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Geographical isolation and restricted gene flow drive speciation of *Aegla singularis* (Decapoda: Anomura: Aeglidae) in southern South America

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Geographical isolation is a key element in allopatric speciation. If gene flow is interrupted for long enough by geographical barriers, populations can evolve independently and eventually form distinct species. *Aegla singularis* provides an ideal model to study this process due to the characteristics of the geographical area that it occupies and its limited dispersal ability. *Aegla singularis* inhabits streams of the Uruguay and Paraná River basins in the Neotropical region of South America. The basins are separated by the Sierra Central Mountains. Here we studied the speciation of *A. singularis* resulting from geographical isolation by using molecular and morphometric data. Individuals of *A. singularis* were analysed using geometric morphometrics and genetic data (*COII* and *EFα1*). We found significant differences in shape and genetics between *A. singularis* populations from the two basins. These differences suggest ongoing divergence due to restricted gene flow caused by the geographical barrier of the Sierra Central Mountains, indicating that the populations of the Parana and Uruguay River slopes are undergoing divergence.

ADDITIONAL KEYWORDS: Aeglidae – allopatry – geometric morphometrics – molecular evidence – phylogeography – speciation.

INTRODUCTION

Little or no gene flow among populations is a key element in speciation (Dobzhansky, 1937). Geographical isolation affects gene flow among individuals in different ways, potentially leading to allopatric speciation. Morphological diversification and population structuring due to a cessation or reduction in gene flow could be the first steps towards speciation (Wiens, 2004; Dutech *et al.*, 2005). There is strong evidence supporting the role of geographical isolation in the process of speciation in crustacea (Barr and Holsinger, 1985; Mashiko, 2000; Stevens and Hogg, 2004; Marchiori et al., 2014; Worsham *et al.*, 2017).

From a biological point of view, molecular and phenotypic evidence can be studied to assess differentiation of populations that seem isolated. Phenotypic expression has an important evolutionary history component (Dutech *et al.*, 2005; Barría *et al.*, 2011) and quantitative shape analysis can help to define intraspecific and interspecific variation (Giri & Collins, 2004; Marchiori *et al.*, 2014; Karanovic *et al.*,

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2018). On the other hand, knowledge of intraspecific molecular genealogies allows us to infer how palaeoclimatic processes have affected the dynamics of populations and to determine current genetic structure (Avise, 2009). Additionally, molecular markers are very useful in identifying cryptic species (Xiao *et al.*, 2010).

Geographical barriers have played a very important role in the diversification of South American freshwater fauna (Darwin, 1859). Despite the limited ability of aeglids to move and migrate (López, 1965; Xu *et al.*, 2009), their radiation and speciation throughout the Cenozoic up to the present day are evidence of successful adaptation and colonization of freshwater habitats (Bueno *et al.*, 2016). This has resulted in a huge diversity of species (Moraes *et al.*, 2016); 82 species have been identified and many others are under study (Santos *et al.*, 2017). Aeglidae freshwater crabs have characteristics that make them of particular interest for evolutionary studies: it is the only anomuran family entirely restricted to freshwater environments, their taxonomic position is disputed and many of their species are threatened (Pérez-Losada *et al.*, 2004; Tumini *et al.* 2019).

The genus *Aegla* provides a good model to apply morphometric analysis because it has a well-preserved morphology and the carapace can vary both within and between species (Martin & Abele, 1988; Giri & Loy, 2008; Hepp *et al.*, 2012). Several studies have described the size and shape of these organisms. Hepp *et al.* (2012) studied different populations of *A. plana* and found that morphological variation was related to environmental variables. Fernandes & Bichuette (2013) found morphological differences between *A. schmitii* individuals inhabiting epigean streams and caves. Marchiori *et al.* (2014) analysed populations of *A. longirostri* in six rivers from southern Brazil and found significant differences in carapace shape among all populations, which could indicate the existence of cryptic species or an incomplete process of speciation. Furthermore, Giri & Loy (2008) studied *A. neuquensis* and *A. riolimayana* populations and found sex and interspecific differences, especially among lake and river populations. Giri & Collins (2014) observed a clinal pattern throughout the distribution of *A. uruguayana*, which could be attributed to genetic drift and geographical isolation of the basins in which it is found, differences in environmental characteristics, and the low dispersal ability of these organisms. Phenotypic plasticity could also explain the size and shape differences (Valladares *et al.*, 2006).

Molecular studies have also been carried out in Aeglidae to better understand their evolution. Pérez-Losada *et al.* (2004) used multiple genes to study the biogeography and phylogenetic relationships within *Aegla*. Giri *et al.* (2014) also used molecular markers (inter simple sequence repeats) to analyse the genetic structure of *A. uruguayana* and found that different populations, inhabiting different sub-basins, have independent histories. The eastern population presents a different genetic structure from the western populations. Thus, the geomorphological history of the La Plata Basin provides evidence for the isolation or reduced gene flow among these populations. Pérez-Losada *et al.* (2011) compared phylogeographical patterns in *A. alacalufi* and *A. neuquensis* from Patagonia and found that their populations diverged differently due to different isolation processes. Crivellaro *et al.* (2018) carried out a molecular study to test the monophyly of *A. longirostri* and observed the presence of two major clades (North and South), which, although geographically close, were genetically very distinct from each other. However, Zimmermann *et al.* (2018) did not find cryptic species in *A. platensis*, a broadly distributed aeglid with low genetic diversity.

Aegla singularis inhabits the streams of the Uruguay and Paraná River basins (Del Plata River system) in Paraná province, a Neotropical region of South America. In the area of the species distribution, the Paraná and Uruguay Rivers are separated by a mountain chain, the Sierra Central (Amsler, 2000), and the two rivers converge more than 100 km downstream from the study site. The populations of the two basins are thus geographically isolated, given that there is little or no gene flow among them. In this scenario, mechanisms such as genetic drift and directional selection are expected to be more pronounced, because populations are subject to independent evolutionary mechanisms that could increase phenotypic variation (Hoffmann & Shirriffs, 2002; Wiens, 2004) due to the absence or very low gene flow. Although *A. singularis* shows a restricted distribution, its phylogeography and evolution have not yet been studied. The presence of a continuous mountain chain and the lack of connectivity between the Uruguay and Paraná basins are potential barriers to gene flow and could induce speciation. The aim of this work was to evaluate the process of speciation driven by geographical isolation as a main factor using molecular and morphmetric data. The evidence presented here could help to understand the evolutionary processes in other aeglid species and generally provide scenarios that explain the great diversity seen within the genus the *Aegla*. It could also provide a model to answer questions regarding other freshwater decapods in which evolutionary processes have not been sufficiently studied.

