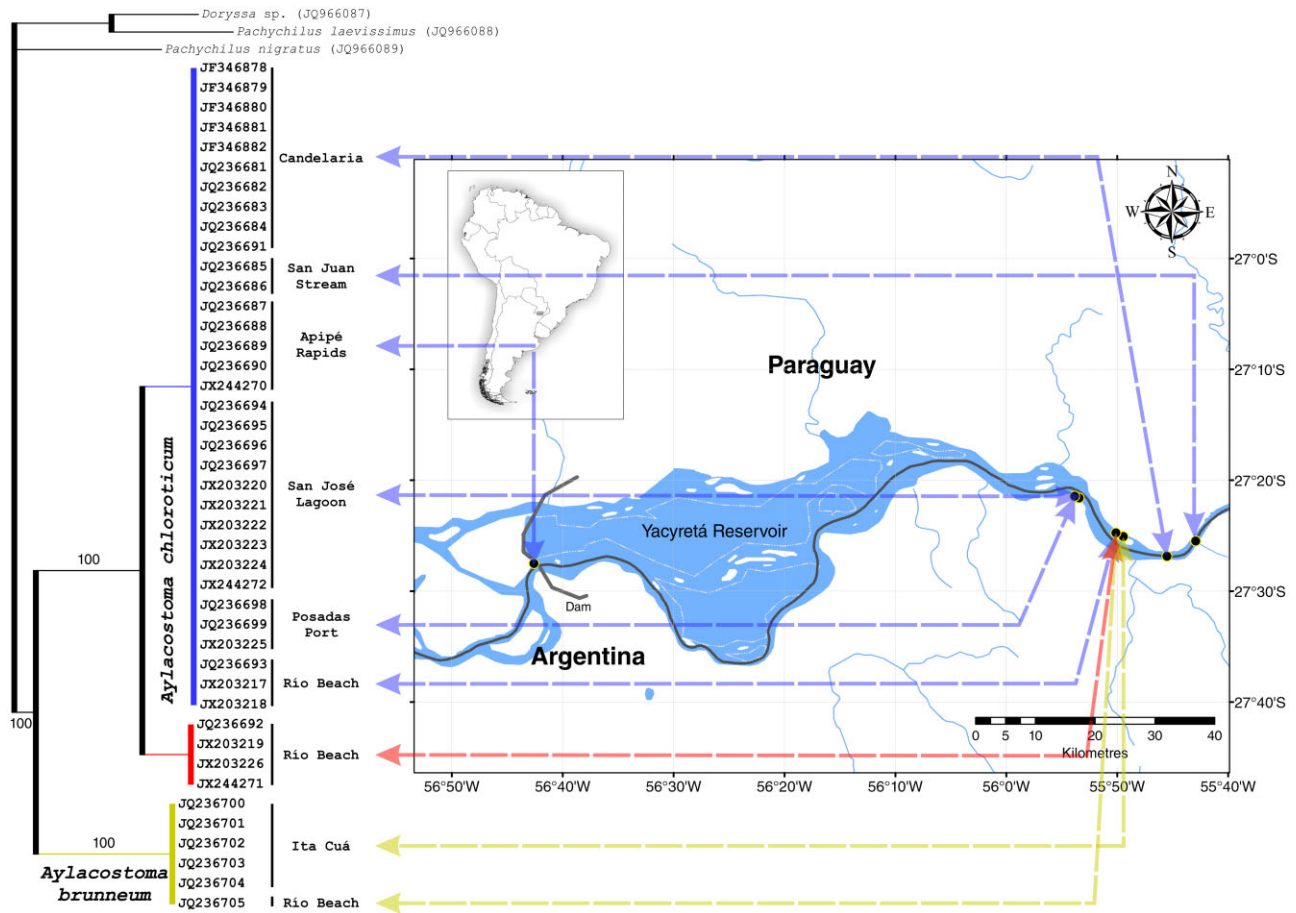
Figure 4. Maximum-likelihood tree of *Aylacostoma* specimens based on 658 nucleotides of the partial cytochrome oxidase subunit I gene and the spatial distribution of geographical populations in the High Paraná River. Bootstrap values are shown above and below the branches. Numbers within clades are GenBank accession numbers. References to localities are given in Table 1.

though no morphological differentiation among the haplotype lineages has been observed (Vogler *et al.*, 2014), the apparent persistence of these lineages since the Pleistocene may be indicative of an incipient speciation event.

