Quaternary refugia and secondary contact in the southern boundary of the Brazilian subregion: comparative phylogeography of freshwater fish

María C. Bruno [1]*, Jorge R. Casciotta [2,5], Adriana E. Almirón [2], Fernando L. Ricillio [3] & Marta S. Lizarrade [4]

[1] Departamento de Biología, Facultad de Ciencias Exactas y Naturales, Universidad Nacional de Mar del Plata, Argentina, Dean Funes 3350, B7602AYL, Mar del Plata, Argentina — [2] División Zoología Vertebrados, Universidad Nacional de La Plata, Paseo del bosque s/n, B1900FWA Argentina — [3] Cátedra de Histología y Embriología Animal, Facultad de Ciencias Naturales y Museo, Universidad Nacional de La Plata, 122 y 60, B1900FWA, Argentina — [4] Centro Regional de Estudios Genómicos, Facultad de Ciencias Exactas, Universidad Nacional de La Plata, Blvd. 120 No. 1459, B1900FWA, Argentina — [5] Comisión de Investigaciones Científicas de la Provincia de Buenos Aires. Camino General Belgrano s/n, 1900 La Plata, Argentina — * Corresponding author: María Cecilia Bruno: mcecibruno(at)gmail.com

Abstract

Freshwater fish are an ideal model to illustrate how climate-tectonic changes affect the distribution of genetic variation. Freshwater bodies are extensively affected by environmental changes, with streams even changing their courses in the most extreme cases. Fortunately, this situation is reflected in the genetic composition of populations and may currently be inferred from the study of mitochondrial DNA molecular markers. Here we analyze and compare the phylogeographic patterns of the species *Corydoras paleatus* and *Jenynsia multidentata* at the southern limit of the Brazilian subregion. These basins are isolated in the current hydrogeographic pattern due to geologic and paleoclimatic changes. Our results support a concurrent pattern for both species. Some lineages have persisted in the area under adverse climate conditions, possibly in environmental refuges, while other lineages may have colonized the area later by means of paleodrainages connections. In addition, the presence of independent, greatly diverging lineages, even within the same watercourses, suggests secondary contact between these lineages. This work represents a first approach to understand how geologic and paleoclimatic changes have affected the distribution of genetic variation in the Southern Pampean Area.

Key words

Introduction

The distribution of genetic variation in freshwater organisms is affected by the configuration and the geologic evolution of the basins where they occur. Freshwater fishes show strong genetic structure as a result of their confinement to given hydrologic systems (Faulks *et al.* 2010; Loxterman & Keeley 2012).The diverse factors which influence basin morphology and dynamics may be major barriers that limit the dispersal capacity of freshwater organisms. When such barriers arise, they restrict gene flow, thus increasing the differentiation of populations. In contrast, when no barriers are present, gene flow tends to homogenize the populations (Hutchinson & Templeton 1999; Allendorf & Luikart 2007; Loxterman & Keeley 2012). The use of DNA markers in combination with
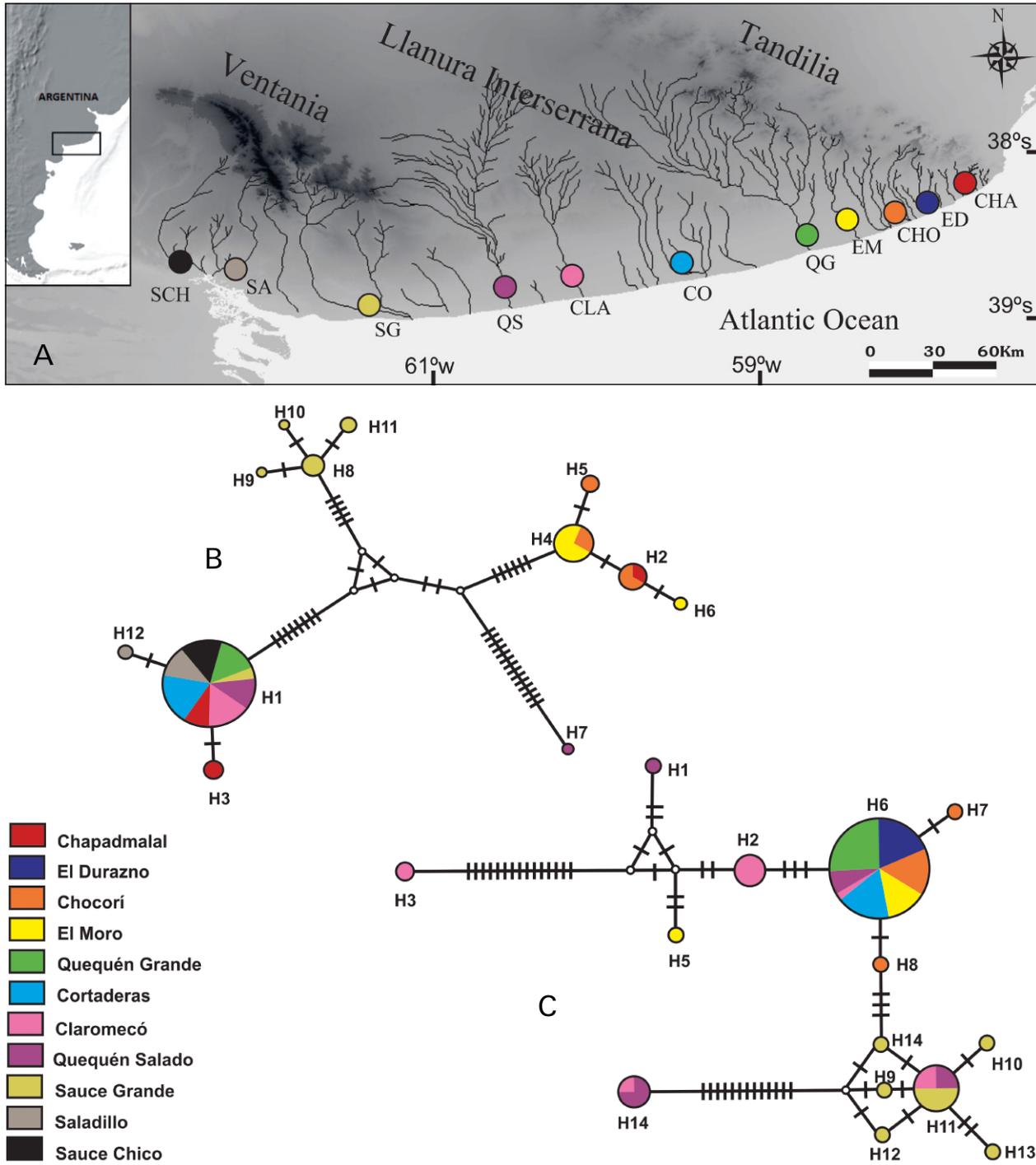
Fig. 1A. Southern Pampean Area including sample localities.CHA: Chapadmalal. ED: El Durazno. CHO: Chocorí. EM: El Moro. QG: Quequén Grande. CO: Cortaderas. CLA: Claromecó. QS: Quequén Salado. SG: Sauce Grande. SA: Saladillo. SCH: Sauce Chico. **B.** Median-Joining haplotype network for *Jenynsia multidentata*. **C.** Median-Joining haplotype network for *Corydoras paleatus*. Circle sizes are proportional to haplotype frequencies. The crossed marks are nucleotide substitutions inferred in that branch. White circles represent hypothetical haplotypes. H1–H14: haplotypes.

