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Pattern of differentiation in the annual killifish genus *Austrolebias* (Cyprinodontiformes: Rivulidae) from a biosphere reserve site in South America: a multidisciplinary approach

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The annual killifish genus *Austrolebias* includes approximately 38 species distributed throughout the Paraná-Plata basin and Patos-Merín system. Within the *Austrolebias adloffi* species complex, the Uruguayan populations of *Austrolebias charrua* were considered as an intergradation between *A. adloffi* and *Austrolebias viarius* populations. *Austrolebias charrua* presents an intermediate phenotype between both taxa and high levels of morphological and chromatic variability. In the present study, we incorporate different methodological approaches (molecular, morphology, and gamete ultrastructure) to elucidate the pattern of differentiation among the parapatric taxa (*A. charrua*, *Austrolebias reicherti*, *A. viarius*) distributed in a Biosphere Reserve Site. Analyses of cytochrome *b* sequences show high values of DNA polymorphism, in particular for *A. charrua*. This is in accordance with both morphological and gametic variation. Using a statistical parsimony network based on these sequences and analysis of morphological data, past fragmentation and range expansion involving perhaps secondary contact between *A. charrua* and *A. reicherti* could be proposed. Coloration pattern and morphometric analyses showed an unexpected higher similarity between the most distantly-related taxa, *A. viarius* and *A. charrua*. This could be the result of retention of ancestral polymorphisms, especially in *A. charrua* populations from ponds of higher elevation, or to directional selection acting in similar ecological environments. Because these annual killifish species are considered endangered, our work reinforces the high priority need to include them in a conservation programme.

ADDITIONAL KEYWORDS: gametic ultrastructure – morphometry – past fragmentation – phylogeography.

INTRODUCTION

The annual killifish genus *Austrolebias* includes approximately 38 species distributed in the Parana-Plata basin and the Patos-Merin coastal lagoon system (Costa, 2006). Although many taxonomic changes were recently made in the genus (Costa, 1998, 2002, 2006), the *Austrolebias adloffi* species complex remains as a well-supported clade (Costa, 2006; García, 2006). Species of this complex are distributed along Los Patos-Merín system lowlands, which include 'Bañados del Este', an area from eastern Uruguay (Fig. 1) that has been declared a Biosphere Reserve Site and a Ramsar Site (Probides, 1999). This is a vast area of Atlantic coastal wetlands of low relief and liable to seasonal flooding. It includes lagoons and several rivers origi-

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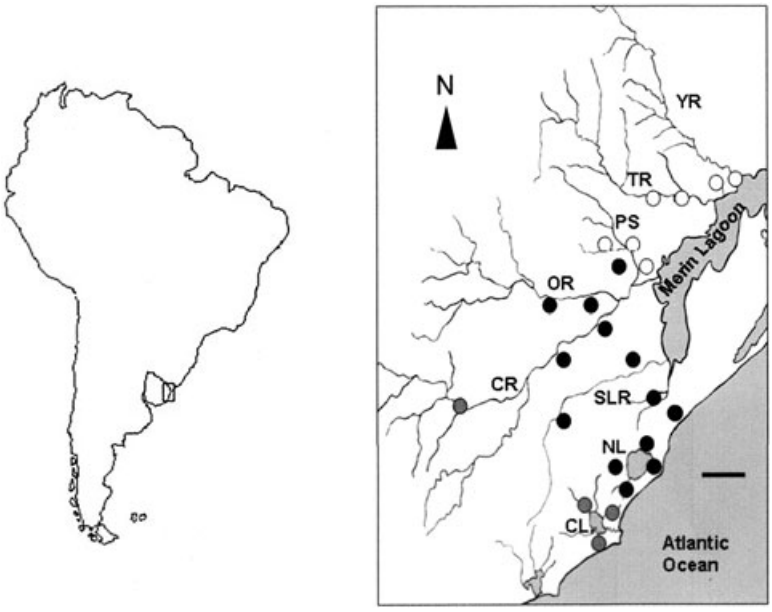

Figure 1. Distribution map of *Austrolebias charrua* (black circles), *Austrolebias reicherti* (white circles), and *Austrolebias viarius* (grey circles). Water bodies are indicated by capital letters: YR, Yaguarón river; TR, Tacuarí river; PS, Parao stream; OR, Olimar river; CR, Cebollatí river; SLR, San Luis river; NL, Negra lagoon; CL, Castillos lagoon. Scale bar = 20 km.

nated during the Quaternary marine transgressions (Iriondo, 2004).

Currently, the *A. adloffi* species complex comprises: *A. adloffi* Ahl 1922; *Austrolebias charrua* Costa & Cheffe 2001; *Austrolebias minuano* Costa & Cheffe 2001; *Austrolebias nigrofasciatus* Costa & Cheffe 2001; *Austrolebias reicherti* Loureiro & García 2004 (Loureiro & García, 2008); *Austrolebias arachan* Loureiro, Azpelicueta & García 2004; and *Austrolebias natchtigalli* Costa & Cheffe 2006.

The Uruguayan populations of *A. charrua* were named previously as *A.* sp., *A. adloffi-2* or *A.* cf. *adloffi* (García, Wlasiuk & Lessa, 2000; García, Alvarez-Valín & Gómez, 2002; García, Claramunt & Lalanne, 2004). This species was considered as an intergradation between the *A. adloffi* and *Austrolebias viarius* Vaz-Ferreira, Sierra & Scaglia (1964) populations because it presents an intermediate phenotype, high levels of variation in colour patterns and morphological variability (Vaz-Ferreira & Melgarejo, 1984). *Austrolebias charrua* is parapatrically distributed in its western and southern limit with *A. viarius,* a species closely related to the *A. adloffi* species complex (Costa, 2006; García, 2006), with some populations separated only by a distance of 70 m between ponds. It is also parapatrically distributed in its northern limit with *A. reicherti*, which is distributed from the Parao stream basin to the southern Río Yaguarón basin (Fig. 1).

Based on molecular nuclear markers, García *et al.* (2004) proposed that populations of the *A. adloffi* complex from Uruguay (including *A. charrua* and *A. reicherti*) would represent an ancient homoploid hybrid ancestry between *A. viarius* and *A. adloffi* followed by its divergence during the Quaternary environmental changes. More recently, a phylogeographic approach based on mitochondrial cytochrome *b* (cyt *b*) haplotypes analysis and chromosome data have supported a different hypothesis (García, 2006). This suggested a multiple simultaneous speciation process and possible reticulation events in the *A. adloffi* complex, followed by divergence.

A recurrent theme in the speciation literature is how to discriminate between primary differentiation (associated with isolation by distance) versus secondary contact as a result of hybridization and introgression of previously fragmented lineages (Endler, 1977). Hybridization and its attendant complications represent a major stumbling block for many species concepts, both in theory and in practice (Hull, 1997).

In the present study, we incorporate different methodological approaches (molecular, morphology, and gamete ultrastructure) to elucidate the pattern of differentiation among these parapatric taxa (*A. charrua*, *A. reicherti*, and *A. viarius*) distributed in the Bañados del Este Biosphere Reserve, emphasizing the importance of conservation strategies in this fish group.

MATERIAL AND METHODS

Specimens examined

A total of 258 adult specimens of *Austrolebias viarius, A. charrua, and A. reicherti* from 29 localities were collected during the rainy season (May to August, 2001–06) in annual ponds from Rocha, Treinta y Tres, and Cerro Largo Departments, Uruguay (Fig. 1). Fishes were sacrificed with an overdose of 2-Phenoxyethanol and fixed in 10% formalin or alcohol 95%. For microanatomy analyses fishes were kept in the laboratory in 30 l aquaria, filled with continuously aerated and dechlorinated tap water (pH 7–7.5), and exposed to natural light. Water was partially changed every 5 days. Water temperature was (19 ± 1) C. Fishes were fed once a day with live *Tubifex* sp. Spawning occurred daily from fish pairs isolated in aquaria that had containers with peat moss on the bottom. Eggs were collected with a pipette from the peat moss and they were subsequently cleaned by rolling them with a soft paintbrush on filter paper moistened with aquarium water.

