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POPULATION GENETIC STRUCTURE OF THE FRESHWATER ANOMURANS *AEGLA URUGUAYANA* SCHMITT, 1942 (DECAPODA, AEGLIDAE) IN THE CENTRAL REGION OF ARGENTINA

BY

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ABSTRACT

Aeglidae is the only freshwater family in the infraorder Anomura. *Aegla uruguayana* Schmitt, 1942 is one of the most widely distributed species in southern South America and is found in different environments, which makes it an interesting object for population genetic studies. The main objective of this work was to analyse the genetic population structure of *A. uruguayana* along a sea distance gradient for four populations that were studied in the La Plata Basin with an 1100-km range in relation to an east-west transection. The studied populations were the Río Tercero Reservoir, the Setúbal Lagoon, the Doll Stream and the Urquiza Stream. Aeglids DNA was extracted using a commercial kit that was amplified with ISSR markers. Of the 10 primers tested, we selected four that showed the best resolution and reproducible results. Our studies revealed a H_e of 0.3479 ± 0.1383 (mean ± SD) and a global F_{ST} of 0.3583 ($p<0.0001$), demonstrating genetic differentiation among populations with low gene flow. The Urquiza Stream population showed a genetic structure clearly different from the other populations. However, the Río Tercero, Setúbal and Doll populations were well grouped with one effective connection among them. The geomorphologic history of the basin provides evidence for the isolation hypothesis. These data demonstrate the importance of geoclimatic history in the study region and the importance of using complete population distribution data where the species live. These data permit us to interpret that different populations have independent histories that are delineated by the geomorphologic events that occurred on earth.

RESUMEN

Aeglidae es la única familia dulciacuícola del infraorden Anomura. *Aegla uruguayana* Schmitt, 1942 es una de las especies más ampliamente distribuidas en el sur de América del Sur y se las

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encuentra en diferentes tipos de ambientes generando esto gran interés por su estudio de genética de poblaciones. El objetivo principal de este trabajo fue analizar la estructura genética de poblaciones de *A. uruguayana* a lo largo de un gradiente de distancia al mar para cuatro poblaciones que son estudiadas en la región de la cuenca del Plata separadas con un rango de 1100 km en una transecta este - oeste. Las estaciones estudiadas fueron: reservorio Río Tercero, laguna Setúbal, arroyo Doll, arroyo Urquiza. El ADN del aéglido fue extraído usando un kit comercial que fue amplificado con marcadores ISSR. De los 10 "primers" testeados, nosotros seleccionamos cuatro que mostraron las mejores resoluciones y resultados reproducibles. Nuestro resultado tuvieron un valor de H_e de 0,3479 $\pm$ 0,1383 (media $\pm$ SD) y un total F_{ST} de 0,35832 ($p<0,0001$), mostrando que existe diferenciación genética entre las poblaciones mientras que el flujo de genes es bajo. La población del arroyo Urquiza mostró una estructura genética y una clara diferenciación de las otras poblaciones. Sin embargo, las poblaciones de Río Tercero, Setúbal y Doll fueron observadas bien agrupadas, indicando una efectiva conexión entre ellas. La historia geomorfológica de la Cuenca provee evidencia para hipotetizar el aislamiento. Esto muestra la importancia del conocimiento de la historia geoclimática en la región de estudio y la importancia de usar, como evidencia, poblaciones en la distribución completa donde la especie vive. Estos datos permiten interpretar que las diferentes poblaciones tienen, en menor o mayor grado, relativa independencia en la historia delineada por los eventos geomorfológicos ocurridos en la tierra.

INTRODUCTION

The presence and permanence at any site as a single unit of a species population depends on several intrinsic and extrinsic factors. At first and in the short term, genetic variation is important to identify distant populations. However, in the long term, variability allows for recognition of potential adaptations in front to variations in environmental conditions (Frankel & Soulé, 1981; Booy et al., 2000; Schulz et al., 2004). *Aegla uruguayana* Schmitt, 1942 is one of the three Aeglidae family species with a wide distribution. This is the only anomuran that inhabits freshwater environments and is restricted to southern South America. It contains approximately 70 species, and some of them have been described (Bond-Buckup et al., 2010). *Aegla uruguayana* inhabits a great diversity of environments such as lagoons, lakes, small rivers and mountain streams with different degrees of connection among them or, in some cases, in aquatic habitats that have been isolated (Giri & Collins, unpubl.). In this study, the species selected had been exposed to several environmental disturbances over time, mainly marine transgressions, which affected the distribution area to different degrees (Lundberg et al., 1998). Thus, based on simple stochastic evolution, the isolated populations in small and/or initial ponds or rivers would exhibit lower levels of genetic variation than large populations, which were in older bodies of water that were open to migration (Busack, 1988). Environmental stability could also affect polymorphisms and heterozygosity (Maynard Smith, 1998), which is lower in more stable environments and higher in more variable environments. These characteristics make *A. uruguayana* an interesting taxa for studying genetic and geographic variability, as posited by Daniels (2003) for cosmopolitan species.