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MATERIAL AND METHODS

Sample collection

We collected 165 specimens of *A. singularis* (Parana: 58 females and 62 males; Uruguay: 19 females and 26 males) from 11 tributaries of the Paraná and Uruguay River basins located in Misiones province, Argentina (Figure 1; Table 1). At the time of sampling, the tributaries were shallow, with different degrees of transparency and water velocity, and had little submerged vegetation. The bed of the streams was composed mainly of clay, rocks and sand. Specimens were sampled in 2009, 2011 and 2016 and are now housed in the collection of Macrocrustacean Laboratory (INALI-CONICET-UNL). Before DNA extraction and photography, the specimens were cryo-anaesthetized (the temperature of the specimens were reduced in cold water of 0 °C) until they no longer responded to stimuli.

Genetic analyses

Genomic DNA was extracted from gill and muscle tissue of *A. singularis* specimens using an AccuPrep Genomic DNA Extraction Kit. The extracted DNA was used to amplify fragments of 500 bp of the mitochondrial cytochrome oxidase subunit II gene (*COII*; Pérez-Losada *et al.*, 2002) and a single-copy nuclear intron (*EFα1*; Xu *et al.*, 2009). A total of 99 specimens of *A. singularis* were amplified, 54 for *COII* and 41 for *EFα1*. A sequence of the mitochondrial gene *COII* from GenBank of a Brazilian specimen (Pérez-Losada *et al.*, 2004; accession code: AY595739.1 GI: 46989080) was also added to the analysis. PCRs were performed using 1 U of Taq polymerase (Promega), dNTPs (0.25 mM each), $MgCl_2$ (2.5 mM), 1× Taq buffer (Promega), primers (0.48 mM each) and 1 μL of DNA (50 ng) in a final volume of 25 μL. The amplification conditions included an initial denaturation step at 94 °C for 3 min, followed by 35 cycles of 1 min at 45 °C (*COII*)/49 °C (*EFα1*) for annealing, and a final extension step at 72 °C for 10 min. The amplicons were sequenced by Macrogen Inc. (www.macrogen.com) using Sanger sequencing and the same primers as those used in the amplification reactions.

The DNA sequences were aligned using the ClustalW algorithm (Li, 2003) implemented in the MEGA7 software (Kumar *et al.*, 2016). The alignment was performed using default settings and adjusted by manual optimization when necessary. Genetic diversity was assessed using the nucleotide diversity (π) and haplotype diversity (h) estimators in DNASP (Rozas *et al.*, 2017). These estimators provide evidence of demographic changes that have occurred within populations (Avise, 2000). Analysis of molecular variance (AMOVA) was carried out in Arlequin 3.5 (Excoffier, 2006).

Phylogeographical analyses

Evolutionary relationships among *Aegla* haplotype variants were estimated using Bayesian phylogenetic inference via the Network software (http://www.fluxus-engineering.com/), and haplotype networks were calculated. Phylogenetic trees were then constructed. The best-fit model of nucleotide substitution was estimated using MrModelTest 2.2 (Nylander, 2004). The HKY+I model was chosen for posterior phylogenetic inference in MrBayes (Ronquist *et al.*, 2003). We ran two analyses with four chains, 1 000 000 initial generations of the Markov chain Monte Carlo (MCMC) and 5 000 000 posterior generations. A cluster analysis was constructed using the 'Bayesian approach to phylogeographic clustering' (BPEC) package (Manolopoulou *et al.*, 2016) implemented in the R package (R Development Core Team, 2012) to test gene flow between current populations and the most likely ancestral geographical locations (parameters, ds = 0, maximum number of migrations = 5 and 200 million iterations).

Geometric morphometric analyses

The images used for geometric morphometric analyses belong to the photograph database of the Macrocrustacean Laboratory (INALI) and were captured with a Sony Cyber-Shot DSC-W210 12.1-Mp camera. Photographs were taken from the dorsal part of the carapace at a distance of 30 cm from the specimen. We only photographed adult specimens (according to Viau *et al.*, 2006) of *A. singularis* (Table 1).

We identified 19 landmarks on the dorsal carapace to use as a baseline configuration (Fig. 2). The photographing and digitization of landmarks were made via the software tpsDig2 v.2.30 (Rohlf, 2017). The coordinates of the landmarks in the specimens were superimposed on a common coordinate system using Generalized Procrustes Analysis (GPA) to remove translation, rotation and scale effects (Rohlf & Slice, 1990). Errors in photographing and landmark digitization were estimated through Procrustes ANOVA with MorphoJ (Klingenberg, 2011). We also evaluated sexual dimorphism using a leave-one-out cross-validated method [based on linear discriminant analysis (LDA)], to evaluate within-group differences between males and females. Allometry, using lineal regression between the two groups (Parana vs. Uruguay basin populations), was performed to evaluate the relationship among size and shape. A multivariate analysis of covariance (MANCOVA) was then used to test the slopes among groups using TPSReg (v.1.45; Rohlf, 2017); if the interaction did not show any statistically significant differences, we

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Figure 1. Sites sampled for *Aegla singularis* in the province of Misiones, Argentina. Stream references are shown in Table 1. 'Brazil' indicates the location of the Brazilian specimen available in GenBank.

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Table 1. Information regarding the populations used in the different analysis

Map code	Basin	Stream	Coordinates	Tree code	mtDNA			nDNA			GM
					n	*Nh*	*Hd*	*n*	*Nh*	*Hd*	*n*
1	Paraná	Ñacaguanzú	27°7′14.50″S 55°22′22.97″W	Nac	6	1	0	5	4	0.9	16
2		Isabel	27°31′0.54″S 55°27′0.15″W	Isa	5	1	0	4	2	0.5	11
3		Mártires	27°31′21.84″S 55°10′45.17″W	Mar	10	3	0.688	2	2	1	24
4		Anyico	27°39′876″S 55°36′560″W	Any	8	5	0.785	7	5	0.857	33
5		Liso	27°40′26.92″S 55°37′13.67″W	Lis	4	3	0.833	7	4	0.714	21
6		Coaty	27°41′9.60″S 55°39′18.00″W	Coa	4	3	0.833	1	1	1	12
7	Uruguay	Los Muertos	27°22′18.66″S 54°24′25.56″W	LMu	–	–	–	2	0	1	6
8		Shancay	27°22′18.66″S 54°40′39.54″W	Sha	5	5	1	3	2	0.667	14
9		Panambí	27°44′46.05″S 54°59′59.12″W	Pan	8	3	0.464	8	2	0.535	13
10		Intersección	27°47′52.20″S 55°8′34.14″W	Int	–	–	–	1	0	–	4
11		Santa María	27°54′13.92″S 55°22′19.20″W	SMa	4	1	0	1	1	1	8

Number of individuals (*n*), haplotype number (*Nh*), haplotypic diversity (*Hd*) and stream codes used in phylogenetic trees and maps. mtDNA, mitochondrial DNA; nDNA, nuclear DNA; GM, geometric morphometrics.