Molecular population analysis

Sampling and DNA extraction

A total of 83 individuals used in the phylogeographic study (see Appendix: technique SA) were collected from 25 natural ponds covering the geographic range of *A. viarius, A. reicherti*, and *A. charrua* in Uruguay and *A. natchtigalli* from R. Grande do Sul, Brazil (Fig. 1). Tissues and voucher specimens were deposited in the Sección Genética Evolutiva and in the fish Collection of Facultad de Ciencias, Universidad de la República, Montevideo, Uruguay.

Genomic DNA was isolated from liver tissue of freshly sacrificed animals (fixed in ethanol 95%) using extraction with sodium chloride protein precipitation, followed by ethanol precipitation, as modified from Medrano, Aasen & Sharrow (1990).

Mitochondrial cyt B sequences

A 815-bp fragment of cyt *b* was amplified using primers CB3-H and Gludg-L (Palumbi *et al.*, 1991) using the polymerase chain reaction (PCR) cycle profile: 94 °C for 1 min, 45 °C for 1 min, and 72 °C for 1 min for 30 cycles). PCR products were cleaned with CONCERT Kit rapid PCR purification System (Life Technology), and amplified segments were sequenced in both strand directions using the same amplification primers with a Perkin-Elmer ABI Prism 377 automated sequencer. Sequence alignments were performed using CLUSTAL X (Thompson *et al.*, 1997).

Statistical analysis of mitochondrial cyt B sequences

Nucleotide composition and substitution patterns were calculated using the software MEGA (Kumar *et al.*, 2001) and DNASP4 (Rozas *et al.*, 2003). The corrected estimates of pairwise sequence divergence were obtained using the two-parameter algorithm (K2P) of Kimura (1980) implemented in MEGA. Within a population, DNA polymorphism was measured calculating the proportion of segregating sites (*S*), the haplotype diversity (Nei, 1987: 179), and the nucleotide diversity p (Nei, 1987: 257) using ARLEQUIN (Schneider, Roessli & Excoffier, 2000) and DNASP4 (Rozas *et al.*, 2003) software. Tajima's *D* (Tajima, 1989) was performed to test the significant excess of low-frecuency haplotypes aiming to evaluate the hypothesis of population expansion using ARLEQUIN software (Schneider *et al.*, 2000).

Analysis of molecular variance and nested clade analysis

To examine the genetic structuring among taxa included in the *A. adloffi* species complex and *A. viarius*, the variance components among hierarchical partitions in the data set were assessed by analysis of molecular variance (AMOVA; Excoffier, Smouse & Quattro, 1992). The Euclidean metric of Excoffier *et al.* (1992) was used to construct the matrix of pairwise distances. Because of the high phylogenetic relationship among *A. adloffi* species complex, each taxon was assigned to different groups and subregions. The genetic variation was partitioned into three components: among groups, among populations within groups, and among individuals, disregarding either their original populations or their groups.

Population subdivision and the level of genetic isolation among parapatric populations of these taxa were measured assuming an infinite sites model (Kimura & Crow, 1964). Pairwise estimates of Φ_{ST} were calculated using ARLEQUIN (Schneider *et al.*, 2000) to generate indirect pairwise estimates of gene flow: $N_f m½ [(1/F_{ST}) - 1]$ (Wright, 1951).

A nested clade phylogeographic analysis (NCA; Templeton, Routman & Phillips, 1995) was implemented to assess the historical and contemporary components of mitochondrial DNA variability among *A. adloffi* complex taxa. The parsimony network was reconstructed using an algorithm described by Templeton, Crandall & Sing (1992). This cladogram included the haplotypes belonging to the parapatric and sister taxa *A. charrua*, *A. reicherti,* and *A. nachtigalli* and was reconstructed using the software TCS, version 1.06 (Clement, Posada & Crandall, 2000). The final nested clade topology was obtained manually *sensu* Crandall & Templeton (1993, 1996). To test the null hypothesis that the haplotypes or clades nested within a high level nesting clade show no geographical associations given their overall frequencies, we used the software GEODIS, version 2.0 (Posada, Crandall & Templeton, 2000). This analysis produced

statistics related to the data distances: internal clade distance (D_c), between clades (D_n) and between interior and tip clades (I–T). We performed 1000 permutations, which is the number recommended for a 5% level of statistical significance. Results obtained from GEODIS were then interpreted using the revised inference key of Templeton (2004) to elucidate the alternative historical scenarios of taxa differentiation.

Molecular clock for cyt B sequences

A substitution rate of 2% per million years based on K2P divergence rates (only considering T_v = transversions) was assumed, as proposed for the genus *Cynolebias* (García *et al.*, 2000, 2002) and tested in other cyprinodontiforms (Mateos, Sanjur & Vrijenhoek, 2002). Although this molecular calibration is very approximate and is subject to considerable uncertainty, it was consistent with the complex Quaternary geological history of the southern Atlantic coastal wetlands (Sprechman, 1978; Montaña & Bossi, 1995).

Morphology

Meristics and pigmentation

Meristic variables analysed (N = 258; Appendix, technique MA) and their correspondent abbreviations were: dorsal fin rays (D), anal fin rays (A), caudal fin rays (C), pectoral fin rays (PC), pelvic fin rays (PV), lateral series of scales (LS), scales around the caudal peduncle (PS), predorsal series of scales (PRS), supraorbital scales (SS), and number of neuromasts in the supraorbital series (SN). Because vertical dark bands in males are characteristic of these fishes and their thickness is highly variable, the ratio between band thickness and band interspace was also calculated (for comparative purposes *A. adloffi* was included in this analysis).

Geometrical morphology

Shape variation was analysed through a geometric morphometry using the thin plate spline (Bookstein, 1991; Monteiro & dos Reis, 2000), methodological details *sensu* D'Anatro & Loureiro (2005). A discriminant function analysis was performed over the partial warp matrix and the uniform component scores generated by the software tpsRegr, version 1.24 (Rohlf, 2003) to examine the extent to which landmarks reveal phenetic clusters of individuals of different shape. Canonical axes were demonstrated in shape space by multivariate regression of partial warps, as well as uniform component scores on canonical scores of the two roots obtained with tpsRegr.

Because of a pronounced sexual dimorphism, data of males and females were analysed separately. Statistical differences between groups centroids was tested using multivariate analysis of variance (P = 0.05).

Gamete ultrastructural analysis

Scanning electron microscopy (SEM) studies of ripe oocytes obtained from females of the three species (see Appendix: technique GU) were processed *sensu* Arezo, Pereiro & Berois (2005). Oocyte surfaces were examined and photographed with a JEOL JSM 5900 LV scanning electron microscope.

From the SEM photographs, the thickness of 1800 filament bases (600 per species) belonging to eggs and females from at least three ponds per species was measured with the software IMAGEJ, version 1.38x (http://rsb.info.nih.gov/ij/). Given that the measures from the filaments of each egg did not fit a normal distribution, the medians were compared using the Kruskal–Wallis test among the intraspecific eggs. Measurements of all the eggs of each species were grouped as a single distribution. Normality was tested and the Kruskal–Wallis test applied again. A Mann–Whitney-Wilcoxon nonparametric test was performed among the species. $P < 0.05$ was considered statistically significant for all tests.

For transmission electron microscopy (TEM) analyses, three adult males of each of the three species were previously anesthetized with 2-phenoxyethanol 3 : 1000 and then killed by decapitation. Fragments of fresh testes tissue were fixed overnight at 4 °C in a 4% solution of paraformaldehyde and 2.5% glutaraldehyde in buffer phosphate 0.1 M (pH 7.4) and post-fixed in a 1% solution of osmium tetroxide in phosphate buffer, for 1 h at room temperature. The samples were processed by the standard procedure for TEM.

Observations were made with a JEOL JEM 1010 transmission electron microscope operating at 80 kV. Images were captured with a Hamamatsu C-4742-95 digital camera.

RESULTS

Mitochondrial cyt B variation among *A. adloffi* species complex and *A. viarius*

The present study included 83 mitochondrial cyt *b* sequences belonging to three taxa from the *A. adloffi* species complex and *A. viarius*. The GenBank accession numbers are provided in the Appendix.