paleoclimatic reconstruction provides new information on the evolution of species during the climatic oscillations of the Quaternary. Such analyses can identify genetic subdivision and its geographical pattern across a species range, and provide attenuated signals of past demographic and geographic changes (HEWITT 2004).

The Neotropical region is divided into the Austral and Brazilian subregions according to fish distribution (CASCIOTTA *et al*. 1999). The richest ichthyofauna occurs in the Brazilian subregion, extends to the drainages south of Buenos Aires province, Argentina. The Southern Pampean Area, in the Buenos Aires province

(Fig. 1A) is the southernmost limit of the Brazilian sub-region. The area is an extensive plain grassland whose general monotony is interrupted by two northwest-southeast-trending sierras ranges. These ranges are Ventania (Sierras Australes) and Tandilia (Sierras Septentrionales). The plain between both ranges is known as Llanura Interserrana Bonaerense (Bonarean Intersierran Plain) (CASCIOTTA *et al*. 1999). Currently, the water courses within the Southern Pampean Area run parallel, and are therefore unconnected to each other; and they are also to the basins that limit this area. The fish fauna of these basins is one of the last assemblies of the Brazilian subregion; despite this, knowledge of the ichthyofauna of this area and of the possible causes that led to its isolation is still fragmentary. The hydrogeographic pattern of this region has been subjected to the Quaternary climate cycles, which have influenced the morphology of the drainage network (TONNI & CIONE 1997; ARAMAYO *et al*. 2002; QUATTROCCHIO *et al*. 2008). Paleoclimatic reconstructions suggest an arid-semiarid climate for this area during much of the Quaternary, associated in part with glacial and interglacial periods (TONNI *et al*. 1999; QUATTROCCHIO *et al*. 2008). This probably affected the flow of many water courses, with the smaller ones even disappearing during the driest periods (NAGLE & SIMONS 2012). The geologic and paleoclimatic changes that resulted in the current isolation of the studied basins probably also influenced the distribution of the fish fauna.

The general goal of this work is to analyze and compare the phylogeographic pattern of two teleostean species that are co-distributed in the basins of the Southern Pampean Area: *Jenynsia multidentata* and *Corydoras paleatus*. The sequences for *J. multidentata* are those of BRUNO *et al*. (2013) with the addition of new individuals. Both species are widely distributed and have documented fossil records within the area. In addition, we examine the relationship between these patterns and the geomorphological and paleoclimatic history of the region. Thus, this approach aims to infer if such geological and paleoclimatic conditions have influenced the distribution of gene lineages in the ichthyofauna of the Southern Pampean Area.

Materials and methods

Study area and sample collections

Specimens were collected in the localities El Durazno, Chocorí, El Moro, Cortaderas streams, Quequén Grande river, Claromecó stream, Quequén Salado and Sauce Grande rivers, Sauce Chico and Saladillo streams (Fig. 1A). For the purpose of these analyses, each locality was considered as a population.

Part of the pectoral fin of each specimen was removed and used for the molecular analyses. Tissue samples were deposited in the tissue bank at the Molecular Ecology Laboratory (Laboratorio de Ecología Molecular, CREG, UNLP).

Molecular analyses

Total DNA was extracted from the preserved tissue samples following the protocol outlined by ALJANABI & MARTÍNEZ (1997). The entire control region of the mitochondrial DNA was amplified by the Polymerase Chain Reaction (PCR), from the specimens of *C. paleatus* using the primers FLR-L (5'−AACTCCCAAAGCTAGGATTC−3') and FLR-H (5'−GCTCGTGGAACTTTCTAGG−3') which were specifically designed for this study using the software Gene Runner v 3.01 (SPRUYT & BUQUICCHIO 1994). The amplification reaction was carried out in a total volume of 25 µl, with a final concentrations of 0.25 µl of Taq DNA polymerase, 1.5 µl of Cl_2Mg 3 mM, 0.4 µl of dNTP's 50 mM, 0.25 µl of each primer 10 mM and1 µl of DNA template. The reaction was made under the following conditions: initial denaturation at 95 °C during four minutes, followed by 30 cycles of denaturation at 95 °C during 45 seconds, annealing at 59 °C during 45 seconds and extension at 72 °C during 1.15 minutes, followed by a final extension of 5 minutes.

In the case of *J. multidentata* it was possible to amplify eleven individuals, which were added to the preexisting ones, using the primers and under the conditions described in BRUNO *et al*. (2013). Negative controls were performed in all cases to verify the absence of contamination.

The amplification products were purified by lithium chloride precipitation and absolute ethanol precipitation (SAMBROOK *et al*. 1989). The amplicons were sequenced in a capillary sequencer ABI 3100 (Macrogen Inc., Korea). Chromatograms were edited using the software Proseq (FILATOV, 2002) and aligned using Clustal W (THOMPSON *et al*. 1994).

Population analyses

Genetic variation

Genetic variation was assessed using two estimators: nucleotide diversity (π) and haplotype diversity (h) in Arlequin v 3.5 (EXCOFFIER & LISCHER 2010). These estimators provide evidence of past demographic changes occurred within populations (GRANT & BOWEN 1998; AVISE 2000).