In freshwater decapods, population genetic evidence studies suggest different patterns that indicate structural levels according to distance or population separation in time or space, or variations according to habitat area, habitat age, and the immigration potential of the locality (Fuller & Lester, 1980; Daniels et al., 1999; Daniels, 2003; Schubart & Huber, 2006; Shih et al., 2006; Xu et al., 2009; Klinbunga et al., 2010; Barber et al., 2011, 2012).

Phylogeographic studies based on the Aeglidae family were performed mainly using DNAmt (Pérez-Losada et al., 2002, 2004; Jara et al., 2003). Aeglids population variability by molecular or genetic evidence is scarce (D'Amato & Corach, 1997a, b; Santos et al., 2009; Xu et al., 2009; Barber et al., 2011, 2012). Here, we applied the Inter Simple Sequence Repeat (ISSR) marker, which is a relatively recent technique (Bornet & Branchard, 2001), to evaluate variation in microsatellite regions. One of the advantages of ISSR is that the primers amplify DNA universally in many animals (Machkour-M'Rabet et al., 2009). Classical genetic variation studies were performed using random amplification of polymorphic DNA (RAPD), but some authors observed that ISSR markers are a better choice for polymorphism detection (Abbot, 2001; Qian et al., 2001).

The aim of the present study was to analyse the population genetic structure of one of the most widely distributed species of Aeglidae along a distance gradient from the sea. The genetic diversity and structure of *A. uruguayana* were studied in a central Argentinian area (West-East gradient). This is the first ISSR molecular marker study in this taxon, thus permitting an understanding of colonisation and re-colonisation history after geo-climatic events that occurred in South America, which could have provoked population displacements.

MATERIAL AND METHODS

Sample collection and DNA extraction

Aegla uruguayana specimens were collected from four localities in the central Argentinian La Plata Basin encompassing two different sub-basin hydrographic systems (fig. 1). Three populations (Río Tercero Reservoir, Setúbal Lagoon and Doll Stream) belonged to the Paraná River system (region A), and the remaining population (Urquiza Stream) belonged to the Uruguay River system (region B). We chose these localities because they represent the species distribution along a West-East gradient in the del Plata Basin.

In the present study, each population was represented by 9 specimens, although Sinclair et al. (2004) proposed that 5 individuals per population would be adequate to detect gene flow (fig. 1).