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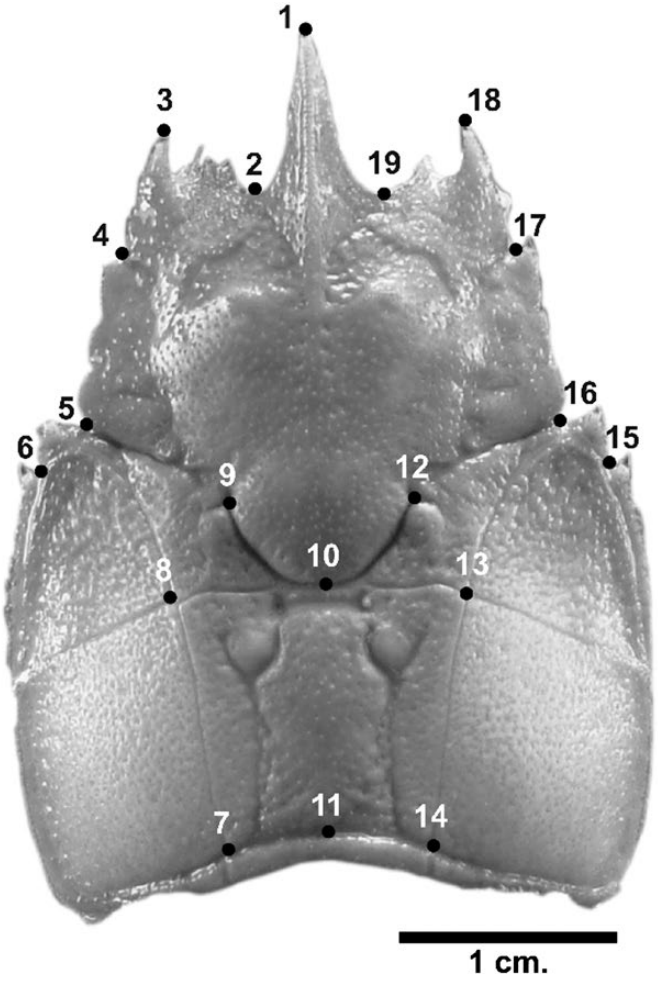

Landmark location	Landmark number
Tip of the *rostrum*	1
Orbital sine	2 and 19
Anterolateral spinal ends	3 and 18
Union between the first and second hepatic lobe	4 and 17
Union between the third and fourth hepatic lobe	5 and 16
Union between the epibranchial tooth and the *linea aeglica lateralis*	6 and 15
Posterior extreme of the longitudinal dorsal line	7 and 14
Posterior and anterior extremes of the bar line	8 and 13
Cervical groove (gastric área)	9 and 12
Areola center	10
Centre-posterior extremes of the cephalothorax	11

Figure 2. Carapace in dorsal view. Landmarks configuration and description.

use the residuals of the regression, whereas if the interaction was statistically significant the original data were used. To evaluate size among specimens of the two groups, ANOVAs were used. Principal components analysis (PCA) was used to evaluate the presence of patterns within groups fromn the two river basins. A leave-one-out cross-validated methods (based on LDA) with permutation test (10 000 permutations) was performed in MorphoJ (Klingenberg, 2011).

RESULTS

GENETICS

A 493-bp fragment of the mitochondrial *COII* gene was sequenced for 55 individuals. A total of 53 polymorphic sites were identified and 19 haplotypes were defined based on these polymorphic sites. The analysis of genetic diversity for all samples showed high nucleotide diversity (π: 0.04109) and high haplotype diversity (h: 0.834). A 250-bp fragment of the *EFα1* gene was sequenced for 45 individuals. A total of 109 polymorphic sites were identified and 18 haplotypes were defined based on these polymorphic sites. The analysis of genetic diversity for all samples showed high nucleotide diversity (π: 0.11684) and high haplotype diversity (h: 0.820). F_{ST} values revealed differences among river basins, both for *EFα1* (0.3251, $P = 0.0001 \pm 0.0001$) and for *COII* (0.9325, $P < 0.0001 \pm <0.0001$).

PHYLOGEOGRAPHY

The *COII* phylogenetic tree separated individuals from the two river basins into two distinct haplogroups connected by a long tree branch. The *EFα1* tree depicted the same two haplogroups found in the *COII* tree, but divergence between them was lower and two individuals were not segregated according to their river of origin. Haplotype networks and phylogenetic trees are shown in Figure 3 for *COII* and Figure 4 for *EFα1*.

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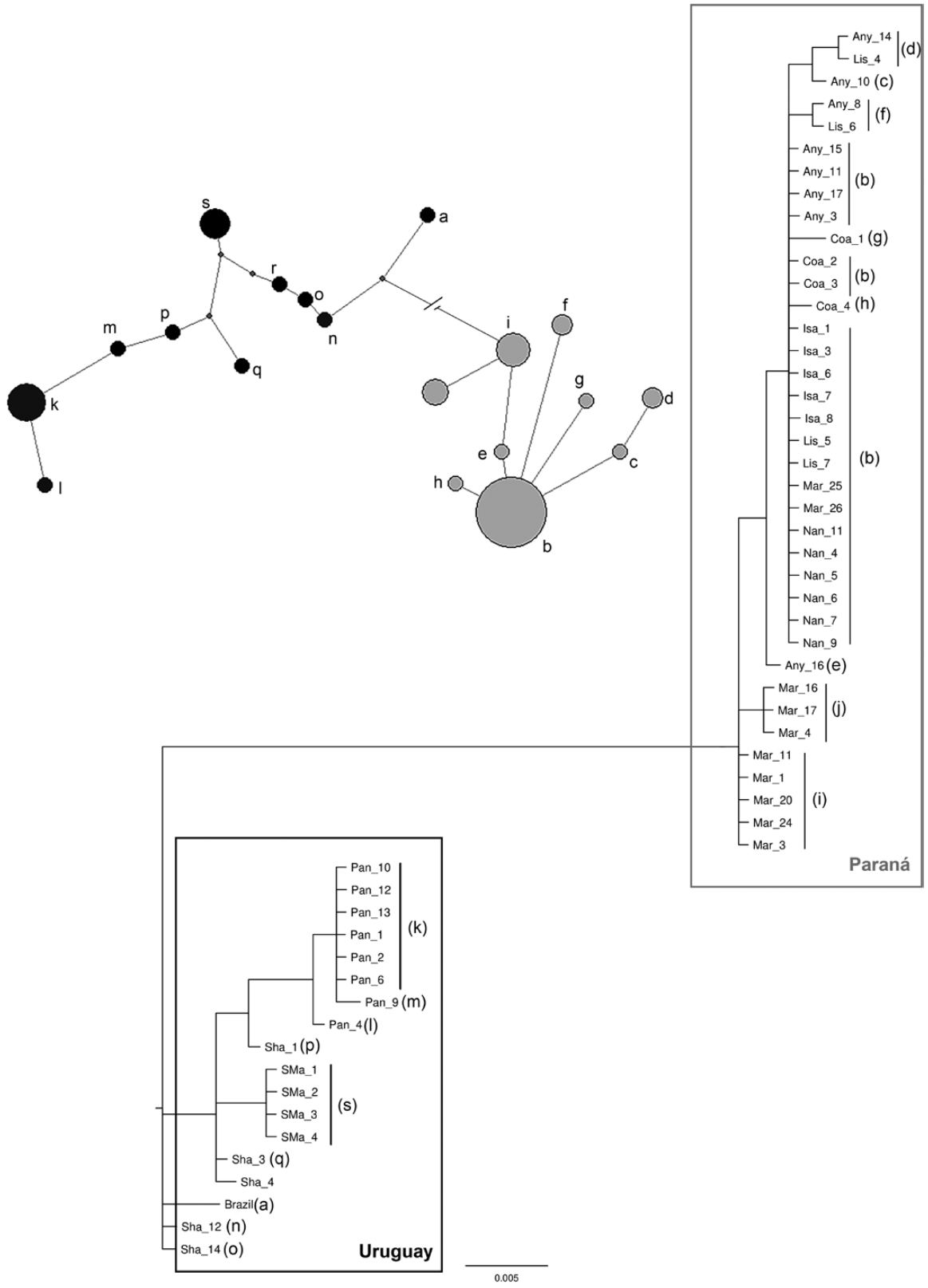