Table 1 shows variable and phylogenetically informative sites among the 815 bp cyt *b* sequenced, as well as the number of haplotypes found in each taxon in samples of at least two individuals. High values of haplotype diversity have been found in the range 0.76–0.80 within the *A. adloffi* species complex and *A. viarius* (Table 1). Except for the most common haplo-

Table 1. Estimates of DNA polymorphism in three taxa: *Austrolebias viarius*, *Austrolebias reicherti*, and *Austrolebias charrua*

	Variables sites	Phylogenetically informative sites sites sites	S	Number of Haplotype diversity	Haplotype diversity	π	Kimura 2-P distance ($T_v + T_s$)	*D*
Austrolebias viarius	110	32	0.039	8	0.800 (0.092)	0.016 (0.006)	0.020 (0.008)	–1.4477 $P > 0.10$ (NS)
Austrolebias reicherti	104	24	0.029	12	0.900 (0.059)	0.013 (0.003)	0.032 (0.011)	–2.0717 $P < 0.05$*
Austrolebias charrua	125	36	0.044	11	0.761 (0.082)	0.020 (0.004)	0.034 (0.009)	–2.2036 $P < 0.05$*

S, proportion of segregating sites; Haplotype (gene) diversity (Nei 1987), π = nucleotide diversity (Nei, 1987). The mean (SD) of the corrected Kimura 2-P (1980) distances is shown. *D*, neutrality test (Tajima, 1989).
*Statistical significance ($P < 0.05$).

Table 2. Demographic inferences from nested geographic distance analysis for taxa of the *Austrolebias adloffi* species complex (*sensu* Templeton, 2001, 2004)

Clade	Inference chain	Inferred pattern
Clade 1-11	1-19- No	Allopatric fragmentation
Clade 1-12	1-2-3-5-6-13-14-No	Long distance colonization and/or past fragmentation (not necessarily mutually exclusive).
Clade 2-1	1-19-20-2-12-13-21-Yes	Long distance colonization Coupled with subsequent fragmentation or past fragmentation followed by range expansion.
Clade 2-2	1-2-3-5-15-21-Yes	Past fragmentation followed range expansion involving secondary contact.
Clade 2-3	1-2-3-5-15-No	Past fragmentation followed range expansion

types (haps1 and 2 in *A. charrua*; haps 1 and 3 in *A reicherti* and hap 3 in *A. viarius*), the remaining haplotypes showed a frequency of 1/*N*, representing rare alleles restricted to one collecting site and explaining the high observed haplotype diversity. The proportion of segregating sites (*S*) ranged from 0.029 in *A. reicherti* to 0.044 in *A. charrua*. The nucleotide diversity (*p*) ranged from 1.3% in *A. reicherti* to 2.0% in *A. charrua*. The average of the corrected K2P sequence divergence (Table 1) ranged from 2.0% (SE = 0.008) in *A. viarius* to 3.4% in *A. charrua* showing the highest value (SE = 0.009).

The pairwise K2P sequence divergence among the taxa of the *A. adloffi* species complex and *A. viarius* showed the greatest divergence between *A. viarius* and *A. reicherti* (21.5%, SE = 0.023), whereas the most genetic similarity was found between *A. charrua* and *A. natchigalli* at 5.7% (SE = 0.008).

NCA, AMOVA, AND POPULATION DEMOGRAPHIC PARAMETERS

Gene genealogy reconstruction resulted in a cladogram (Fig. 2) that could be connected with 95% confidence level using the statistical parsimony approach implemented in TCS (Templeton, Boerwinkle & Sing, 1987). Among 27 clades obtained for step-clades from 1–3, no significant distances were found (D_c, D_n or *I–T*) excepting for two one-step clades (clades 1-11 and 1-12) and three two-step clades (2-1, 2-2 and 2-3) (Table 2). These two-step clades group ten haplotypes from *A. charrua* (2-1 clade), whereas all haplotypes from *A. reicherti* were associated with the 2-2 and 2-3 clades. Most of the species have one or more central haplotypes conforming star-like topologies. Within *A. charrua* (2-1 clade), the central and ancestral haplotype 11 from pond number 66 (Fig. 2), which was restricted to upland ponds, connected through five missing haplotypes to the more frequent haplotype 1, which shows an extensive geographic distribution throughout the Atlantic coastal wetlands. Also, only five mutations separated haplotype 11 from its sister taxon, *A. natchigalli*. Remarkably, the haplotype 11 of *A. charrua* connected with haplotype 6 of *A. reicherti* through nine missing haplotypes. Based on present NCA, the most geographically distant haplotype 12 of *A. reicherti* shows the

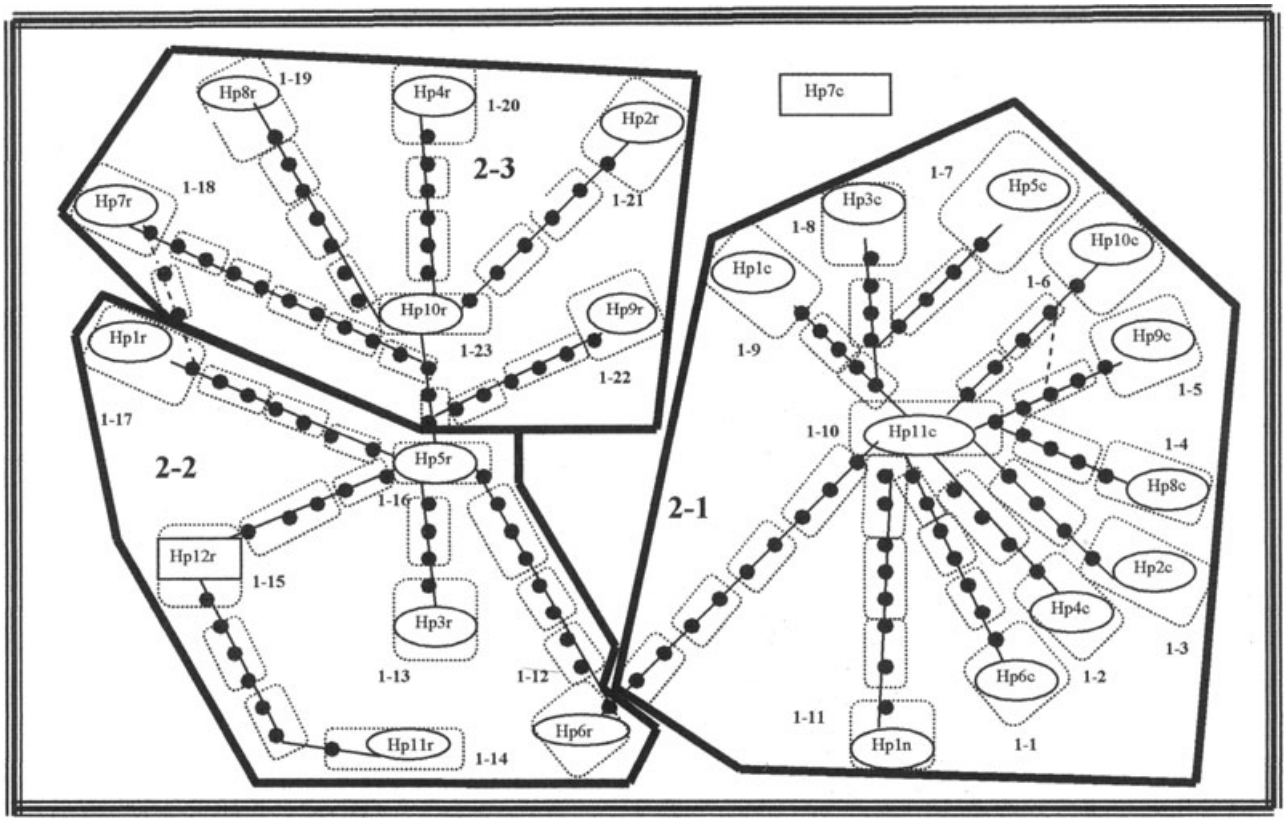

Figure 2. Maximum parsimony network and the corresponding nested design for cytochrome *b* haplotypes for the sister taxa of the *Austrolebias adloffi* species complex: *Austrolebias charrua* (step-clade 2-1), *Austrolebias reicherti* (step-clades 2-2 and 2-3), and *Austrolebias nachtigalli* (step-clade 1-11). The cladogram was estimated under the 95% statistical limits of parsimony using the algorithm described in Templeton *et al.* (1992). Oval includes haplotypes number (0-step clades). Solid circles represent hypothetical haplotypes. Thin-lined polygons enclose one-step clades; thick-lined polygons enclose two-step clades and the thick line includes the total cladogram. Specimens and the respective haplotype are listed in the Appendix.

highest outgroup probability (Fig. 2). Haplotype 5 and 10, which represent the central haplotypes at clades 2-2 and 2-3, respectively, were separated through three missing haplotypes. Except haplotype 1, which was distributed in four ponds from the southern and northern Tacuarí River, the remaining haplotypes inhabit different and single ponds.