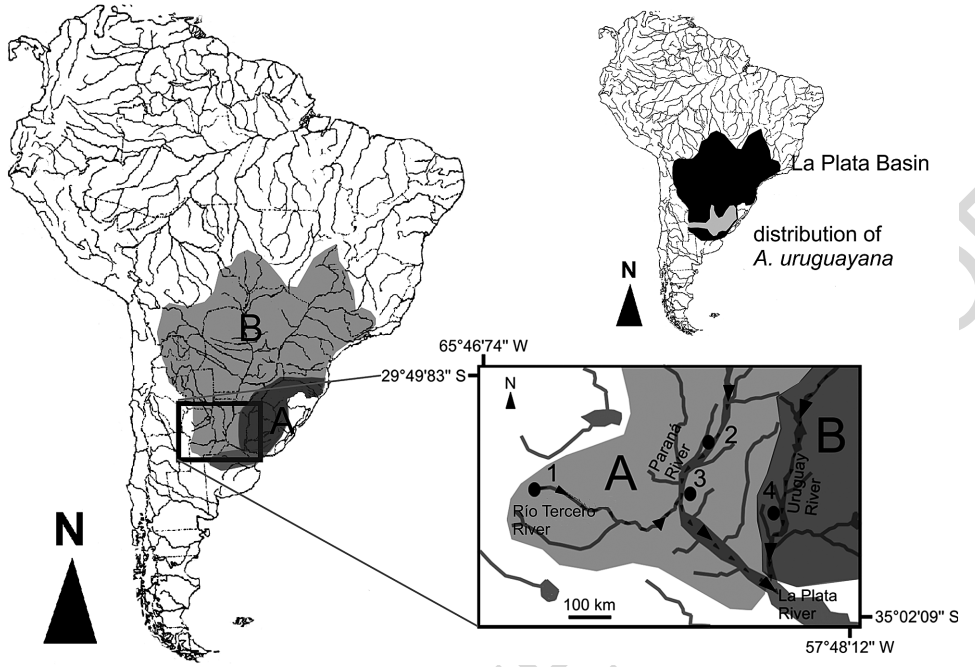

Fig. 1. South America with the location of La Plata Basin and (A) the Uruguay and (B) the Paraná River sub-basins. Moreover, the distribution of *Aegla uruguayana* Schmitt, 1942 and sample sites in the two sub-basins with references of the main rivers. The art line indicates the population connection through river corridors. The arrows indicate direction of water flow. Sample sites: 1, Río Tercero Reservoir; 2, Setúbal Lagoon; 3, Doll Stream; 4, Urquiza Stream.

The specimens were first frozen in liquid nitrogen and then fixed and stored in 70% ethanol. The samples were identified following the protocol of Bond-Buckup & Buckup (1994). Genomic DNA was extracted from pereiopods according to the AccuPrep® Genomic DNA Extraction Kit protocol. DNA was quantified on 0.8% agarose gels and stained with GelGreen® (Biotium).

ISSR-PCR amplification

To select the ISSR primers that would be useful for revealing polymorphisms, a set of 10 UBC series primers (Operon®) was tested. Of the 10 primers tested, we selected four that showed the best resolution and reproducible results. Each reaction was repeated at least twice. PCR reactions were conducted individually in an MPI® Thermal-Cycler in 15 μl reaction volumes as follows: 1× buffer, 2.5 mM $MgCl_2$, 20 μM dNTPs, 0.5 μM each primer, 1 U *Taq* (PB-L®, Universidad de Quilmes, Buenos Aires, Argentina) and 50 ng genomic DNA. The PCR protocol was performed was as follows: initial denaturation at 94°C for 4 min; 40 cycles each of 94°C for 1 min, primer annealing temperature (range 54-57°C) for 1.5 min,

and 72°C for 2 min; and a final extension at 72°C for 10 min. The primers selected were P7 (AGA GAG AGA GAG AGA GT), P8 (AGA GAG AGA GAG AGA GC), P10 (GAG AGA GAG AGA GAG AT) and P34 (AGA GAG AGA GAG AGA GYT).

Analysis of amplified PCR products

The PCR products were first visualised on 2% agarose gels and stained with GelGreen® (Biotium). To obtain higher definition, we analysed PCR products by electrophoresis on 4% polyacrylamide gels (33 cm × 39 cm) at 2200 V and 75 W in 0.5× TBE buffer and staining with silver nitrate solution (Bassam et al., 1991). Stained gels were photographed with an Olympus C5000 digital camera. Binary matrices were made with data obtained from polyacrylamide gels.

Data analysis

Basic genetic diversity indices were calculated using TFPGA 1.3 (Miller, 1997). Allele frequencies were used to estimate genetic variability levels in each population using the population-expected heterozygosity (H_e) (Nei, 1972) and percentage of polymorphic loci (P). We also used the Mantel test to observe the correlation between hydrographic and genetic distances by means using TFPGA 1.3 software. We calculated genetic distances by applying the correction formula $F_{ST}/(1 - F_{ST})$. All analyses were done assumed that populations are in Hardy-Weinberg equilibrium (Aagaard et al., 1998).