Figure 3. Bayesian phylogenetic analysis of *COII* haplotypes. Parana River (grey box) and Uruguay River specimens (black box). Scale 0.005 changes.

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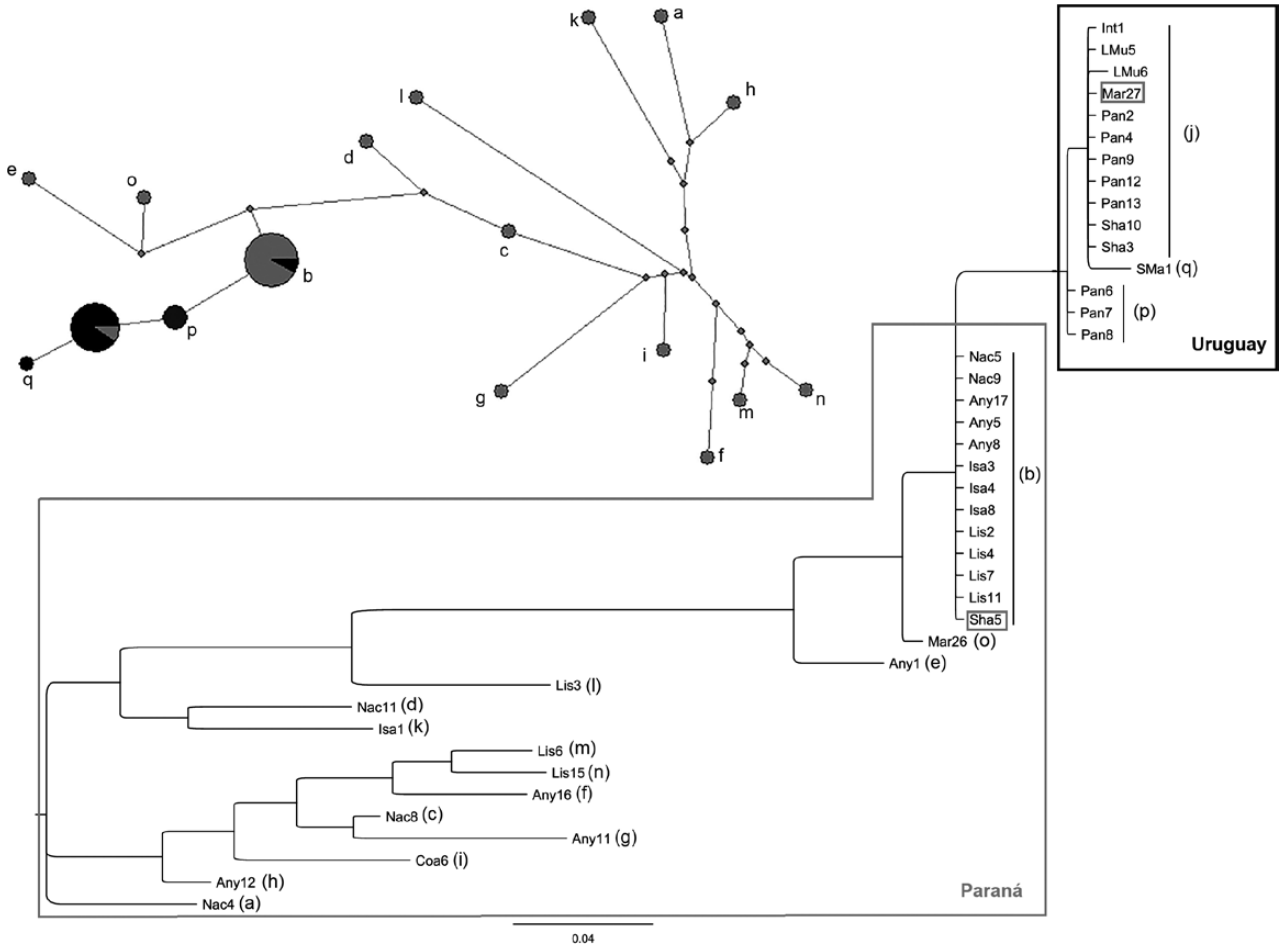

Figure 4. Bayesian phylogenetic analysis of *EFα1* haplotypes. Parana River (grey box) and Uruguay River (black box) specimens. The Uruguay Mar27 haplotype is depicted in the Parana basin, while the Parana haplotype Sha5 is depicted in the Uruguay basin. Scale 0.04 changes.

Three more well-defined clusters were observed in the BPEC analysis: one for the Paraná River basin and two for the Uruguay River basin. These three clusters overlapped and fell in the middle of the two river basins, indicating possible past gene flow between them (Fig. 5).