The analyses of molecular variance of all taxa indicates that most of the genetic variation among the cyt *b* haplotypes was distributed among groups (84.73%), suggesting a high level of population structure among these taxa. Low levels of variation were found within population (15.20%).

Table 1 shows a significant excess of low-frequency haplotypes in *A. charrua* ($D = -2.2036$, $P < 0.05$) and *A. reicherti* ($D = -2.0717$, $P < 0.05$).

The indirect estimate of gene flow from mitochondrial haplotypes reveals the existence of no genetic exchange among *A. charrua*, *A. reicherti*, and *A. viarius*. Remarkably, within each taxon, we estimated a highly variable level of gene flow. Some populations of *A. charrua* (ponds 6 and 8–9) and of *A. viarius* (ponds 10–16 and Pirarajá-Retamosa) remain partially isolated from the rest. The indirect estimate of gene flow suggests genetic exchange between haplotypes of *A. nachtigalli* and *A. charrua* in pond 32 at the Southern Merin Lagoon.

Molecular clock estimate

We suggest that lineage differentiation among the studied taxa occurred in a 'wave' of cladogenesis events. The most recent events are estimated to have occurred 450 000 years ago and 1.0 Mya (mid and late Pleistocene) and resulted in the split of *A. nachtigalli* and *A. reicherti* from *A. charrua* populations. A second and basal event, as old as 3 Mya (early Pleistocene), represents the divergence between *A. viarius* and the

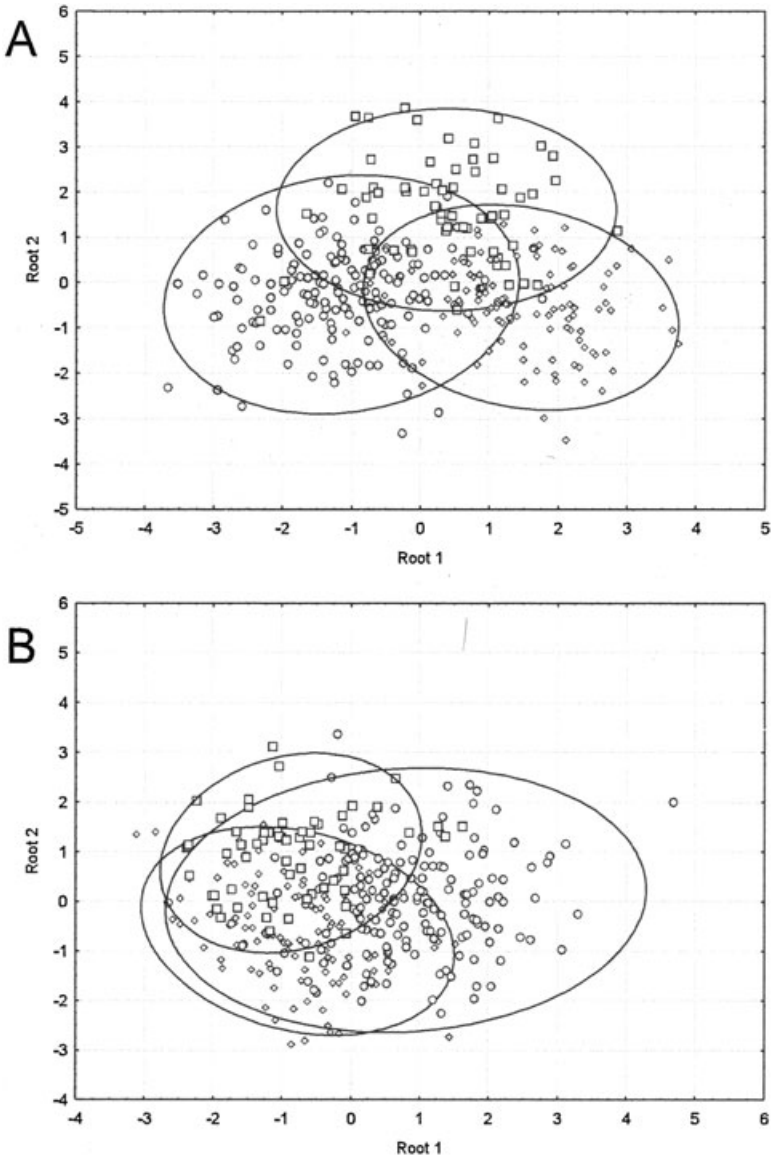

Figure 3. Discriminant analysis of meristic characters of the species analysed. A, Males. B, Females. Circles, *Austrolebias charrua*; diamonds, *Austrolebias viarius*; squares, *Austrolebias reicherti*.

A. adloffi species complex. These time computations are tentative and cannot be indirectly verified because of the lack of a fossil record for Rivulidae.

MORPHOLOGY

Meristics and pigmentation

The canonical analysis of meristic data showed a high overlap between species in both sexes (Fig. 3); however, the overlap was greater in females where the variability of *A. charrua* includes that of the other two species. Concerning the coloration design analysed (ratio band/interband space), variability was greatest in *A. viarius* males, followed by *A. charrua*. In *A. reicherti*, as well as in *A. adloffi* (used for comparative purposes), variability of this character was similar and extremely low (Fig. 4)

Morphometric analysis

Analysis of males showed partial discrimination among species (8% of total individuals misclassified), although group centroids were significantly different ($F = 6.33$; $P = 0.00$). Squared Mahalanobis distances between group centroids are shown in Table 3. Root 1 discriminated between *A. reicherti* and the other two species, with shape changes associated with an elongation of the head and an anterior position of

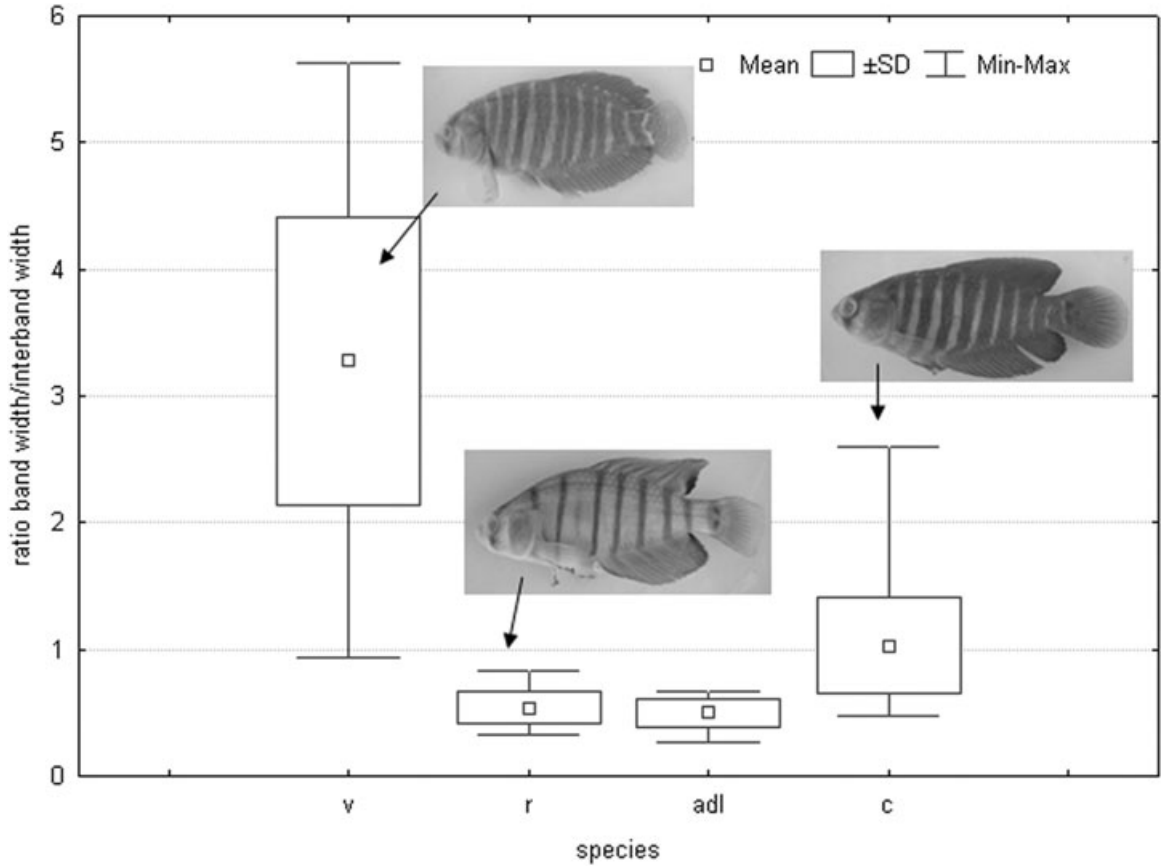

Figure 4. Box plot of band thickness and inter band space ratio. v, *Austrolebias viarius*; r, *Austrolebias reicherti*; adl, *Austrolebias adloffi*; c, *Austrolebias charrua*.