Genetic differentiation among all population pairs was estimated using the F_{ST}. The F_{ST} value significance was assessed through 10 000 permutations under the hypothesis of an absence of population subdivision with ARLEQUIN 3.11 software (Excoffier et al., 2005). Population structure was studied by analysis of molecular variance (AMOVA) using Arlequin 3.11. The migrant number (N_m) was calculated using the equation $N_m = 0.25 \times ((1/F_{ST}) - 1)$, as proposed by Slatkin (1994). The tests were adjusted with the Bonferroni correction according to Rice (1989). To assess the population structure, we also used the Bayesian clustering method that was implemented in Structure 2.3.1 software (Pritchard et al., 2000) to infer the most likely number of individual clusters (K). Four independent runs of 100 000 Markov Chain Monte Carlo (MCMC) cycles for burn-in and 100 000 for data collection were performed for K values from 1 to 6, assuming that allele frequencies among populations were correlated, thus allowing for admixture (i.e., gene flow) and setting the allele frequency prior parameter λ to 1. The membership coefficients for each individual (Q_{indiv}), indicating the individual genome proportion that originated from each cluster, and the mean membership of each predefined population in each inferred cluster (Q_{pop}) were

TABLE I

Analysis of genetic variability with ISSR markers of *Aegla uruguayana* Schmitt, 1942 in four populations studied

Site	H_e	P
Río Tercero Reservoir	0.17934	49.5327
Setúbal Lagoon	0.20524	53.271
Doll Stream	0.20768	52.3364
Urquiza Stream	0.38458	90.6542

H_e, expected heterozygosity; P, percentage of polymorphic loci.

calculated for the highest K value. The Q values obtained for the five MCMC runs were combined using the program Clumpp 1.1 (Jakobsson & Rosenberg, 2007) and turned into graphs using Distruct (Rosenberg, 2004).

A Mantel Test, performed with TFPGA 1.3 software (Miller, 1997), was used to assess correlations between genetic and hydrographic distances considering the distance through to the river ways and the paleo-basins since the last sea transgression.

RESULTS

Analysis of genetic variability with ISSR markers

The studies performed with ISSR markers had a total H_e value of 0.3479 $\pm$ 0.1383 (mean $\pm$ SD). Each *Aegla uruguayana* population demonstrated differences in H_e: Urquiza had the highest He value and the highest polymorphic loci percentage (P), followed by Setúbal and Doll, both with similar H_e and P values. Finally, the Rio Tercero population had the lowest H_e and P values (table I).

The AMOVA analysis showed that the variation among populations (V_a: 35.8%) was lower than within populations (V_b: 64.2%) (table II). The high variability observed in each sample site demonstrated the genetic structure in some sites.

TABLE II

AMOVA among populations (V_a) and within populations (V_b) of *Aegla uruguayana* Schmitt, 1942

Source of variation	df	Sum of squares	Variance components	% variation
Among populations (V_a)	3	433.167	7.29575	35.83
Within populations (V_b)	68	888.444	13.06536	64.17

$F_{ST} = 0.3583$; $p<0.0001$, after Bonferroni corrections from 107 loci.

TABLE III

Pairwise values comparisons of *Aegla uruguayana* Schmitt, 1942

Pair	Genetic distance	Hydrographic distance	$F_{ST} = G_{ST}$ value	N_m
Río Tercero-Setúbal	0.1721	500	0.2405*	0.7895
Río Tercero-Doll	0.1770	450	0.3578*	0.4487
Río Tercero-Urquiza	0.1918	1100	0.4379*	0.3209
Setúbal-Doll	0.0359	100	0.2310*	0.8322
Setúbal-Urquiza	0.0247	750	0.3522*	0.4598
Doll-Urquiza	0.0300	600	0.4174*	0.3489

Sites were compared in genetic distances (Nei, 1972), hydrographic distances, F_{ST} and N_m.
* $p<0.001$, after Bonferroni corrections from 107 loci.

Population structure analyses

The four populations showed a significant highly ordered structure along the studied area. We observed existing differentiation and scarce gene flows among the studied populations ($F_{ST} = 0.3583$; $p<0.0001$). The total migration rate (N_m) was 0.4477, demonstrating low gene flows among the sampled sites.

A detailed analysis between sample sites showed a pattern of genetic similarity, and it was related to hydrographic distance among the sampled populations (e.g., Setúbal-Doll; Urquiza-Doll; Doll-Río Tercero) (table III). The nearest populations (i.e., Setúbal and Doll) were closest genetically, and the most distant populations, Rio Tercero and Urquiza, were the most different (fig. 2). However, all of the population pairwise assessed displayed evidences of low gene flow.

The relationship between the hydrographic distance and population pairwise gene flow reflects an inverse correlation between hydrographic distance and gene flow in each pairwise studied, suggesting isolation by distance (fig. 2).