Genealogic relationships among haplotypes

To evaluate phylogenetic relationships among the haplotypes of the studied species, we used analyses based both on evolutionary methods (Maximum Parsimony and Bayesian Inference) and on genetic distances (Neighbour

Table 1. Genetic diversity and historical demography of *Jenynsia multidentata* in the Southern Pampean Area. N = number of individuals. S = number of polymorphic sites. NH = number of haplotypes. h ± SD = haplotype diversity ± standard deviation. π ± SD = nucleotide diversity ± standard deviation. D_T: Tajima's D. Fs_{FU}: Fu's F. SSD: Sum of square deviations. rg: Raggedness Index. − : Not estimated. * = P < 0.05.

Locality	N	NH	S	h ± DS	π ± DS	D_T	Fs_{FU}	SSD	rg
Chapadmalal (CHA)	11	3	17	0.58 ± 0.14	0.007 ± 0.004	− 0.181	5.71	0.096	0.194
Chocorí (CHO)	10	3	2	0.71 ± 0.008	0.001 ± 0.0009	0.830	0.253	0.030	0.217
El Moro (EM)	11	2	2	0.18 ± 0.14	0.00004 ± 0.0005	− 1.429*	0.506	0.075	0.735
Quequén Grande (QG)	11	1	—	—	—	—	—	—	—
Cortaderas (CO)	13	1	—	—	—	—	—	—	—
Claromecó (CLA)	11	1	—	—	—	—	—	—	—
Quequén Salado (QS)	10	2	19	0.20 ± 0.15	0.004 ± 0.003	− 1.999*	6.348	0.057*	0.720
Sauce Grande (SG)	11	5	17	0.81 ± 0.08	0.008 ± 0.005	0.697	2.831	0.130	0.150
Saladillo (SA)	11	3	2	0.47 ± 0.10	0.0006 ± 0.0006	− 0.778	− 0.658	0.014	0.169
Sauce Chico (SCH)	11	1	—	—	—	—	—	—	—
Global	110	12	37	0.54 ± 0.05	0.0096 ± 0.005	− 0.098	− 5.909	0.352*	0.184

Joining). The software JModelTest (Posada & Buckley 2004; Posada 2008) with Akaike's information criterion (Akaike, 1974) was used to select the nucleotide substitution model that best fit the data. For *J. multidentata* the resulting model was *TPM1uf + G*. For *C. paleatus*, the resulting nucleotide substitution model was HKY + G. The parameters obtained for the nucleotide substitution models were used to optimize the data in the phylogenetic reconstruction.

A Maximum Parsimony (MP) analysis was implemented in PAUP* 4.0b10 (Swofford 2002), performing a heuristic search with 100 random stepwise addition and tree bisection and reconnection (Tbr) branch swapping. Secondly, a Bayesian Inference (BI) analysis was performed using the software Mr. Bayes (Ronquist & Huelsenbeck 2003). Four independent runs of 3×10^6 generations were made for the two species. The first 40,000 trees were discarded as burn-in for *J. multidentata*, and the first 30,000 for *C. paleatus*. The Neighbour-Joining (NJ) algorithm was implemented in PAUP* 4.0b10 (Swofford 2002). Because of *TPM1uf + G* is not a model available in PAUP, the GTR model was selected in *J. multidentata* analyses. Node support for the MP and NJ analyses was tested using 1,000 bootstrap replicates (Felsenstein 1985). Sequences of *Poecilia latipinna* (GenBank accession number DQ445680.1) and *Corydoras rabauti* (GenBank accession number 29570739.1) were used as outgroup in the *J. multidentata* and *C. paleatus* analyses respectively.

Alternatively the evolutionary relationships between haplotype were analyzed by constructing a haplotype network for each species, applying the *Median Joining* algorithm (Bandelt *et al*. 1999) implemented in the software Network 4.5.1 (http://www.fluxus-engineering.com).

Historical demography

Historical demography was assessed by means of *Fu*'s (1997) and *Tajima*'s (1989) neutrality tests. These analyses were made both globally for the pooled samples of each species, and for each locality. Their significance was tested using 1,000 coalescent simulations in Arlequin v 3.5 (Excoffier & Lischer 2010).

To evaluate for a sudden demographic expansion model a Mismatch distribution analysis (Rogers & Harpending 1992) were conducted for the different populations and for the pooled samples using the software Arlequin v 3.5 (Excoffier & Lischer 2010). To test the validity of the population expansion model of the observed vs. the expected *mismatch distribution* we used a goodness of fit test based on square sum deviation (SSD) and Harpending's Raggedness index (*rg*) (Harpending 1994). These analyses were also carried out using the software Arlequin v 3.5 (Excoffier & Lischer 2010).

Population structure

A description of the genetic structure was obtained from an Analysis of Molecular Variance (AMOVA) (Excoffier *et al*. 1992) using Arlequin v 3.5 (Excoffier & Lischer 2010). For this analysis, three hierarchical population groupings were established on the basis of the geological province where each water course was located: Eastern or Tandilia group, Central or Bonaerean Intersierran Plain group, and Western or Ventania group. The statistical significance was assessed using 1,000 permutations.

In order to evaluate the existence of migration- genetic drift equilibrium at regional scale, we used the approach proposed by Hutchinson & Templeton (1999). Under equilibrium conditions, genetic distances are expected to increase with the increase of geographic distance between populations, in a pattern known as isolation by distance (Wright 1943). The absence or weakness of one or both of these relationships would indicate lack of regional equilibrium. The relationship between geographic and genetic distances (F_{ST}) was evaluated by means of a Mantel test (Mantel 1967). Statistical significance was assessed using 1,000 permutations.

Table 2. Genetic diversity and historical demography of *Corydoras paleatus* in the Southern Pampean Area. N = number of individuals. S = number of polymorphic sites. NH = number of haplotypes. $h \pm SD$ = haplotype diversity ± standard deviation. $\pi \pm SD$ = nucleotide diversity ± standard deviation. D_T: Tajima's D. Fs_{FU}: *Fu*'s F. SSD: Sum of square deviations. *Rg*: Raggedness Index. – : Not estimated. * =P< 0.05.