Table 3. Squared Mahalanobis distances between group centroids of the geometrical morphometric analysis

	Austrolebias viarius	*Austrolebias charrua*	*Austrolebias reicherti*
Austrolebias viarius	–	17.8	12.30
Austrolebias charrua	7.9	–	9.12
Austrolebias reicherti	12.8	9.9	–

Females over diagonal; males below diagonal.

dorsal fin (Fig. 5). Root 2 discriminated between *A. charrua* and the other species, with shape changes associated with a decrease in head size and an elongation of the base of the anal fin (Fig. 5). Females showed more discrimination in shape than males (6% of total individuals misclassified), as well as significant differences among groups' centroids.

($F = 9.01$, $P = 0.00$). Squared Mahalanobis distances between group centroids are shown in Table 3. *Austrolebias viarius* was located in the negative values of Root 1, associated with a head size increase and a posterior displacement of pelvic and anal fins; *A. charrua* was located in the positive values (with opposite shape changes), whereas *A. reicherti* presented an intermediate shape (Fig. 6). Furthermore, Root 2 discriminated *A. reicherti* from the other two species; shape changes were associated with a decrease in body depth (Fig. 6).

Gamete ultrastructure

Vitelline envelope surface

SEM analyses of eggs' envelopes of the three species show a rough outer surface ornamented by dense hair-like projections of different thickness. The filaments of all species have an overall cone-shaped morphology (Fig. 7A).

The quantitative data resulting from measurements of the filament bases are summarized in box-and-whisker plots (Fig. 7B, C, D, E). Individuals of the same species showed statistically significant dif-

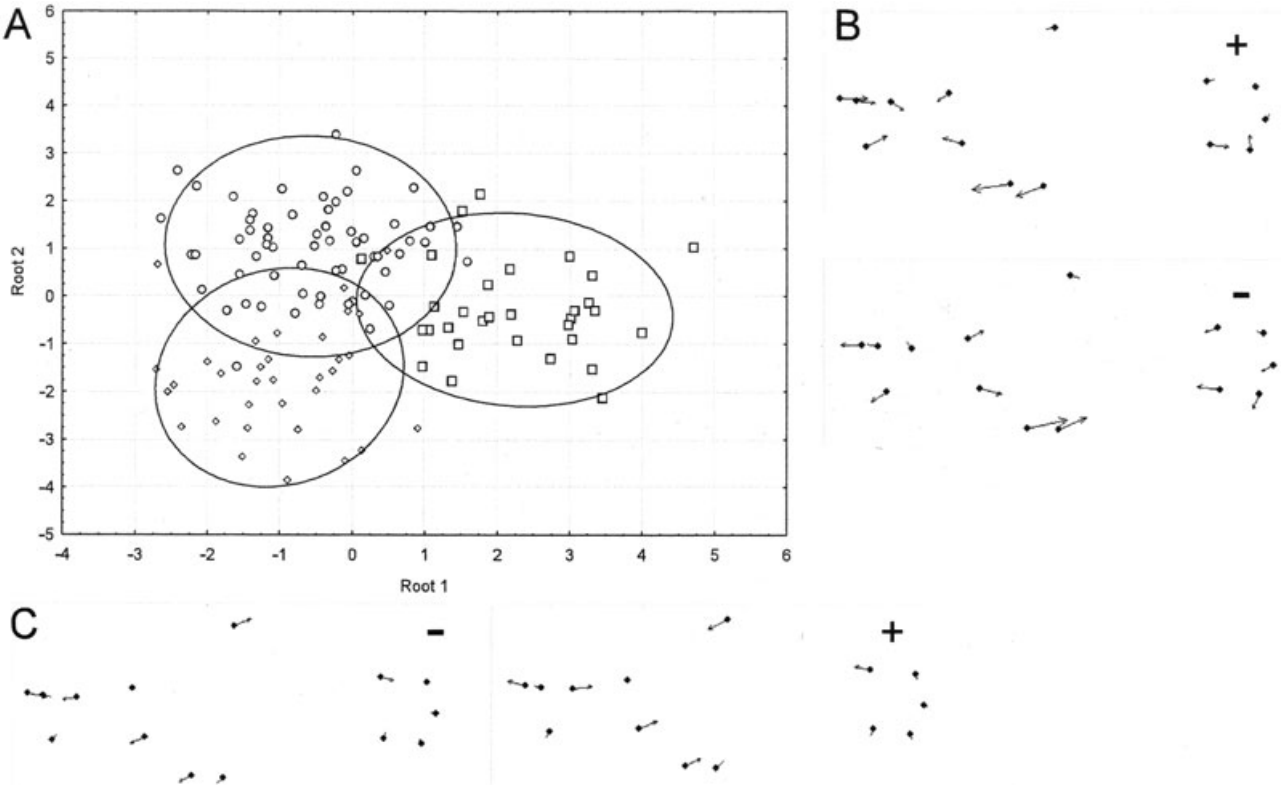

Figure 5. A, discriminant analysis of male morphometry of the species analysed. B, shape changes associated with Root 2. C, shape changes associated with Root 1. Circles, *Austrolebias charrua*; diamonds, *Austrolebias viarius*; squares, *Austrolebias reicherti*. +, positive values; –, negative values. The magnitude of arrows is magnified three times.

ference among the medians (*A. charrua*: $W = 145.966$, $P = 0$; *A. reicherti*: $W = 29.9626$, $P < 0.01$; and *A. viarius*: $W = 167.617$, $P = 0$). The test comparing the species produced the same results ($W = 12.4893$, $P < 0.01$). When the Mann–Whitney Wilcoxon test was applied, a statistically significant difference appeared between *A. viarius*/*A. reicherti* ($W = 192\,197.00$, $P = 0.0414$) and *A. viarius*/*A. charrua* ($W = 201\,314.00$, $P < 0.01$). Nevertheless, there is no statistically significant difference between the medians of *A. reicherti* and *A. charrua* ($W = 188\,173.00$, $P = 0.1714$).

Sperm flagellum

The three species exhibit sperms with a long flagellum with wing-like lateral periaxonemal fins, which show differences among species. In *A. reicherti* (Fig. 8A) and *A. viarius* (Fig. 8B), most spermatozoa have two lateral fins. Nevertheless, in *A. reicherti*, it is possible to occasionally find some gametes with three lateral extensions. In *A. charrua*, the number of these projections is especially variable, demonstrating from two to four fins in the same specimen (Fig. 8C).

DISCUSSION

Phylogeographic pattern and historical demographic scenarios in the *A. adloffi* species complex

The cyt *b* analyses supports the high levels of DNA polymorphism, in particular in *A. charrua* (Table 1), previously reported for *Austrolebias* (García, 2006). The levels of polymorphism are similar to those published for species of *Cyprinodon* from Mexico based on mitochondrial D-loop sequences (Strecker, 2006).

Nested clade phylogeographic analysis yielded a cladogram, with a 95% degree of confidence, in which ten *A. charrua* haplotypes (including that of *A. nachtigalli*) and all of *A. reicherti* haplotypes remain connected (Fig. 2). Nevertheless, the results of the AMOVA strongly support the genetic distinctiveness among these taxa, supporting the validity of their taxonomic status of the recently described species.