Genetic distance indicated that Urquiza, in addition to presenting the highest H_e and P values, showed the most genetic differentiation compared with the

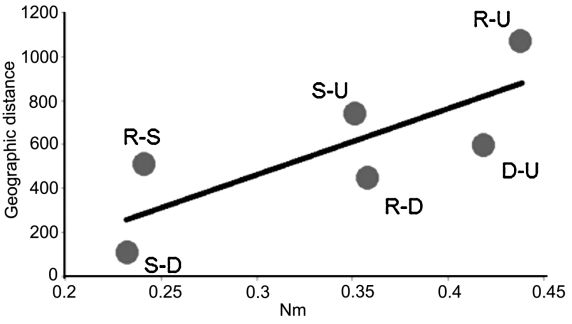

Fig. 2. Correlations between N_m and hydrographical distances between pairs of populations of *Aegla uruguayana* Schmitt, 1942.

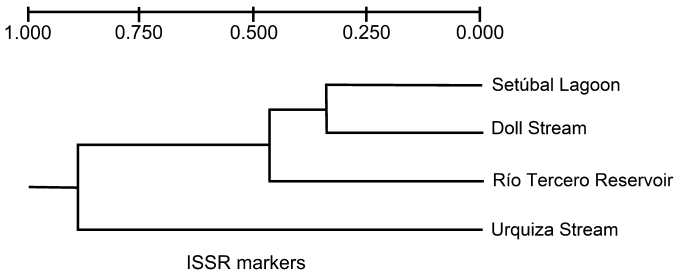

Fig. 3. Dendrogram performed by UPGMA tree based on the genetic distances for the populations studied of *Aegla uruguayana* Schmitt, 1942.

other populations (fig. 3). Finally, the most likely number of individual clusters (K) estimated by the Bayesian clustering method was four ($K = 4$). The Urquiza Stream population was integrated by two different genetic groups, while the Doll stream was integrated by another. However, Río Tercero Reservoir and Setubal lagoon constituted one single cluster. The sampled sites were genetically structured between Uruguay and Paraná sub-basins and within the Paraná sub-basin (fig. 4).

DISCUSSION

Analysis of genetic variability with ISSR markers

The *Aegla uruguayana* populations that were the furthest apart demonstrated the greatest difference in H_e and P, with Urquiza Stream having the highest and Río Tercero Reservoir the lowest values, while the Doll stream and Setúbal lagoon populations were nearest and had very similar H_e and P values (table III). In freshwater or sea decapods, the heterozygosity levels are variable according to observations by Busack (1988), Nguyen et al. (2005), and Weber & Levy (2000). In agreement with this result, we found evidence of two scenarios; the first was represented by the population age (younger or older). For example, in two crayfish (*Procambarus acutus* (Girard, 1852) and *P. clarkia* (Girard, 1852)), the more ancestral species had a marked heterozygosity compared with the younger populations (Busack, 1988), and it was hypothesised that the *P. clarkii* radiation was relatively recent. The second scenario is related to the decreased allelic diversity by low genetic drift and was also observed in freshwater crayfish (Hedgecock et al., 1979; Avery & Austin, 1997; Crandall, 1997; Nguyen et al., 2005). In *A. uruguayana*, each population studied had different H_e and P levels, suggesting that this species' variability was most likely related to habitat area and age as well as the immigration potential of the locality (Fuller & Lester, 1980) and habitat stability. Some populations such Río Tercero Reservoir have not had