Geometric morphometrics

Photographing and digitization errors were negligible. The discriminant analysis test for sexual dimorphism did not reveal statistical differences among individuals of either group ($P > 0.05$). Instead, allometry was detected (predicted = 2.3861%, $P = 0.0001$), but MANCOVA revealed that the interaction was statistically significant, after which we ran all analyses using non-corrected data. In this way, regression evidence showed that Uruguay River crabs were larger than the Parana River crabs (Fig. 6). ANOVA on centroide size corroborated this ($F = 83.67$, d.f. = 1, $P < 0.0001$). The Parana basin populations (predicted = 3.0586%, $P = 0.0006$) and Uruguay basin populations (predicted = 4.0808%, $P = 0.0586$) revealed, as shown above, different patterns of allometry that indicate group divergence. Thereafter, we performed analysis without allometry correction. Shape differences were also found in the PCA (PC1, PC2 and PC3 explained 23.11, 15.56 and 10.07% of the variation in shape, respectively) among Paraná and Uruguay groups (Fig. 7). Different PCs revealed patterns of shape variation supporting the separation among groups; in PC1, specimens belonging to the Parana and Uruguay populations showed virtually no overlap (Fig. 7A). PC2 revealed that shape variance observed in Parana specimens was larger than for Uruguay specimens (Fig. 7B). PC3 showed differences among the two groups, similar to those observed in PC1, but with more overlap. Discriminant analysis of the two river basin populations revealed statistically significant differences (leave-one-out cross-validation:

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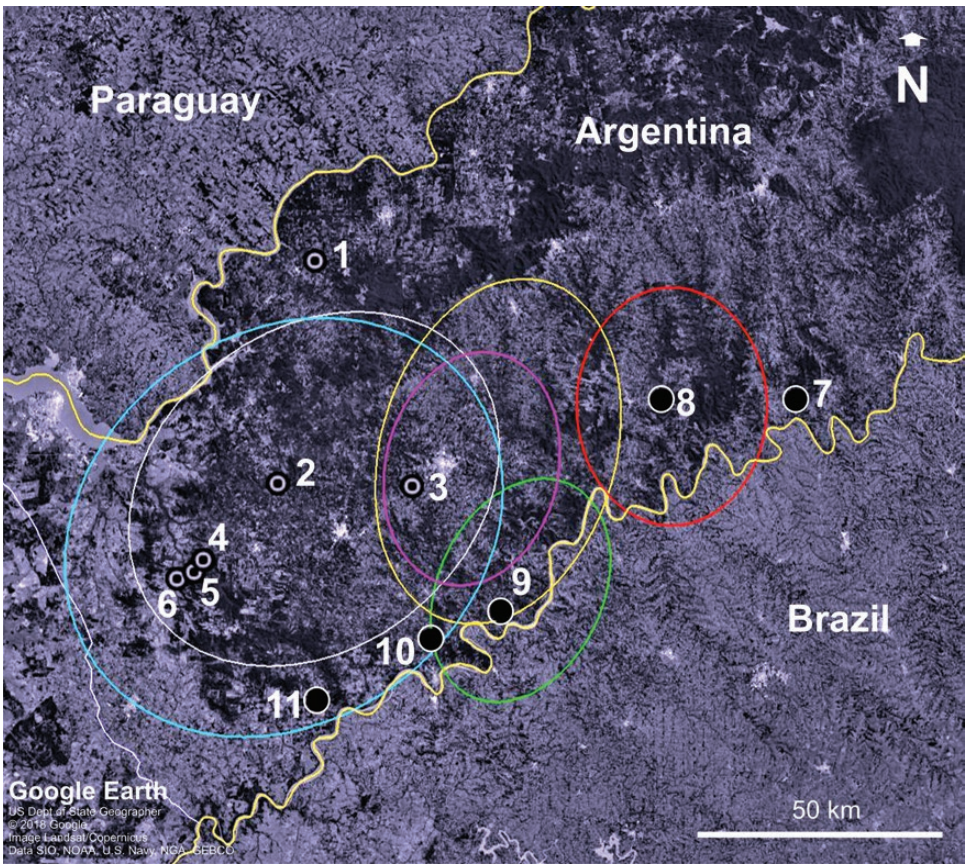

Figure 5. Cluster analysis map built by BPEC software using the *COII* gene region. Colours represent different clusters and landmarks represent sampling sites. Stream references are given in Table 1.

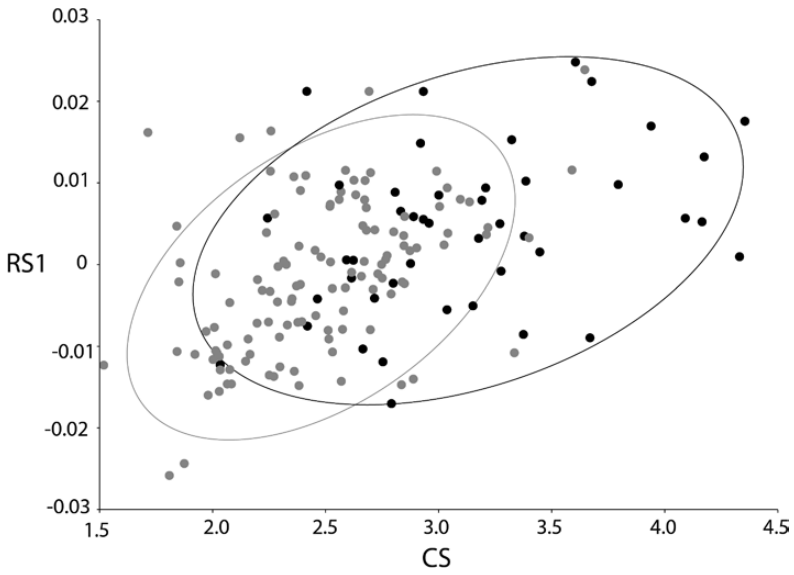

Figure 6. Allometry, and shape changes related to centroid size. Parana River (grey dots) and Uruguay River (black dots).

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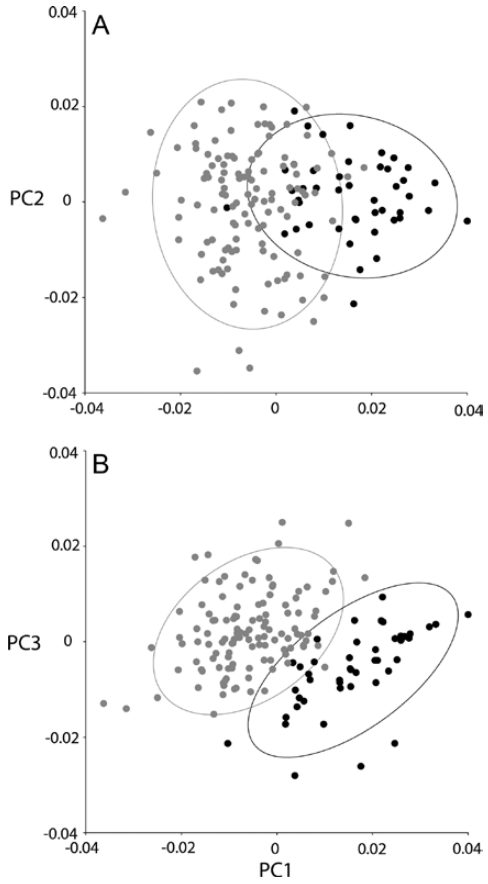

Figure 7. Principal component analysis of shape coordinates from dorsal carapace shape. A, PC1 and PC2; B, PC1 and PC3. Parana River (grey dots) and Uruguay River (black dots).