Locality	N	NH	S	$h \pm DS$	$\pi \pm DS$	D_T	Fs_{FU}	SSD	*rg-P*
El Durazno (ED)	9	1	—	—	—	—	—	—	—
Chocorí (CHO)	9	3	2	0,416 ± 0,190	0,00048 ± 0,0005	−1,088	−1,081*	0,0086	0,168
El Moro (EM)	7	2	7	0,285 ± 0,196	0,002 ± 0,001	−1,434	3,295	0,115*	0,673
Quequén Grande (QG)	11	1	—	—	—	—	—	—	—
Cortaderas (CO)	8	1	—	—	—	—	—	—	—
Claromecó (CLA)	9	5	27	0,805 ± 0,119	0,010 ± 0,006	−0,573	2,960	0,127*	0,295*
Quequén Salado (QS)	10	4	22	0,777 ± 0,090	0,010 ± 0,005	0,684	5,468	0,196	0,461*
Sauce Grande (SG)	9	6	7	0,833 ± 0,126	0,001 ± 0,001	−1,766*	−2,871*	0,004	0,067
Global	72	14	36	0,579 ± 0,066	0,0048 ± 0,0026	−1,540*	−0,281	0,081	0,192

Results

Genetic variation

A 773 base-pair (bp) fragment from the control region was analyzed, using 110 *J. multidentata* individuals (Table 1). Thirty-seven polymorphic sites were identified, 22 of which were transitions and eight, transversions. Likewise, seven *indels* were found, defining a total of 12 haplotypes (GenBank acc number KM086733-KM086735). Total haplotype diversity (*h*) was 0.54 +/- 0.05, while total nucleotide diversity (π) was 0.0096 +/- 0.005, indicating low to moderate genetic diversity for the populations. Values for each population are show in Table 1.

A 923-base pair (bp) fragment of the control region was obtained for *C. paleatus* from 72 individuals. Thirty-six polymorphic sites were observed, including 27 transitions, two transversions and ten *indels*, to define a total of 14 haplotypes (GenBank acc number KM077147 – KM077160). Total haplotype diversity (Table 2) was moderate (0.580 ± 0.066) and so was nucleotide diversity (0.0048 ± 0.0026). Table 2 presents the values for each population.

Genealogical relationships among haplotypes

The phylogenetic relationships among *J. multidentata* haplotypes (Fig. 2) showed similar patterns according to evolutionary and distance methods. In both cases, three major clades were found. One include the widely distributed haplotype as well as haplotypes from the localities Chapadmalal (East) and Saladillo (West). A second clade restricted to the East (or Tandilia region) of the geographical range. The third clade is formed by haplotypes that occur in the West locality (Sauce Grande). The main difference between the MP and BI methods concerned haplotype 7, which was independent from these clades according to MP, while it had a basal position within Clade 1 in the BI tree. In any case, this latter relationship had low support as indicated by the posterior probabil-

ity of the node (data not shown). The haplotype network was highly concordant with the trees obtained previously. The same terminal groups described in the trees were recovered (Fig. 1B). Four major lineages differentiated by several mutational steps can be observed. Of these, one occurs exclusively at Sauce Grande river (haplotypes 8, 9, 10 and 11). Another lineage is formed by a single individual from Quequén Salado river (haplotype 7). A third group was defined by individuals from the populations Chapadmalal, Chocorí, and El Moro (haplotypes 2, 4, 5, 6), which are part of the Tandilia region. Lastly, a widely distributed lineage was recovered, which was observed in all populations with the exception of Chocorí and El Moro. This latter lineage is formed by haplotype 1, and two haplotypes are differentiated from it by one mutational step: one belonging to the Saladillo population and another occurring in both Saladillo and Chapadmalal. Even though signs of local demographic expansion can be observed within each lineage (except for haplotype 7, locality Quequén Salado), the population as a whole shows clear population structure.

In the case of *C. paleatus*, the MP tree (Fig. 3) showed one clade which includes one group formed by the localities El Moro, Claromecó y Quequén Salado, and the widely haplotype (6) and two Chocorí haplotypes occupying basal positions in this clade. Basal to this group and forming a polytomy are the haplotypes from the locality Sauce Grande. Haplotypes 3 and 4 were independently separate. The BI tree showed similar topology, but in this case the first clade shows a split so that the haplotypes from Sauce Grande form an independent clade, although with low support value. Haplotypes 3 and 4 are also differentiated as an independent clade, again with low support (data not shown). The NJ topology was highly concordant with the one obtained through MP.

The haplotype network for *C. paleatus* (Fig. 1C) shows marked population structure, with haplotypes that are unique to some populations such as Claromecó, Quequén Salado and Sauce Grande. According to the divergence observed in terms of mutational steps, three haplogroups or lineages may be differentiated. One group is formed by a widely distributed haplotype (H 6), related with the populations from Chocorí, Sauce Grande
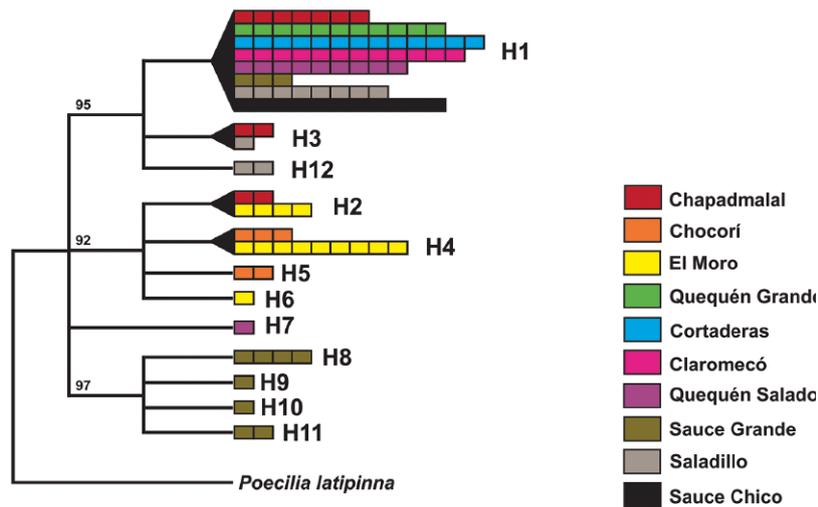
Fig. 2. Phylogenetic strict consensus tree obtained by Maximun Parsimony based on mitochondrial DNA control region of *Jenynsia multidentata*. Numbers on the nodes represents the Bootstrap values. The length branches are proportional to mutations per site. H1−H12: Haplotypes. Each square corresponds to one individual.
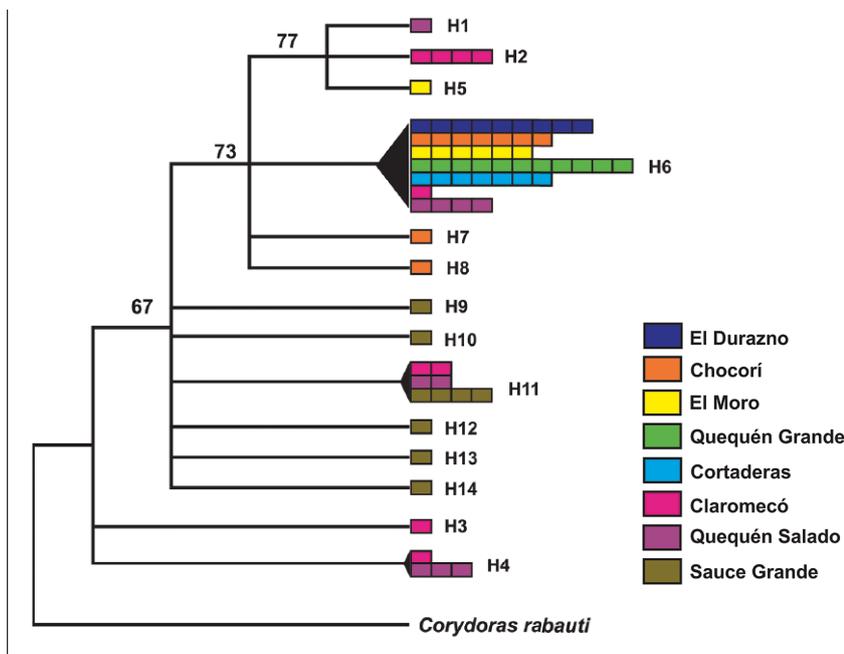
Fig. 3. Phylogenetic strict consensus tree obtained by Maximun Parsimony based on mitochondrial DNA control region of *Corydoras paleatus*. Numbers on the nodes represents the Bootstrap values. The length branches are proportional to mutations per site. H1-H14: Haplotypes. Each square corresponds to one individual.