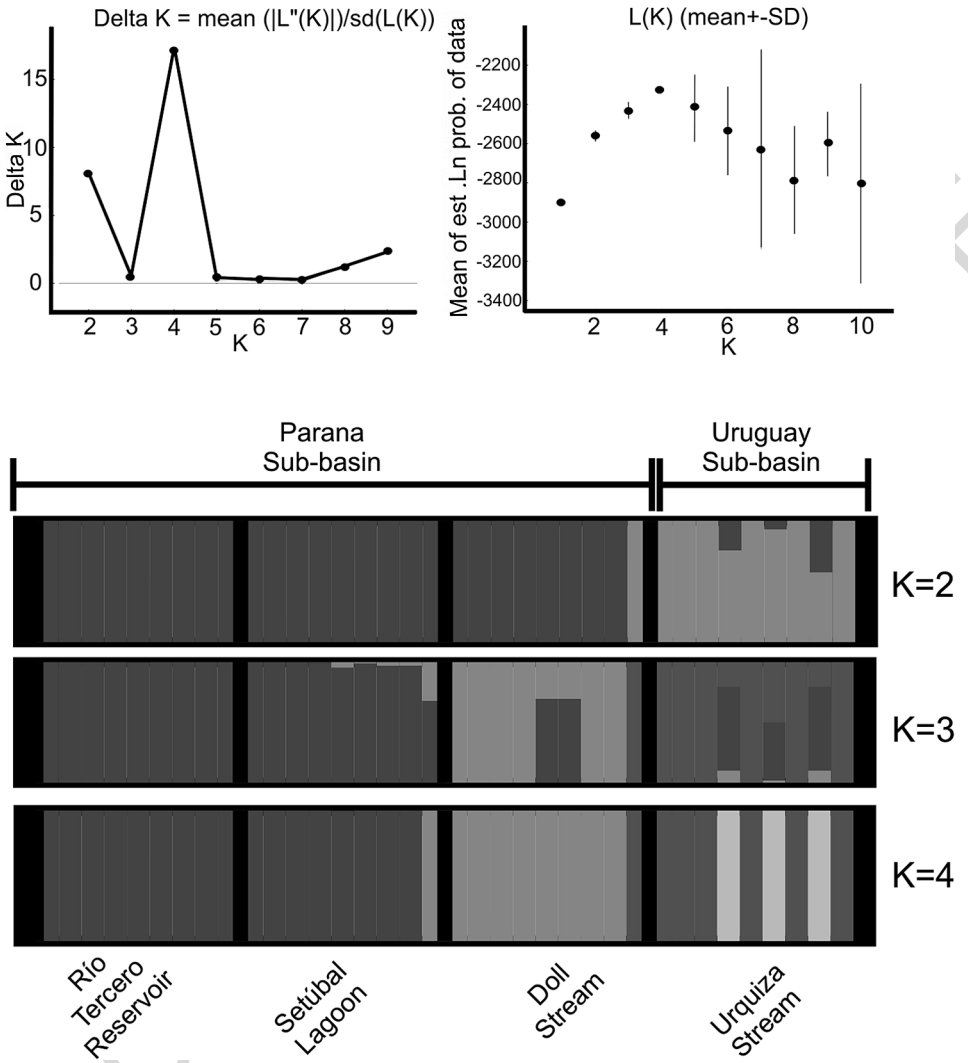

Fig. 4. Analysis showed the four population structure by ISSR of *Aegla uruguayana* Schmitt, 1942.

drastic environmental changes by recent marine transgression because they have not reached these places. However, other populations were affected (e.g., Urquiza Stream) by the last marine transgressions.

In accordance with this observation, the Rio Tercero population was considered to be ancestral by habitat stability, and the site border position in the system added to the low movement capacity of Aeglidae (Lopez, 1965; Maynard Smith, 1998; Xu et al., 2009). Moreover, the geomorphological history of the basin could be added as evidence of isolation; and other factor and relative to the decapods taxa could be the direct development of juveniles without planktonic larval stage, which

also is a factor limiting of the gene flow among populations (Anger, 2013). Species with planktonic larval phases have more homogeneity, unlike the species with direct development that have strong heterogeneity at scarce distances (Burton & Feldman, 1981; Knowlton & Keller, 1986; Weber & Levy, 2000; Weber et al., 2000).

The other three populations (Urquiza Stream, Doll Stream and Setubal Lagoon) show more effective connections among populations due of their geographic-hydrologic positions (e.g., current velocity, turbidity, substrate and depth). Storms provoke downstream drift to individuals of decapod populations, and the flood pulse provoke a high level of connectivity among environments, permitting the gene flow through of passive movements or active migrations (Collins et al., 2006). With special attention, the Urquiza population could be the product of great gene flow by their geographic position. This population could be integrated by specimens from de La Plata River and Paraná River populations or the Rio Negro and from Uruguay River populations, which border *A. uruguayana* distribution sites. Another factor that could have contributed to the high H_e and P levels is environmental instability (Maynard Smith, 1998). The physical and geographic landscape aspects could condition *A. uruguayana* migration because species distribution occurs in great environmental diversity (e.g., mountain streams, floodplain rivers, lagoons and lakes), and each of these have different physical-chemical characteristics.