$P < 0.0001$, in both Procrustes = 0.0237 and Mahalanobis distances = 4.009), based on LDA (Fig. 8). Classification/misclassification among two basins groups was found: 114 (97.5%) in the Paraná group and six (2.5%) in the Uruguay group; 45 (100%) specimens belonging to the Uruguay basin were well classified. Aeglids from the Paraná basin had a thinner carapace and their abdomen and cephalothorax had similar width. Rostrum spines were short and wide. In the consensus of the aeglids from the Uruguay River, the cephalothorax was wider than the rostrum and abdomen. Rostrum spines were also longer, more elongated and thinner (Fig. 9).

DISCUSSION

According to our assumptions (Wiens, 2004; Dutech *et al.*, 2005) and all the evidence presented in this research, we conclude that the two group of aeglids studied are in the process of speciation. Genetic, phylogeographical and morphological evidence show that the Uruguay and Parana basin groups present different characteristics, but also that the pattern of variations is different (e.g. nucleotide diversity, haplotype diversity, phylogeographical clustering, allometry and shape variation).

We have studied the speciation of *A. singularis* in southern South America using molecular and morphometric data. Our results indicate that *A. singularis* populations from the Uruguay and Parana River basins are diversifying due to geographical isolation and consequent gene flow interruption. Both mitochondrial (*COII*) and nuclear (*EFα1*) genes indicate that the *Aegla* populations can be subdivided into two haplogroups, consistent with the study river basins. We also found that body size and carapace shape of *A. singularis* individuals differ significantly ($P < 0.05$) between the Parana and Uruguay populations. This suggests ongoing speciation between populations of the two river basins due to geographical isolation caused by the Sierra Central Mountains, which pose a barrier to migration. The most likely ancestral locations for *A. singularis* were the Coaty, Isabel and Liso streams, all within the Paraná River basin. This is consistent with the fact that the Paraná was the first of the two rivers to originate in the La Plata system (Soldano, 1947).

Previous work in other *Aegla* species found differences in the morphology and genetic structure of populations. Giri & Loy (2008) compared individuals of *A. neuquensis* from lakes and rivers and discovered the presence of two ecotypes associated with each environment. Fernandes & Bichuette (2013) studied specimens of *A. schmitii* from underground caves and surface rivers and found morphological variations associated with environmental conditions. Marchiori *et al.* (2014) revealed morphometric differences in *A. longirostri* populations correlated with geographical distance, thus demonstrating the possible existence of cryptic species and ongoing speciation. Subsequently, Crivellaro *et al.* (2018) corroborated this result using mitochondrial and nuclear molecular markers and showed that this species has a polyphyletic origin. Giri *et al.* (2014) and Giri & Collins (2014) found that in *A. uruguayana*, a broadly distributed aeglid, morphological and genetic divergence were related to geographical distance and that geo-climatic processes (Gondwana separation, Andes uplift, marine transgressions, and glaciations) occurring in South Americn river basins could have led to population isolation. Another study (Crivellaro *et al.*, 2018) of a widely distributed species, *A. longirostri*, from southern Brazil using multiple loci showed high genetic differentiation between populations across two geographically isolated river basins and suggested the presence of cryptic species. Xu *et al.* (2009) examined Pleistocene glacial impacts on the phylogeographical pattern of *A. alacalufi* in Chilean

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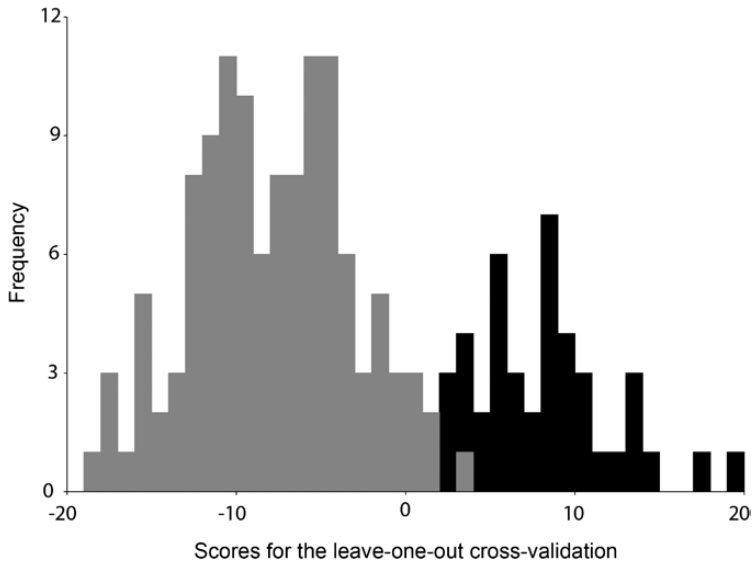

Figure 8. Leave-one-out cross-validation (based on linear discriminant analysis) between the Parana River (grey bars) and Uruguay River (black bars).

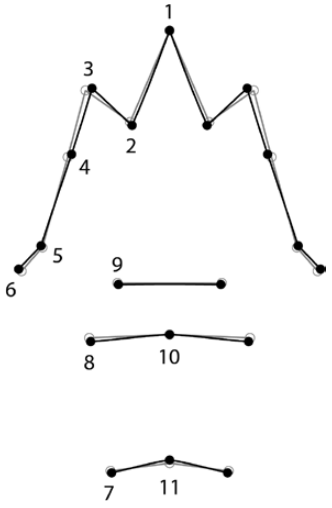

Figure 9. Shape differences between Parana River (grey) and Uruguay River (black) specimens indicated by discriminant function analysis.

Patagonia and found a deep phylogenetic structure in populations on islands thtat were free of ice or at the edge of ice but a shallow one in populations on the glaciated continent as a consequence of glacial cycles. They also suggested vicariance from drainage isolation as an important mechanism for producing the differentiation among the non-glaciated and glaciated populations. In a study of the phylogeography of *A. neuquensis* (Barber *et al.*, 2012), samples from two rivers (Vaca and Telsen) were morphologically similar to *A. neuquensis* although molecular data indicated that they were more closely related to other *Aegla* taxa. Interestingly, these rivers are geographically isolated from other systems and have not been connected to rivers that contain another *A. neuquensis*. A recent study (Zimmermann *et al.*, 2018), however, found low genetic diversity in a widely distributed species, *A. platensis*. They found evidence for only two potentially unrecognized new species from 18 populations under study, although *A. platensis* seemed to be polyphyletic. Similarly, Barría *et al.* (2011) found no morphological differences between *A. araucaniensis* populations from the rivers of the hydrographic system of the Valdivia River, as these rivers are connected to the main basin, thus facilitating gene flow between populations. These results do not fit the phylogeographical pattern seen in most other aeglids, suggesting that the habitat, historical climatic and geological changes and low dispersion capacity of these crabs may impact *Aegla* differentiation and speciation differently.