and Claromecó, as well as with those from Claromecó, Quequén Salado and El Moro. On the other hand, two haplotypes are widely divergent and connected to the network by intermediate haplotypes, which occur in the populations Quequén Salado and Claromecó.

Historical demography

The global *mismatch distribution* for *J. multidentata* showed an erratic curve (Fig. 4a). This result could sug-

gest constant population size, or alternatively a population subdivision for a long period. The goodness of fit tests yielded high values, although not significant in the case of Harpending's Raggedness index (*rg*) (Table 1). Such values confirm that the data do not fit a sudden demographic expansion model. The neutrality tests were positive and not statistically significant, supporting the results of the *mismatch distribution* analysis (Table 1).

In the case of *C. paleatus* the *mismatch distribution* analysis for the global populations also showed an erratic curve that does not fit the expected sudden expansion
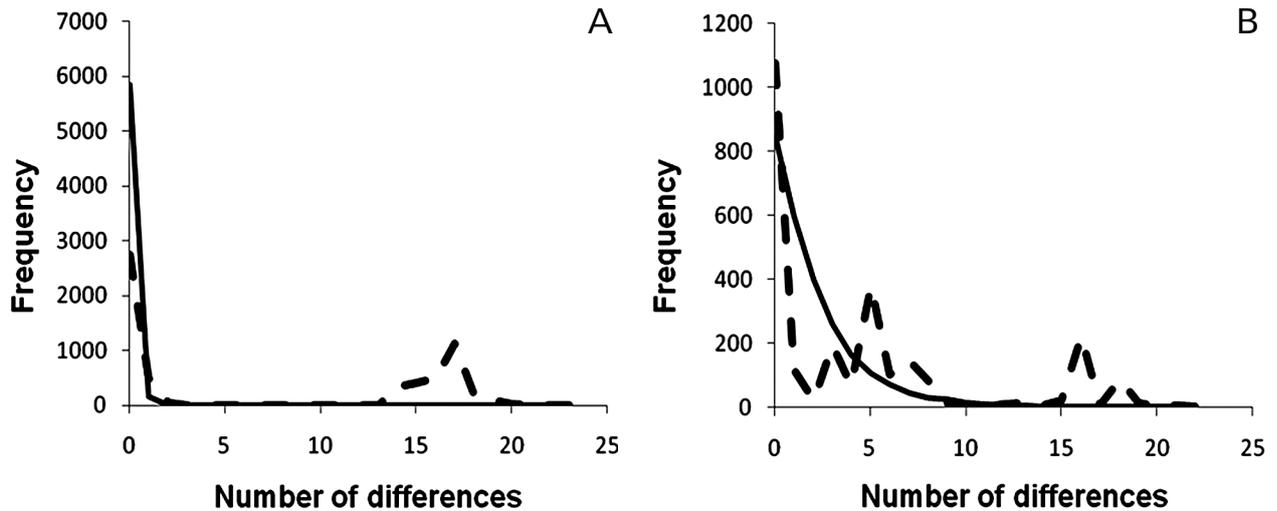
Fig. 4. Mismatch distribution of mitochondrial DNA control region for **a**: *Jenynsia multidentata and* **b** *Corydoras paleatus*. Thick line: observed distribution. Fine line: expected distribution under a sudden population expansion model.

model (Fig. 4b). This result could suggest constant population size, or a population that has been subdivided for a long period. The neutrality tests were negative in both cases, although only statistically significant for Tajima's D (Table 2).

Population structure

The genetic differentiation between *J. multidentata* populations, showed the highest and significant F_{ST} values in the El Moro and Chocorí populations when compared to the populations from Quequén Grande, Cortaderas and Claromecó. Likewise, high F_{ST} values were observed in the comparisons with the Sauce Chico and Saladillo populations (Table 3). In contrast, the lowest values were found in the locality Quequén Salado vs. Chapadmalal and Saladillo (Table 3). The F_{ST} fixation index for the pooled populations was 0.80 (p < 0.001), indicating high degree of population structure.

On the other hand, the AMOVA indicated that most of the variance is explained among groups, while the rest of it is distributed among populations within groups, and lastly, within populations (Table 4).

The Mantel test yielded a correlation coefficient r = 0.28 (P = 0.076). Although some localities were shown to be greatly divergent, this result from the Mantel test suggests that equilibrium between flow and gene drift has not yet been achieved (Fig. 5a).