The NCA shows two major distinct lineages corresponding to *A. charrua* (clade 2-1), including the *A. nachtigalli* haplotype and *A. reicherti*. The later taxon further splits into two main lineages: clades 2-2 and 2-3. These step-clade of level 2 remain connected

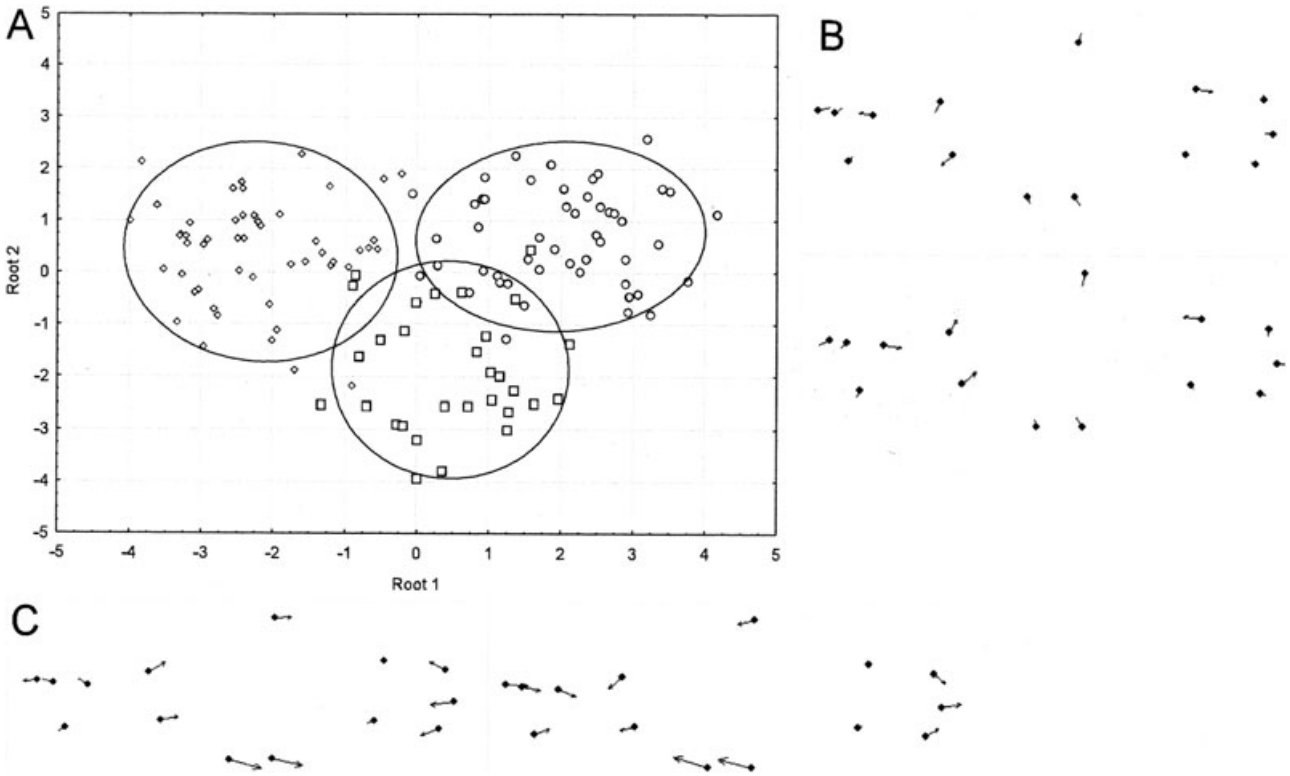

Figure 6. A, discriminant analysis of female morphometry of the species analysed. B, shape changes associated with Root 2. C, shape changes associated with Root 1. Circles, *Austrolebias charrua*; diamonds, *Austrolebias viarius*; squares, *Austrolebias reicherti*. +, positive values; –, negative values. The magnitude of arrows is magnified three times.

through haplotypes 11 of *A. charrua* and six of *A. reicherti*, which inhabit the pond 55 in a border area between the distribution of both taxa (Figs 1, 2).

The results of the present study agree with previous phylogenetic analyses in which *A. viarius* represents the sister taxon to the *A. adloffi* species complex (Costa, 2006; García, 2006), whereas *A. charrua*, *A. nachtigalli*, and *A. reicherti* appear as sister taxa within such species complex (García *et al.*, 2004; García, 2006).

In accordance with the biological key of Posada, Crandall & Templeton (2006), for the majority of clades, we cannot reject the null hypothesis. However, based on the statistical significant values found in any step-clades (Fig. 2; Table 2), allopatric fragmentation, long-distance colonization, and/or past fragmentation could be inferred from the present data set. Subsequently, we incorporated Templeton's supplementary test for secondary contact, which confirmed such hypothesis. *Sensu* Templeton (2001), when secondary contact occurs between previously fragmented populations, haplotypes or clades with very divergent geographical centres can be placed at the same locality or in the neighbouring locality, as could be observed in the present study in relation to clade 2-1 (*A. charrua*) and clade 2-2 (*A. reicherti*) and their peculiar connections through the haplotype 6 from *A. reicherti*. The location of contact between the major lineages (clade 2-1 versus clades 2-2, 2-3) is consistent with two waves of population expansion diverging over the Northern and the Southern Tacuari river, respectively, and subsequently meeting at the Southern one (Fig. 1). A potential demographic expansion scenario is also supported by the star-like topologies around *A. charrua* clades 1-10 and *A. reicherti* clades 1-16 and 1-23 (Fig. 2) and in the significantly negative Tajima's *D*-values found in both taxa (Table 1). Because statistics D cannot discriminate between the patterns of haplotype diversity generated under this model and those generated under selection against slightly deleterious mutations in the cyt *b* gene, both hypotheses are nonmutually exclusive with the present data. Therefore past allopatric fragmentation and range expansion involving perhaps secondary contacts could be the most plausible explanation. This hypothesis is congruent with previous molecular population analyses of the *A. adloffi* species complex (García *et al.*, 2004; García, 2006). Moreover, the current hydrological pattern of flooding in the area could support this scenario. When the Cebollatí river