Population structure analyses

Populations are genetically "well" structured with variable population distances. In a similar study, four populations of the crab *Callinectes danae* Smith, 1869 with different distances among them are considered to have a certain independence, and the individuals represent four distinct subpopulations (Weber & Levy, 2000). This finding determines that there is a population genotype structure with the capacity to modify itself as a consequence of the different environmental conditions and connections (or gene flow) with other populations.

The population structure could be more evident in freshwater animals than those that live in sea or salt-marsh environments, where the abiotic conditions are more stable (Nguyen et al., 2005). However, in freshwater decapods, population genetic studies suggest different population genetic structure patterns (Daniels, 2003). For example, Daniels et al. (1998) showed that *Potamonautes calcaratus* (Gordon, 1929) populations were genetically moderately structured in very close populations. In contrast, other studies demonstrated genetic invariance both within and among *P. parvispina* Stewart, 1997 populations from two geographically isolated drainage systems. Moreover, freshwater crayfish *Austropotamobius*

torrentium (Schrank, 1803) or freshwater African crab *P. perlatus* (Milne-Edwards, 1837) populations have genetic differences among nearby populations (Daniels et al., 1999; Schubart & Huber, 2006), similar to some river crab species in South Africa or from Jamaican rivers (Daniels, 2003; Schubart & Koller, 2005).

According to Weber & Levy (2000), the observed genetic structure in *A. uruguayana* would provide evidence of the differences among populations, thus defining three genetic units (clusters) and distinguishing the Urquiza stream population as the most different. Migrant number (N_m), which ranged from 0.3-0.8 (table III), would be additional evidence of population quasi-independence. Under this model, Wright (1969) proposed that an $N_m > 1$ in each generation is sufficient to counteract drift-associated genetic differentiation.

In our study, the populations are distant from each other (from 100 to 1100 km), with potential corridors and without geographic barriers that interrupt gene flow. The difference among populations agrees with proposals that were realised by other researchers (Hedgecock et al., 1979; Fuller & Lester, 1980; Busack, 1988; Fevolden & Hessen, 1989; Nguyen et al., 2005), where the population structure will be influenced by the fragmentary nature of the freshwater environments, which limits gene flow and favours population divergence.

In the current *A. uruguayana* distribution area, different sea ingression events occurred, which could provoke different population displacement. The Rio Tercero sample site was never affected directly by the transgressions (Lundberg et al., 1998), which suggests that the Rio Tercero population could be relictual and most likely the most ancestral. The other populations demonstrated high heterozygosity values by anomurans gene flow from other relict areas. Horwitz & Knott (1995) indicated that populations could have survived in the aquatic refuges of different geographic zones, and after many sea ingressions, the migration to colonise old or new areas began. Late Miocene (11.8-10 Ma) marine transgressions could have caused extinct at different *A. uruguayana* populations from their geographic distributions. After the transgression, the animals input could have been generated to form new populations that were occupying environments that were previously flooded by the sea. Furthermore, ingressions of the sea have occurred several times, the last of which was approximately 6000-4000 years ago (Fucks et al., 2011), which did not directly influence study sample locations. The main effect occurred only in the main corridors of each sub-basin, 300 km upstream of the La Plata River mouth. Species population isolation because of sea ingression is registered in other related species such as *Callinectes bellicosus* (Stimpson, 1859) (Pfeiler et al., 2005), whose gene flow was interrupted by the sea during the Pleistocene. The populations began to diverge before the river reconnection. A similar example is freshwater crayfish that demonstrated quick postglacial re-colonisation in an

extensive European area (Hewitt, 1999; Schubart & Huber, 2006), indicating that the old haplotypes corresponded to the middle Pleistocene (e.g., 11 000 to 1.8 Ma). These populations survived glaciations in micro-refuges of the Alps (Trontelj et al., 2005; Schubart & Huber, 2006).

Geo-climatic processes occurring in the basin provide evidence to hypothesise that different *A. uruguayana* populations could be isolated. Speciation dynamics were observed, which could have occurred in southern South American of Aeglidae species. These data reflect the importance of understanding geo-climatic history in the study region and the importance of using this evidence to understand distribution populations in where the species live. These data permit us to conclude that different populations have a relatively independent history as delineated by the geomorphologic events that occurred during the Earth's history. These results showed low levels of genetic variation in different populations that varied according to habitat area, habitat age, and the immigration potential of the locality. The above-mentioned studies on freshwater crustaceans have suggested that geographic distribution in fragmented habitats affects the population structure by limiting gene flow.

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