Among the *C. paleatus* populations, the highest significant F_{ST} values were found for the populations Sauce Grande vs. Quequén Grande, Claromecó and Cortaderas, as well as for Claromecó vs. El Durazno, Quequén Grande and Cortaderas. The lowest values corresponded to El Durazno vs. Quequén Grande and Cortaderas, as well as Cortaderas vs. Quequén Grande. Values for the remaining comparisons were medium to low (Table 5).

On the other hand, the AMOVA (Table 4) indicated that most of the variance is accounted for within populations. This result shows the marked differentiation within the *Corydoras paleatus* populations, given by highly divergent haplotypes within a single population (e.g. Claromecó and Sauce Grande). The remaining variance was distributed among groups and lastly, among populations within groups (Table 4).

The Mantel test (Fig. 5b) resulted in a correlation coefficient r = 0.43 (P = 0.0038) which indicates low fit to a pattern of isolation by distance.

Discussion

The analysis of genetic diversity of *C. paleatus* and *J. multidentata* yielded moderate values, both for haplotype and nucleotide diversity. According to AVISE (2000) and GRANT & BOWEN (1998), high values for both estimators suggest a demographically stable population with large effective population size maintained through time. On the contrary, low values for both estimators could suggest a severe population bottleneck or possibly selective sweeps, ultimately resulting in a strong reduction of population size. The moderate values found for both species could suggest that the respective populations are on their way to demographic equilibrium. The patterns shown by the historical demography analyses support a scenario of demographic stability.

The results of the analysis of genealogic relationships were similar for *C. paleatus* and *J. multidentata*. Both taxa comprised, on the one hand, diverging haplogroups that were in some cases confined to small populations, and on the other, a widely distributed haplotype, with the presence of both lineages in the same locality in some

Table 3. Pairwise F$_{ST}$ of *Jenynsia multidentata* in the Southern Pampean Area. * = P value significant at 0.05 level. CHA: Chapadmalal. CHO: Chocorí. EM: El Moro. QG: Quequén Grande. CO: Cortaderas. CLA: Claromecó. QS: Quequén Salado. SG: Sauce Grande. SA: Saladillo. SCH: Sauce Chico.

	CHA	CHO	E M	QG	CO	CLA	QS	SG	SA	SCH
CHA	0									
CHO	0.764*	0								
EM	0.80*	0.12	0							
QG	0.1	0.97*	0.98*	0						
CO	0.12*	0.97*	0.99*	0	0					
CLA	0.1	0.97*	0.98*	0	0	0				
QS	0.002	0.86*	0.88*	0.01	0.03	0.01	0			
SG	0.47*	0.75*	0.76*	0.68*	0.70*	0.67*	0.52*	0		
SA	0.08	0.95*	0.97*	0.06	0.08	0.06	0.007	0.66*	0	
SCH	0.1	0.97*	0.98*	0	0	0	0.01	0.67*	0.066	0

Table 4. AMOVA for *Corydoras paleatus* and *Jenynsia multidentata* considering three hierarchical population groupings: Eastern or Tandilia group, Central or Bonaerean Intersierran Plain group, and Western or Ventania group.

Source of variation		d.f	Sum of squares	Variance components	Percentage of variation
Among groups	*Corydoras paleatus*	2	3.71	0.047 Va	14.99
	Jenynsia multidentata	2	181.09	1.95 Va	42.90
Among populations within groups	*Corydoras paleatus*	5	4.27	0.073 Vb	23.10
	Jenynsia multidentata	7	139.03	1.72 Vb	37.93
Within populations	*Corydoras paleatus*	64	12.58	0.196 Vc	61.91
	Jenynsia multidentata	100	87.28	087 Vc	19.17

cases, suggesting the occurrence of secondary contact between the different lineages.

The lack of fit between mutation-genetic drift equilibrium in *J. multidentata* could be explained by strong effect of genetic drift. According to HUTCHINSON & TEMPLETON (1999), the expected patterns in regions that have not reached equilibrium may be affected both by the time elapsed since colonization of the region, and by the degree of dispersal within said region. If environmental conditions change so that the population that occupies the colonized region becomes fragmented into small isolated populations, genetic drift will have relatively greater influence than gene flow (Case III, HUTCHINSON & TEMPLETON 1999).

In contrast, for *C. paleatus* there was low although significant fit between genetic and geographic distances (Case IV, HUTCHINSON & TEMPLETON 1999). Given these results, it is probable that the most diverging lineages fit an isolation-by-distance pattern, but the presence of a widely distributed haplotype hinders a more defined manifestation of such pattern.

The integration of the information available so far indicates a similar phylogeographic pattern for both species: both possess haplogroups that are greatly divergent among them. In both species one of these haplogroups is widely distributed, while the remaining ones are restricted to a few watercourses.

The geological and paleoclimatic changes that have affected the Southern Pampean Area could account for the distribution of genetic variation in the fish species that inhabit the area. For freshwater fishes, a pattern of wide colonization in an area can be explained in terms of basin connectivity. On the other hand, for population structure to occur, enough time is necessary so that the individuals may accumulate differences through mutation and in absence of gene flow, which entails a certain degree of geographic discontinuity (AVISE, 2000; ALLENDORF & LUIKART 2007). The existence of the phylogeographic patterns found in this study provide an approximation to a geologic and paleoclimatic scenario with alternating connection and disconnection of the basins in this region. Such phenomena have been characteristic of much of the Quaternary in the Southern Pampean Area.