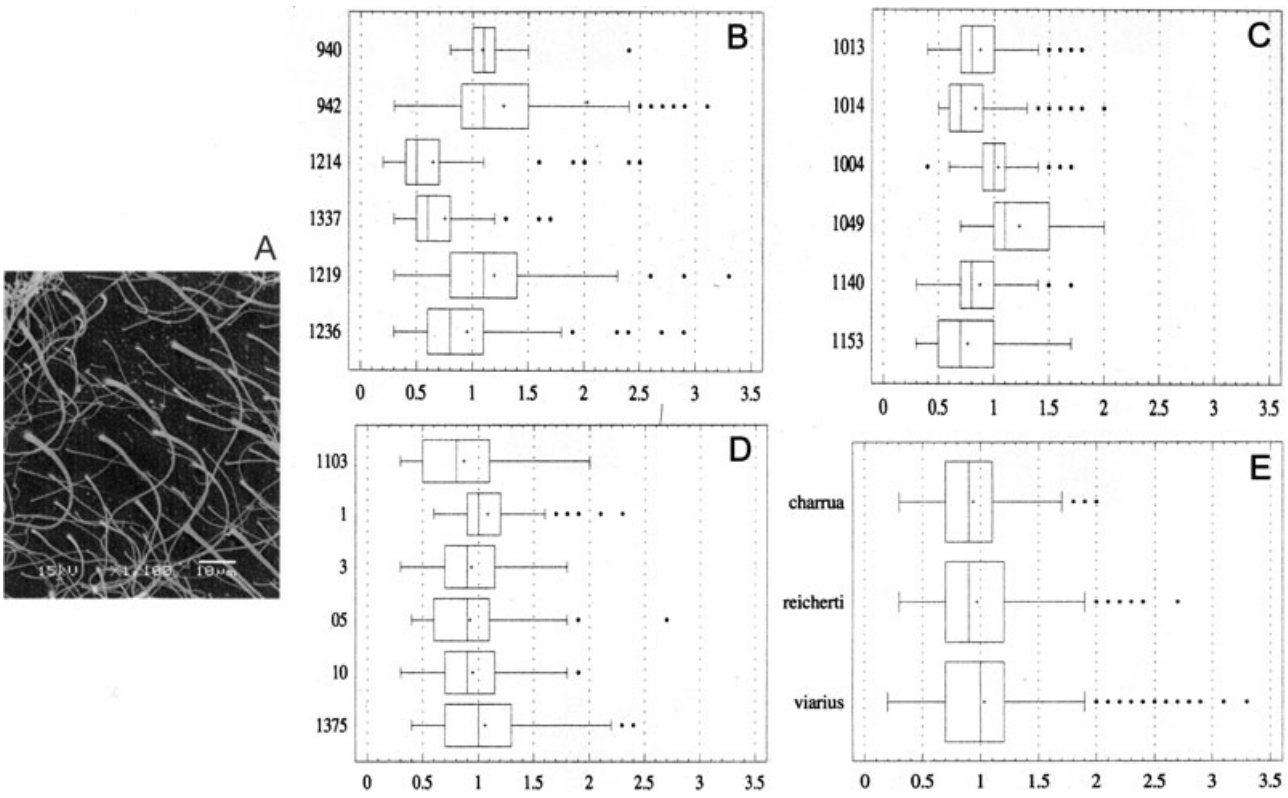

Figure 7. Ultrastructural analyses of the surface of the oocyte. A, scanning electron microscopy of the vitelline envelope of *Austrolebias reicherti* showing the hair-like filaments. The image was selected as representative for the three species. B, C, D, box-and whisker diagram of the distribution of the data for *Austrolebias viarius*, *Austrolebias charrua*, and A. reicherti, respectively. E, box-and whisker diagram showing the distribution of the data for the three species taking every one as a whole. The *y*-axis shows the individuals or species and the x-axis represents the measures (in μm). A cross indicates the mean, the middle line of the boxes indicates the median, the right of box indicates the 75th quartile and the left of box indicates the 25th quartile. Dots represent outliers.

outflows, the excess water flows southward where the remaining localities of *A. charrua* occur, favouring a population dispersion from northern and higher localities to southern and lowland localities. In this context, it has to be considered that lowland localities were colonized more recently because they were the most affected by recent marine transgressions reported in the region (Sprechman, 1978; García-Rodriguez & Witkowaki, 2003).

The indirect estimate of gene flow based on present data set reveals the current lack of genetic exchange among *A. charrua*, *A. reicherti*, and *A. viarius*; therefore, in most cases, the former taxa could represent incipient species formed at a distance. Nevertheless, the wide range of gene flow values within each taxon could indicate that this factor is not homogeneous among ponds. Individuals from pond 66, located in the higher lands of the Merin Lagoon basin, showed a Nm values between 0.5 and 1 (or higher than) in relation to the remaining ponds of the same taxon and to those of *A. reicherti* and *A. nachtigalli*. This might suggest that current gene flow is a weak genetically cohesive factor but probably sufficient to retain genetic relationships among populations of these taxa. Jiggins & Mallet (2000) reported that parapatric populations surveyed by them showed secondary contact between previously allopatric taxa, with all degrees of gene flow depending on the amount of reproductive isolation that had evolved in allopatry. Therefore, based on corrected pairwise genetic distances, NCA (Fig. 2), and the indirect estimates of gene flow, *A. nachtigalli* remains integrated genetically within *A. charrua* populations and no reproductive isolation from this taxon was detected.

However, we cannot rule out that the intermediate haplotype 6 from *A reicherti* located between the range of expansion from both major clades including *A. charrua* and *A. reicherti* haplotypes (2-1 versus 2-2 and 2-3 clades) could reflect an incomplete lineage sorting after the occurrence of past fragmentation and cladogenetic events during the Pleistocene.

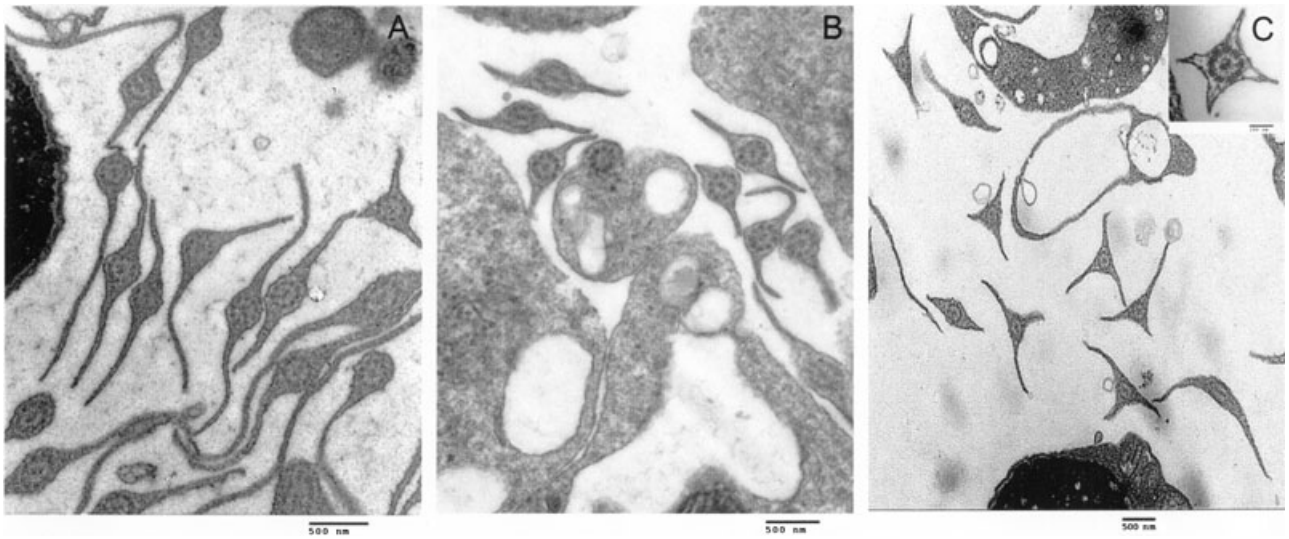

Figure 8. Transmission electron micrographies of transversal sections of sperm flagellum of three *Austrolebias* species: *Austrolebias reicherti* (A), *Austrolebias viarius* (B), and *Austrolebias charrua* (C). The flagellum has the 9 + 2 axonemal doublet configuration. Note that the presence of two lateral membrane projections is the most common character in (A) and (B), whereas, in (C), the flagellum show two up to four (insert) lateral fins. Scale bar in (A, B, C) = 0.5 μm; insert = 0.1 μm.

Evolutionary scenarios for taxa differentiation versus morphological features

The present morphological analyses yielded different possible scenarios for taxa differentiation. Meristic variation observed in females favours a secondary contact event because *A. charrua* variation includes and surpasses the other two species considered. However, the variation in colour patterns in males considered in the present study does not support this view. Although *A. reicherti* and other related species of the *A. adloffi* complex present a pattern of thin dark lateral bands on body, *A. charrua* presents variation of this character similar to the parapatric and more ancient *A. viarius*. An alternative hypothesis about the retention of ancestral characters in *A. charrua* cannot be excluded. Morphometric results in males show the same pattern (i.e. males of *A. charrua* are more similar to *A. viarius* than to *A. reicherti*).

Furthermore, another possible scenario yielding similarities between *A. charrua* and *A. viarius* males could be the shared ecological context. Both species are sympatric and even syntopic in some ponds with other three annual fish species that are considered to be the top predators of temporary ponds. Because males are more exposed to predation as a result of their active courtship behaviour (see below), similarity in body shape and coloration patterns could be a response to predator selective pressures.

Finally, sexual dimorphism in annual fishes involves colour patterns (i.e. males with conspicuous pigmentation in body and fins), morphology (i.e. males with longer unpaired fins), and courtship behaviour (Vaz-Ferreira *et al.*, 1964; Belote & Costa, 2004; García, Loureiro & Tassino, 2008). This also appears to be reflected in differences in morphological discrimination between males and females. At the level of meristic and coloration characters, females are more homogeneous across species (present study) and within species (D'Anatro & Loureiro, 2005) than males. All this evidence points to sexual selection, especially female choice, as representing a strong force in annual fishes evolution. However, when body shape is considered, females are more differentiated than males, as expressed by the lower percentage of misclassification and greater Mahalanobis distances among group centroids. A more conservative body shape in males points to other sexual selection mechanism, such as male competition for mates. This behaviour has already been observed in other species of the genus (Vaz-Ferreira *et al.*, 1964) as well as in *A. reicherti* (B. Tassino, pers. comm.).