In summary, our genetic, phylogeographical and geometric morphometric analyses provide evidence of the ongoing divergence of *A. singularis* populations from the Uruguay and Paraná Rivers due to restricted gene flow, probably caused by the Sierra Central Mountains barrier. Geographical isolation, which restricted gene flow, is probably a recurrent starting point for the speciation process in *Aegla* and the main driver of its species diversity.

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REFERENCES

Adams DC. 1999. Methods for shapes analysis of landmark data from articulate structures. *Evolutionary Ecology Research* **1:** 959–970.

Alberch P, **Gould SJ**, **Oster GF**, **Wake DB. 1979.** Size and shape in ontogeny and phylogeny. *Paleobiology* **5:** 296–317.

Amsler M. 2000. *El río Paraná en su tramo medio: contribución al conocimiento y prácticas ingenieriles en un gran río de llanura*. Centro de publicaciones. Secretaría de Extensión. Universidad Nacional del Litoral 1.

Avise JC. 2000. *Phylogeography: the history and duration of species*. Cambridge, MA: Harvard University Press.

Avise JC. 2009. Phylogeography: retrospect and prospect. *Journal of Biogeography* **36:** 3–15.

Barr TC Jr, **Holsinger JR. 1985.** Speciation in cave faunas. *Annual Review of Ecology and Systematics* **16:** 313–337.

Barber BR, **Xu J**, **Pérez-Losada M**, **Jara CG**, **Crandall KA. 2012.** Conflicting evolutionary patterns due to mitochondrial introgression and multilocus phylogeography of the Patagonian freshwater crab *Aegla neuquensis*. *PLoS One* **7:** e37105.

Barría EM, **Jara CG**, **Sepúlveda RD. 2011.** Morphologic variation in *Aegla* leach (Decapoda: Reptantia: Aeglidae) from Central-Southern Chile: interspecific differences, sexual dimorphism, and spatial segregation. *Journal of Crustacean Biology* **31:** 231–239.

Bueno SL, **Santos S**, **da Rocha SS**, **Gomes KM**, **Mossolin EC**, **Mantelatto FL. 2016.** Avaliação do estado de conservação dos eglídeos (Decapoda: Aeglidae). In: Pinheiro M, Boss H, eds. *Livro vermelho dos crustáceos do Brasil: avaliação 2010–2014*. Porto Alegre: Sociedade Brasileira de Carcinologia, 35–65.

Crivellaro MS, **Zimmermann BL**, **Bartholomei-santos ML**, **Crandall KA**, **Pérez-losada M**, **Bond-buckup G**, **Santos S. 2018.** Looks can be deceiving: species delimitation reveals hidden diversity in the freshwater crab *Aegla longirostri* (Decapoda : Anomura). *Zoological Journal of the Linnean Society* **182:** 24–37.

Darwin, C. 1859. *On the origins of species by means of natural selection*. London: John Murray.

Dobzhansky T. 1937. *Genetics and the origin of species*. New York: Columbia University Press.

Dutech C, **Sork VL**, **Irwin AJ**, **Smouse PE**, **Davis FW. 2005.** Gene flow and fine-scale genetic structure in a wind-pollinated tree species, *Quercus lobata* (Fagaceaee). *American Journal of Botany* **92:** 252–261.

Excoffier L, **Laval G**, **Schneider S. 2006.** *Arlequin ver 3.1-- An Integrated Software Package for Population Genetics Data Analysis*.

Fernandes CS, **Bichuette ME. 2013.** Shape variation of *Aegla schmitti* (Crustacea, Decapoda, Aeglidae) associated to superficial and subterranean stream reaches. *Subterranean Biology* **10:** 17–24.

Giri F, **Collins P. 2014.** Clinal variation in carapace shape in the South American freshwater crab, *Aegla uruguayana* (Anomura: Aeglidae). *Biological Journal of the Linnean Society* **113:** 914–930.

Giri F, **Collins PA. 2004.** A geometric morphometric analysis of two sympatric species of the family Aeglidae (Crustacea, Decapoda, Anomura) from the La Plata basin. *Italian Journal of Zoology* **7:** 85–88.

Giri F, **Loy A. 2008.** Size and shape variation of two freshwater crabs in Argentinean Patagonia: the influence of sexual dimorphism, habitat, and species interactions. *Journal of Crustacean Biology* **28:** 37–45.

Giri F, **Ojeda G**, **Rueda E**, **Amavet P**, **Williner V**, **Collins P. 2014.** Population genetic structure of the freshwater anomuran *Aegla uruguayana* Schmitt, 1942 (Decapoda, Aeglidae) in the central region of Argentina. *Crustaceana* **87:** 414–429.

Hepp LU, **Fornel R**, **Restello RM**, **Trevisan A**, **Santos S. 2012.** Intraspecific morphological variation in a freshwater crustacean *Aegla plana* in southern Brazil: effects of geographical isolation on carapace shape. *Journal of Crustacean Biology* **32:** 511–518.

Hoffmann AA, **Shirriffs J. 2002.** Geographic variation for wing shape in *Drosophila serrata*. *Evolution* **56:** 1068–1073.

Karanovic T, **Lee S**, **Lee W. 2018.** Instant taxonomy: choosing adequate characters for species delimitation and description through congruence between molecular data and quantitative shape analysis. *Invertebrate Systematics* **32:** 551–580.

Klingenberg CP. 2011. MorphoJ: an integrated software package for geometric morphometrics. *Molecular Ecology Resources* **11:** 353–357.

Kumar S, **Stecher G**, **Tamura K. 2016.** MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Molecular Biology and Evolution* **33:** 1870–1874.

Li KB. 2003. ClustalW-MPI: ClustalW analysis using distributed and parallel computing. *Bioinformatics* **19:** 1585–1586.

López M. 1965. Estudios biológicos en *Aegla odebrechtti* paulensis, Schmitt (Crustacea, Decapoda, Anomura). *Boletim de Zoologia da Faculdade de Filosofia, Ciências e Letras da USP* **25:** 301–314.

Mashiko K. 2000. Insights into the mechanism of speciation in gammarid crustaceans of Lake Baikal using a population-genetic approach. *Advances in Ecological Research* **31:** 221–235.

Manolopoulou I, **Hille A**, **Emerson B. 2016.** BPEC: an R package for Bayesian phylogeographic and ecological clustering. *arXiv* preprint arXiv:1604.01617.