In this context, understanding the geographical distribution of genetic diversity in the High Paraná River is vital for identifying and prioritizing areas for protection, so that the genetic representation of *Aylacostoma* in nature can be maximized. Our analysis clearly shows that the entry zone of the Yacyretá Reservoir, and particularly Río Beach, is an area of great interest in terms of conservation. This is where *A. brunneum* was found, and is where the two haplotypes of *A. chloroticum* converge. Thus, this area deserves to be highlighted as a key genetic resource for the *ex situ* conservation of the group. It is important to emphasize that the filling of the Yacyretá Reservoir was completed in 2011. Although the entry zone was less impacted by the new water levels, what little genetic variation has been de-

termined here for this area may have already been lost. This highlights the urgent need to survey the area and fully justifies the *ex situ* efforts to preserve the available diversity of *A. chloroticum* and *A. brunneum*, both of which deserve the highest priorities for conservation.

Finally, by considering our results and taking into account the ongoing efforts of the '*Aylacostoma* Project' in which re-introduction of captive-bred individuals and field translocations are being attempted, we suggest that such actions should be made, whenever possible, in available areas of the entry zone, to preserve as much as possible the future evolutionary potential of the extant diversity.
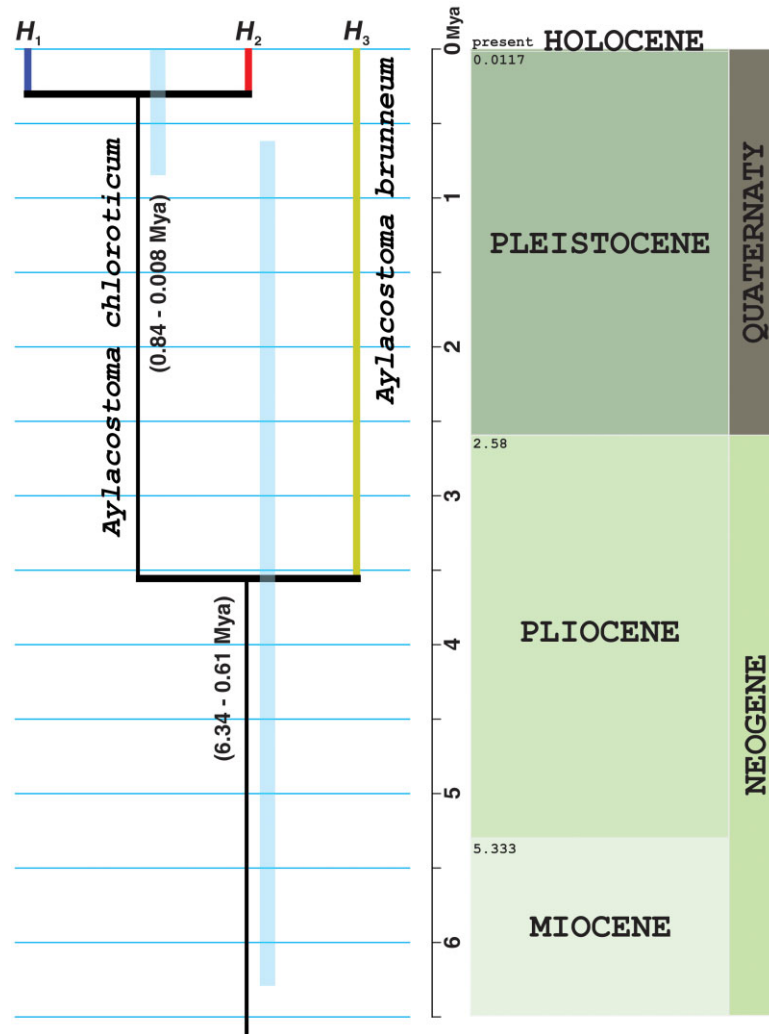
ACKNOWLEDGEMENTS

Figure 5. Divergence times for *Aylacostoma* from the High Paraná River. The nodes in the phylogram are presented according to the mean value of oldest estimated age, assuming different divergence rates and substitution models. Divergence between *A. chloroticum* and *A. brunneum* = 3.60 Mya (K2P); divergence within *A. chloroticum* = 0.38 Mya (GTR+I+G). The bars around each node represent the minimum and maximum estimated age. Time scale adapted from the International Chronostratigraphic Chart (International Commission on Stratigraphy, 2014).

this study. We are especially grateful to María José Molina for providing the pictures of living *Aylacostoma* specimens.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1. Bayesian estimation of divergence times between *A. chloroticum* and *A. brunneum*, and between *A. chloroticum* lineages by applying the mitochondrial invertebrate clock from Wilke *et al*. (2009) of 1.76 ± 0.66 Myr under the GTR+I+G evolution model.

Table S2. Estimation of divergence times between *A. chloroticum* and *A. brunneum*, by applying the mitochondrial invertebrate clock from Wilke *et al*. (2009) under the JC and K2P evolution models.

Table S3. Estimation of divergence times between *A. chloroticum* lineages by applying the mitochondrial invertebrate clock from Wilke *et al*. (2009) under the JC and K2P evolution models.