Gametic isolation is likely to be important in preventing current gene flow in many species. Such isolation probably evolves rapidly, at least in animals, because gametic barriers may be a by-product of sexual selection (Coyne & Orr, 2004).

Studies on sperm and oocyte envelope ultrastructural features have been proposed as useful characters to be considered for identification of different

teleost species (Jamieson, 1991; Li, Wu & Yang, 2000; Esmaeili & Johal, 2005; Gwo *et al.*, 2006). According to the present study, data from gamete ultrastructure might also prove useful for inferring phylogenetic relationships and possible mechanisms of pre-zygotic isolation among parapatric taxa.

SEM analyses of egg envelope in *A. viarius* (Arezo *et al.*, 2005), *A. charrua* (Arezo *et al.*, 2007), and *A. reicherti* (present study) revealed a rough outer surface (sticky *in vivo*) that was ornamented by prominent filaments. The overall morphology was similar in the three species not showing a species-specific pattern. However, quantitative data from filament base thickness indicate statistical significant differences when *A. viarius* was compared with *A. reicherti* and *A. charrua*, respectively. Nevertheless, there were no statistical significant differences between *A. charrua* and *A. reicherti*, suggesting that these two species are more closely related. These results are concordant with the high phylogenetic relationships emerging from the NCA discussed above.

The species of *Austrolebias* studied possess a typical uniflagellate anacrosomal aquasperm. The presence of periaxonemal lateral membrane projection is a frequent trait in the sperm of many species of fishes and a general condition for Atherinomorpha (Jamieson, 1991). It was suggested that the occurrence of this character most likely contributes to increase the efficiency of flagellar movement (Thiaw *et al.*, 1986). Although *A. viarius* and *A. reicherti* agree with the general pattern of two flagellar projections, *A. charrua* presents a high level of polymorphism, demonstrating from two up to four wings even in the same individual. Similar results were previously reported for seven genera and 15 species of teleostean fishes of the family Cyprinodontidae, which shows a wide diversity of structure of the spermatic flagellum (Thiaw *et al.*, 1986). Sperm with two and sometimes three lateral fins in the same specimen were also detected in *A. reicherti* but at a lower frequency than in *A. charrua.* This pattern of lateral fins shared by both taxa suggests their close relationship. These findings imply that the morphology of the sperm tail may be an additional character to suggest a phylogenetic relationship among *Austrolebias* species.

Gamete isolating mechanisms should be common among closely-related taxa, especially geographical isolated populations of a single species. This type of isolation may entail more than one type of evolutionary change (Coyne & Orr, 2004). Conversely, morphological gamete similarities detected in the present study between *A. reicherti* and *A. charrua* (*A. adloffi* complex) support the idea that these highly-related taxa have emerged from more recent cladogenetic events.

CONCLUSIONS

The results from the combined analyses developed in the present study suggest the existence of chronological events in the differentiation of the studied parapatric taxa.

First, past allopatric fragmentation and range expansion possibly involving secondary contact could be a plausible hypothesis of differentiation among the *A. adloffi* species complex. Events of explosive speciation involving *A. charrua* and *A. reicherti* likely occurred during the Late Pleistocene (1.25 Mya to 450 000 years ago) in agreement with a molecular clock estimate based on cyt *b* sequences and in accordance with the geological scenarios in this region. Gametic morphology similarities support their more recent separation. Two waves of population expansion of *A. reicherti*, diverging over the Northern and the Southern Tacuari river and meeting *A. charrua* secondarily in the lower Ceboll-latí River basin, are proposed in the present study. This area could represent a parapatry/sympatry intergradation zone reflecting a current localized introgression.

Alternatively, the observed phylogeographic pattern could be explained through an incomplete lineage sorting after explosive cladogenetic events during the Pleistocene. Therefore, these species within the *A. adloffi* complex show evidence of hybridization, lineage sorting, or both.

Additionally, the lowlands where these species are distributed are subject to strong impacts. The demand for land for agricultural activities such as rice crops leads to wetland draining, with the potential of completely drying out the temporary ponds that comprise their habitat. The present study reinforces the inclusion of the relictual group of *A. charrua* populations from the highland ponds, which preserve ancestral polymorphisms, in a priority conservation program.

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APPENDIX

Appendix 1. Detailed list of localities, geographic coordinates, GenBank access numbers, and Facultad de Ciencias Vertebrates Collection Institutional Code (ZVC-P) of specimens used in this article

Species	Locality	Latitide/longitude	GenBank accession number	ZVC-P	Analysis
Austrolebias charrua	Dept. Rocha (Uruguay)				
	Road 19 km 6.5–7 (ch33)	33°41′19″S/53°31′36″W	AY724390, AY724391, AY724379-AY724380	900	S, M
	Road 9 km 272 (ch6)	34°12′54″S/53°46′18″W	AY724377	4178	S, M
	Road 13 and Road 16 (ch8)	34°03′34″S/53°51′17″W	AY724390-AY724409	4135	S, M
	Road 14 km 489.5 (ch28)	33°54′09″S/53°40′38″W	AY724400	3886	M
	Road 9 km 302	34°01′39″S/53°35′35″W		3954	S, M
	Road 9 km 336.5 (ch32)	33°45′19″S/53°26′09″W	AY724399, AY724378-AY724410, AY724404	3952	S, M, G
	Road 19 km close to San Luis stream (ch35)	33°36′03″S/53°43′29″W		3943	M, G
	Road 15 km 151.5 (ch37)	33°35′04″S/54°04′10″W	AY724381, AY724406, AY724405	3953	S, M
	Road 16 km 34.5 (ch38)	33°59′48″S/53°49′03″W		3948	M, G
	Road 15 km 173 (ch44)	33°28′13″S/53°52″11″W		4137	S, M
	Road 9 km 272 (ch50)	34°12′18′S/53°46′28″W		4134	S, M
	Dept. Treinta y Tres (Uruguay)				
	Road 91, close to Corrales del Parao stream (ch54)	33°00′06″S/53°52′22″W	AY724390, AY724390-AY724389	4175	S, M
	Road 8, close to treinta y tres city (ch66)	33°13′32″S/54°23′45″W			S
	Road 91 10 km to the North of Charqueada town (ch53)	33°09′04″S/53°52′27″W		4171	S, M
Austrolebias viarius	Dept. Rocha (Uruguay)				
	Road 10 km 250 (ch1)	34°28′35″S/53°59′50″W	AY724408	3967	S, M
	Road 10 km 253.5 (ch3)	34°27′09″/53°56′37″W		3965	M, G
	Road 16 km 2.5 (ch11)	34°16′18′S/53°47′58″W	AY724383, AY724384, AY724408, AY724382	3969	S, M
	Road 10 km 266.5 (ch4)	34°22′09′S/53°50′32″W	AY724387, AY724386	4173	S, M
	Road 9 km 228.5 (ch17)	34°24′10′S/54°07′26″W		3970	M, G
	Road 9 km 272 (ch23)	34°12′55′S/53°43′16″W	AY724385	3975	S, M
	Road 9 km 272 (ch49)	34°12′44″/53°46′31″W		4181	M, G
	Road 10 and Road 16 (ch5)	34°16′24′S/53°47′51″W	AF245456	4209	S, M
	Dept. Lavalleja (Uruguay)				
	Road 8 close to Pirarajá stream	33°42′31′S/54°42′47″W			S
Austrolebias reicherti	Dept. Treinta y Tres (Uruguay)				
	Road 18 km 369.5 (ch42)	32°46′51′S/53°38′40″W	AY724392, AY724401, AY724402, AY724403	4177	S, M, G
	Road 18 close to Vergara town (ch43)	32°55′21′S/53°54′49″W	AY724398, AY724396	3933	S, M, G
	Road 91, 39 km North of Charqueada town (ch55)	33°01′34′S/53°52′46″W	AY724397, AY724395, AY724393, AY724394	4176	S, M
	Road 18 km 369.5 (ch56)	32°46′51′S/53°38′40″W		3934	M, G
	Dept. Cerro Largo (Uruguay)				
	Road to Lago Merín town (ch58)	32°42′04′S/53°18′32″W		4182	M
	Road 26 5 km from Rio Branco city (ch59)	32°34′34′S/53°26′20″W		4337	M
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	Road BR 116 km 15		AY724407		S

S, sequence analyses; M, morphological analyses; G, gamete ultraestructural analyses.