During the middle Pleistocene, the southeastern Buenos Aires province was characterized by arid conditions with marked eolic influence (ARAMAYO *et al*. 2002; QUATTROCCHIO *et al*. 1993, 2008). The sea level drop during the last glacial maximum (approx. 22.000 years AP, PONCE *et al*. 2011) produced great eastwards expansion of the coastal line. In turn, this expansion favored changes in the distribution of rivers and the integration of the drainage network. By the late Pleistocene (16.000 – 12.000 AP), during dry periods, the main courses were discontinuous and accessory drainage networks, both permanent and semi-permanent, were established in association with shallow lakes, and their extension underwent marked seasonal fluctuations (ZAVALA *et al*. 2005). These adverse geologic and climatic conditions could have caused, on the one hand, local extinctions, and on the other, the survival of some populations in those courses that remained active during climate fluctuations. Assuming this paleoclimatic scenario, it is possible that the highly genetically differentiated populations of *J. multidentata* and *C. paleatus* may have occupied environments that remained active during their evolution. Such populations could have achieved population differentiation in absence of or with restricted gene
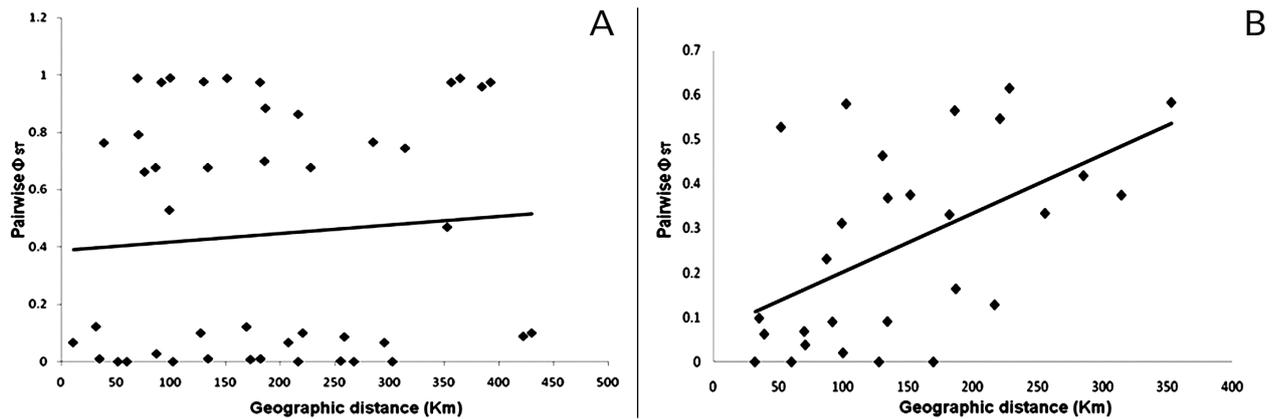
Fig. 5. Relationship between geographic and genetic distances for **a**: *Jenynsia multidentata* and **b**: *Corydoras paleatus.*

Table 5. Pairwise F_{ST} of *Corydoras paleatus* in the Southern Pampean Area. * = P value significant at 0.05 level. D: Durazno. CHO: Cho-corí. EM: El Moro. QG: Quequén Grande. CO: Cortaderas. CLA: Claromecó. QS: Quequén Salado. SG: Sauce Grande.

	D	CHO	EM	QG	CO	CLA	QS	SG
D	0							
CHO	0.062	0						
EM	0.038	0	0					
QG	0	0.090	0.068	0				
CO	0	0.463	0.020	0	0			
CLA	0.546*	0.331*	0.375*	0.580*	0.527*	0		
QS	0.334*	0.128	0.164	0.368*	0.231*	0.098	0	
SG	0.583*	0.375*	0.418*	0.615*	0.564*	0.090	0.116*	0

flow, in a drainage network that was partially disintegrated due to the deactivation of given water courses (Bruno *et al.* 2013). In fact, both genera occur in the middle Pleistocene fossil record of the study area (Deschamps 2005; Bogan *et al.* 2009). Nevertheless, although they have persisted to the present, it is possible that the populations of these lineages underwent drastic size decreases due to habitat fragmentation brought about by the effects of the above-mentioned paleoclimatic conditions on the drainage network. This could account for the current population structure. Furthermore, some environments could represent environmental refuges, such as the Quequén Salado river where both species present lineages that are quite divergent with respect to the others. In addition, both species presented exclusive or unique haplotypes in Sauce Grande river. The star-shaped topology observed in the haplotype networks for this locality suggests demographic expansion. Similarly, both species showed the highest values of genetic distance measured as pairwise F_{ST}. It is evident that the characteristics of Sauce Grande river have resulted in the population differentiation of these species.

More benign climatic conditions have been suggested to occur around 9,000 years BP, during the Holocene sequence (Quattrocchio & Borromei 1998; Zavala & Quattrocchio 2001; Aramayo *et al.* 2002). Due to the higher temperature and consequent increase of precipi-

tations, medium-sized interconnected lakes were formed in the area (Aramayo *et al.* 2002). Given this scenario, it is possible that some haplotypes, such as the widely distributed one, entered the system after the LGM, due to the favorable connections of the drainage network. This situation may explain the presence of at least one widely distributed haplotype in both species.

Thus, we find lineages that have remained in the area under adverse climatic conditions, in potential environmental refuges, together with lineages that may have colonized the area later due to the more benign climate conditions, as the result of the formation of the modern hydrographic system (Bruno *et al.* 2013), showing evidence of secondary contact in some localities.

The geologic and paleoclimatic history of the region has largely influenced the distribution of genetic variation in the studied species, promoting population differentiation in some cases. Some water courses, such as the Sauce Grande river, host a considerable genetic diversity, which should be taken into account when formulating conservation plans in the area. Future studies of the ichthyofauna that occurs in basins outside the Southern Pampean area would contribute to enhance the knowledge of factors at a regional scale, that could have shaped the distribution and diversity of the fish fauna in the southern limits of the Brazilian subregion.

Acknowledgements

References

Akaike, H. (1974): A new look at the statistical model identification. – IEEE Transactions on Automatic Control, **19**: 716–723.

Aljanabi, S.M. & Martinez, I. (1997): Universal and rapid salt-extraction of high quality genomic DNA for PCR-based techniques. – Nucleic Acids Research, **25**: 4692–4693.

Allendorf, F.W. & Luikart, G.H. (2007): Conservation and the Genetics of Populations. Blackwell Publishing Ltd.

Aramayo, S., Schillizzi, R. & Gutiérrez Téllez, B. (2002) Evolución paleoambiental del Cuaternario en la costa atlántica del sur de la provincia de Buenos Aires, Argentina. Revista de la Sociedad Geológica de España, **15**: 95–104.

Avise, J.C. (2000). Phylogeography: the history and formation of species. Harvard University Press.

Bandelt, H.-J., Forster, P. & Röhl, A. (1999): Median-joining networks for inferring intraspecific phylogenies. – Molecular Biology and Evolution, **16**: 37–48.

Bogan, S., De Los Reyes, M.L. & Cenizo, M.M. (2009): Primer registro del género *Jenynsia* Günther, 1866 (Teleostei: Cyprinodontiformes) en el Pleistoceno Medio tardío de la provincia de Buenos Aires (Argentina). – Papéis Avulsos de Zoologia (São Paulo), **49**: 81–86.