Marchiori AB, **Fornel R**, **Santos S. 2014.** Morphometric variation in allopatric populations of *Aegla platensis* (Crustacea: Decapoda: Anomura): possible evidence for cryptic speciation. *Zoomorphology* **134:** 45–53.

Martin JW, **Abele LG. 1988.** External morphology of the genus *Aegla* (Crustacea: Anomura: Aeglidae). *Smithsonian Contributions to Zoology* **453:** 1–46.

Moraes JCB, **Terossi M**, **Buranelli RC**, **Tavares M**, **Mantelatto FL**, **Bueno SLDS. 2016.** Morphological

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and molecular data reveal the cryptic diversity among populations of *Aegla paulensis* (Decapoda, Anomura, Aeglidae), with descriptions of four new species and comments on dispersal routes and conservation status. *Zootaxa* **4193:** 1–48.

Muñoz-Ortiz A, **Velásquez-Álvarez ÁA**, **Guarnizo CE**, **Crawford AJ. 2015.** Of peaks and valleys: testing the roles of orogeny and habitat heterogeneity in driving allopatry in mid-elevation frogs (Aromobatidae: Rheobates) of the northern Andes. *Journal of Biogeography* **42:** 193–205.

Nylander, JAA. 2004. *MrModeltest version 2.* Distributed by the author. Uppsala: Evolutionary Biology Centre, Uppsala University.

Pérez-Losada M, **Bond-Buckup G**, **Jara CG**, **Crandall KA. 2004.** Molecular systematics and biogeography of the southern South American freshwater 'crabs' *Aegla* (Decapoda: Anomura: Aeglidae) using multiple heuristic tree search approaches. *Systematic Biology* **53:** 767–780.

Pérez-Losada M, **Jara CG**, **Bond-Buckup G**, **Porter ML**, **Crandall KA. 2002.** Phylogenetic position of the freshwater anomuran family Aeglidae. *Journal of Crustacean Biology*. **22:** 670–676.

Pérez-Losada M, **Xu J**, **Jara CG**, **Crandall KA. 2011.** Comparing phylogeographic patterns across the Patagonian Andes in two freshwater crabs of the genus Aegla (Decapoda: Aeglidae). *Phylogeography and Population Genetics in Crustacea*. CRC Press, Boca Raton, pp. 291–303.

Pigliucci M. 2003. Phenotypic integration: studying the ecology and evolution of complex phenotypes. *Ecology Letters* **6:** 265–272.

R Development Core Team. **2012.** *R: A language and environment for statistical computing, reference index version. 2(0).* Vienna: R Foundation for Statistical Computing.

Rohlf F. 2017. *TpsDig2*. Stony Brook: Department of Ecology and Evolution, State University of New York.

Rohlf FJ, **Slice D. 1990.** Extensions of the Procrustes method for the optimal superimposition of landmarks. *Systematic Biology* **39:** 40–59.

Ronquist F, **Huelsenbeck JP. 2003.** MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* **19:** 1572–1574.

Rozas J, **Ferrer-Mata A**, **Sánchez-DelBarrio JC**, **Guirao-Rico S**, **Librado P**, **Ramos-Onsins SE**, **Sánchez-Gracia A. 2017.** DnaSP 6: DNA sequence polymorphism analysis of large data sets. *Molecular Biology and Evolution* **34:** 3299–3302.

Santos S, **Bond-Buckup G**, **Gonçalves AS**, **Bartholomei-Santos ML**, **Buckup L**, **Jara CG. 2017.** Diversity and conservation status of *Aegla* spp. (Anomura, Aeglidae): an update. *Nauplius* **25:** e2017011.

Schott RK, **Evans DC**, **Goodwin MB**, **Brown CM**, **Horner JR**, **Longrich NR. 2011.** Cranial ontogeny in *Stegoceras validum* (Dinosauria: Pachycephalosauria): a quantitative model of pachycephalosaur dome growth and variation. *PLoS One* **6:** e21092

Soldano FA. 1947. *Régimen y aprovechamiento de la red fluvial argentina*. Editorial cimera.

Stevens MI, **Hogg ID. 2004.** Population genetic structure of New Zealand's endemic corophiid amphipods: evidence for allopatric speciation. *Biological Journal of the linnean Society* **81:** 119–133.

Tumini, G, **Giri F**, **Williner V**, **Collins PA**, & **Morrone JJ. 2019.** Selecting and ranking areas for conservation of *Aegla* (Crustacea: Decapoda: Anomura) in southern South America integrating biogeography, phylogeny and assessments of extinction risk. *Aquatic Conservation: Marine and Freshwater Ecosystems* **29:** 1–13.

Valladares F, **Sánchez- Gómez D**, & **Zavala MA. 2006.** Quantitative estimation of phenotypic plasticity: bridging the gap between the evolutionary concept and its ecological applications. *Journal of Ecology* **94:** 1103–1116.

Viau VE, **López Greco, LS**, **Bond-Buckup G**, & **Rodríguez, EM. 2006.** Size at the onset of sexual maturity in the anomuran crab, *Aegla uruguayana* (Aeglidae). *Acta Zoologica* **87:** 253–264.

Wiens JJ. 2004. What is speciation and how should we study it? *The American Naturalist* **163:** 914–923.

Wu J, **Shen W**, **Sun W**, **Tueller PT. 2002.** Empirical patterns of the effects of changing scale on landscape metrics. *Landscape Ecology* **17:** 761–782.

Worsham ML, **Julius EP**, **Nice CC**, **Diaz PH**, **Huffman DG. 2017.** Geographic isolation facilitates the evolution of reproductive isolation and morphological divergence. *Ecology and Evolution* **7:** 10278–10288.

Xiao JH, **Wang NX**, **Li YW**, **Murphy RW**, **Wan DG**, **Niu LM**, **Hu HY**, **Fu YG**, & **Huang, DW. 2010.** Molecular approaches to identify cryptic species and polymorphic species within a complex community of fig wasps. *PLoS One* **5:** e15067.

Xu J, **Pérez-Losada M**, **Jara CG**, **Crandall KA. 2009.** Pleistocene glaciation leaves deep signature on the freshwater crab *Aegla alacalufi* in Chilean Patagonia. *Molecular Ecology* **18:** 904–918.

Zimmermann BL, **Crivellaro MS**, **Hauschild CB**, **Bartholomei-santos ML**, **Crandall KA**, **Pérez-losada M**, **Giri F**, **Collins P**, **Santos S. 2018.** Phylogeography reveals unexpectedly low genetic diversity in a widely distributed species : the case of the freshwater crab *Aegla platensis* (Decapoda : Anomura). *Zoological Journal of the Linnean Society* **123**(3): 1–15.

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