Bruno, M.C., Casciotta, J.R., Almiron, A.E. & Lizarralde, M.S. (2013): Phylogeographic pattern of *Jenynsia multidentata* (Cyprinodontiformes: Anablepidae) in the southern boundary of the Brazilian Subregion, Argentina. – Neotropical Ichthyology, **11**: 477–486.

Casciotta, J., Almirón, A., Cione, A. & Azpelicueta, M. (1999): Brazilian freshwater fish assemblages from Southern Pampean Area, Argentina. – Biogeographica, **75**: 67–78.

Deschamps, C.M. (2005): Bio-cronoestratigrafía de mamíferos del Cenozoico tardío en el sudoeste de la provincia de Buenos Aires, Argentina. – Ameghiniana, **42**: 733–750.

Excoffier, L. & Lischer, H. E. (2010): Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. – Molecular Ecology Resources, **10**: 564–567.

Excoffier, L., Smouse, P.E. & Quattro, J.M. (1992): Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. – Genetics, **131**: 479–491.

Faulks, L.K., Gilligan, D.M. & Beheregaray, L.B. (2010): Islands of water in a sea of dry land: hydrological regime predicts genetic diversity and dispersal in a widespread fish from Australia's arid zone, the golden perch (*Macquaria ambigua*). – Molecular Ecology, **19**: 4723–4737.

Felsenstein, J. (1985): Confidence limits on phylogenies: an approach using the bootstrap. – Evolution, **39**: 783–791.

Filatov, D.A. (2002): ProSeq: a software for preparation and evolutionary analysis of DNA sequence data sets. – Molecular Ecology Notes, **2**: 621–624.

Fu, Y.X. (1997): Statistical tests of neutrality of mutations against population growth, hitchhiking and background selection. – Genetics, **147**: 915–925.

Grant, W. & Bowen, B. (1998): Shallow population histories in deep evolutionary lineages of marine fishes: insights from sardines and anchovies and lessons for conservation. – Journal of Heredity, **89**: 415–426.

Harpending, H. (1994): Signature of ancient population growth in a low resolution mitochondrial DNA mismatch distribution. – Human Biology, **66**(4): 591–600.

Hewitt, G. (2004.):Genetic consequences of climatic oscillations in the Quaternary. – Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences, **359**: 183–195.

Hutchison, D.W. & Templeton, A.R. (1999). Correlation of pairwise genetic and geographic distance measures: inferring the relative influences of gene flow and drift on the distribution of genetic variability. – Evolution, **53**: 1898–1914.

Loxterman, J.L. & Keeley, E.R. (2012): Watershed boundaries and geographic isolation: patterns of diversification in cutthroat trout from western North America. – BMC Evolutionary Biology, **12**: 38.

Mantel, N. (1967): The detection of disease clustering and a generalized regression approach. – Cancer Research, **27**: 209–220.

Nagle, B.C. & Simons, A.M. (2012): Rapid diversification in the North American minnow genus Nocomis. – Molecular Phylogenetics and Evolution, **63**: 639–649.

Ponce, J.F., Rabassa, J., Coronato, A. & Borromei, A. (2011): Palaeogeographical evolution of the Atlantic coast of Pampa and Patagonia from the last glacial maximum to the Middle Holocene. – Biological Journal of the Linnean Society, **103**: 363–379.

Posada, D. (2008): jModelTest: phylogenetic model averaging. – Molecular biology and evolution, **25**: 1253–1256.

Posada, D. & Buckley, T.R. (2004): Model selection and model averaging in phylogenetics: advantages of Akaike information criterion and Bayesian approaches over likelihood ratio tests. – Systematic Biology, **53**: 793–808.

Quattrocchio, M.E. & Borromei, A.M. (1998): Paleovegetational and paleoclimatic changes during the late Quaternary in southwestern Buenos Aires Province and southern Tierra del Fuego (Argentina). – Palynology, **22**: 67–82.

Quattrocchio, M.E., Borromei, A.M., Deschamps, C.M., Grill, S.C. & Zavala, C.A. (2008): Landscape evolution and climate changes in the Late Pleistocene–Holocene, southern Pampa (Argentina): evidence from palynology, mammals and sedimentology. – Quaternary International, **181:** 123–138.

Quattrocchio, M.E., Kostadinoff, J., Martinez, G. & Prieto, A. (1993): Evidencias de neotectónica en el Río Sauce Chico, Prov. de Buenos Aires. – Revista de la Asociación Geológica Argentina, **49**: 297–305.

Rogers, A.R. & Harpending, H. (1992): Population growth makes waves in the distribution of pairwise genetic differences. – Molecular Biology and Evolution, **9**: 552–569.

Ronquist, F. & Huelsenbeck, J.P. (2003): MrBayes 3: Bayesian phylogenetic inference under mixed models. – Bioinformatics, **19**: 1572 – 1574.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989): Molecular cloning. – Cold spring harbor laboratory press New York.

Spruyt, M. & Buquicchio, F. (1994): Gene Runner Version 3.05. Hastings Software. Inc.

Swofford, D. (2002): PAUP* version 4.0. Phylogenetic analysis using parsimony (and other methods). Version 4. Sinauer Associates, Sunderland, Massachusetts.

Tajima, F. (1989): Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. – Genetics, **123**: 585 – 595.

Thompson, J.D., Higgins, D.G., & Gibson, T.J. (1994): CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. – Nucleic Acids Research, **22**: 4673 – 4680.

Tonni, E. & Cione, A. (1997): Did the Argentine Pampean ecosystem exist in the Pleistocene. – Current research in the Pleistocene, **14**: 131 – 133.

Tonni, E.P., Cione, A.L. & Figini, A.J. (1999): Predominance of arid climates indicated by mammals in the pampas of Argentina during the Late Pleistocene and Holocene. – Palaeogeography, Palaeoeclimatology, Paleoecology, **147**: 257 – 281.

Wright, S. (1943): Isolation by distance. – Genetics, **28:** 114 – 138.

Zavala, C., García, L. & Di Meglio, M. (2005): Redes de drenaje y paleoclimas en el Cuaternario del sur de la provincia de Buenos Aires. In: Xvi Congreso Geológico Argentino, Cd-Rom, Artículo.

Zavala, C. & Quattrocchio, M. (2001): Estratigrafía y evolución geológica del río Sauce Grande (Cuaternario), provincia de Buenos Aires, Argentina. – Revista de la Asociación Geológica Argentina, **56**: 25 – 37.