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RESEARCH ARTICLE

A hybrid zone of two toad sister species, *Rhinella atacamensis* and *R. arunco* (Anura: Bufonidae), defined by a consistent altitudinal segregation in watersheds

Una zona de hibridación de dos especies hermanas de anuros, *Rhinella atacamensis* y *R. arunco* (Anura: Bufonidae), definida por una consistente segregación altitudinal en cuencas hidrográficas

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ABSTRACT

Delimiting the spatial extension of a hybrid zone is essential to understand its historical origin and to identify the geographical and/or environmental factors which delimit it. *Rhinella atacamensis* and *R. arunco* are two sister species which together inhabit Chile between 25° and 38° S. Their distribution limits coincide at about 32° S, where recently it was reported that they hybridize in a small watershed (Pupío creek). Although the genetic evidence suggests that these two species form a hybrid swarm, they are not mixed homogeneously in the entire watershed, but rather are spatially segregated: *R. arunco* is found in the lower part of the creek and *R. atacamensis* in the higher part. An extensive exploration north and south of 32° S revealed other instances of hybridization, with the same pattern of spatial segregation within other watersheds. This study describes the hybrid zone combining mitochondrial sequences and nuclear AFLP markers. In the northern part, the hybrid zone is a narrow strip which crosses several watersheds and extends more than 130 km from NW-SE, so that *R. atacamensis* is found at higher altitudes towards the south. However, two points south of this strip show that the hybrid zone is more extensive and complex, and probably extends along the entire border of the mountain chains which form the watershed of the Aconcagua River (32°30' - 33° S). We propose an explanation for the origin of this hybrid zone considering paleoclimatic and orographic information, and briefly discuss the taxonomic implications of these results.

Key words: AFLP markers, Chile, control region, hybridization, toads.

RESUMEN

Definir la extensión espacial de una zona de hibridación es esencial para entender su origen histórico e identificar los factores geográficos y/o ambientales que la delimitan. *Rhinella atacamensis* y *R. arunco* son dos especies hermanas que en conjunto habitan entre los 25 y 38° S a lo largo de Chile. Sus límites de distribución coinciden aproximadamente a los 32° S, donde recientemente se reportó que hibridan en una pequeña cuenca hidrográfica (estero Pupío). Aunque la evidencia genética sugiere que estas especies forman un enjambre híbrido, no se mezclan homogéneamente a lo largo de la cuenca sino que se segregan espacialmente: *R. arunco* se encuentra en la parte baja del estero, mientras que *R. atacamensis* se ubica en la parte alta. Una extensa exploración al norte y sur de los 32° S reveló otras instancias de hibridación donde se mantiene el mismo patrón de segregación espacial dentro de otras cuencas. En este estudio se describe esta zona de hibridación combinando secuencias mitocondriales y marcadores nucleares AFLP. En su parte norte, la zona conforma una estrecha franja que atraviesa varias cuencas extendiéndose más de 130 km en dirección noroeste-sureste, de manera que *R. atacamensis* se encuentra cada vez a mayor altura a medida que se avanza hacia el sur. Sin embargo, dos puntos ubicados al sur de esta franja muestran que la zona de hibridación es más extensa y compleja, y que probablemente se extiende por todo el borde de las cadenas de cerros que conforman la cuenca hidrográfica del río Aconcagua (32°30' - 33° S). Se plantea una explicación para el origen de esta zona de hibridación considerando antecedentes paleoclimáticos y orográficos y se discuten brevemente las implicancias taxonómicas de estos resultados.

Palabras clave: Chile, hibridación, marcadores AFLP, región control, sapos.

INTRODUCTION

Hybrid zones (relatively narrow areas in which two genetically different populations, whether of the same or different species, meet and produce hybrids) are highly idiosyncratic phenomena, since they involve diverse evolutionary and ecological processes which interact at different spatial and temporal scales (Barton & Hewitt 1985, Arnold 1997, Howard et al. 2004). Examination of historical and current distributions of various taxa which co-exist over wide geographic areas has led to suggest that hybridization has been important in the diversification and current composition of those biotas (Swenson 2010, Nieto Feliner 2011). More restricted studies have demonstrated that this phenomenon is frequent in a great variety of taxonomic groups (e.g., Willis et al. 2006, Grant & Grant 2008, Lepais et al. 2009, Fontenot et al. 2011). Although these examples can be considered as exceptional since they are from well-known taxonomic groups or regional biota, they show that natural hybridization is a common and widely distributed phenomenon geographically and taxonomically.

In order to identify the processes involved in the origin and persistence of hybrid zones it is essential to define their spatial extension, which can now be done with high precision using molecular markers. A detailed study of the geography of these zones may contribute to elucidate the historical factors or population events which originated them (e.g., Hofman et al. 2007, Hird et al. 2010, Edwards et al. 2011), and to identify the current physical and environmental conditions which determine their location and extension (e.g., Buckley et al. 2003, Yanchukov et al. 2006, Shields et al. 2010, Hapke et al. 2011).

This study illustrates how an extensive exploration and the combined use of mitochondrial and nuclear markers may reveal the complex geography of a hybrid zone, and how its location and configuration allow the generation of an explanation for its origin. The taxa involved are two sister species of toads, *Rhinella atacamensis* (Ceí, 1962) and *R. arunco* (Molina, 1782) (Méndez 2000, Pramuk 2006), endemic to north-central Chile, whose joint distribution ranges extend from approximately 25° S to 38° S (Ceí 1962, Veloso 2006, Correa et al. 2008). Both species are nocturnal and can

be found in borders of rivers, creeks, lagoons and other water bodies, including canals, dams and other man made environments (Ceí 1962). Until recently, it had been assumed that these two species had allopatric distributions, with their limits located around of 32° S. However, populations of the Pupío watershed (a small creek located at 32° S) were recently described as a possible hybrid swarm of *R. atacamensis* x *R. arunco* (Correa et al. 2012). One of the main findings of that study was that a spatial segregation exists within the Pupío watershed: *R. atacamensis* occupies the higher part of the watershed, whereas *R. arunco* inhabits the lower part. At mid elevations, they hybridize.

The objective of this study was to define the spatial extension of the contact zone of *R. atacamensis* and *R. arunco*, for which we performed an intensive fieldwork north and south of the Pupío watershed. Using a combination of mitochondrial (sequences of the control region) and nuclear (AFLP) markers we mapped the hybrid zone, which allowed us to evaluate whether the two species maintain the same spatial segregation in other watersheds and to identify the areas which need to be sampled to define the zone with greater precision. We also suggest a biogeographical explanation for the origin and present conformation of the hybrid zone, considering the paleoclimatic history and the geographical relief of the study area.

METHODS

Material and study sites

Between years 2007 and 2011 we collected a variable number of samples per locality from 43 localities which represent almost the entire known distribution ranges of *R. atacamensis* (25° to 32° S) and *R. arunco* (32° to 38° S), except for 120 km in the southern range of *R. arunco* (Fig. 1, Table 1). Sampling was more intense between 31° and 33° S, around the Pupío watershed (32° S) in which hybridization was originally described (Correa et al. 2012; Fig. 1). The individuals used were mainly tadpoles, postmetamorphics and juveniles. We also included a few adults, most of which were sampled by excising a small piece of interdigital membrane and released in the place they were collected. The specific identity of the specimens from north of 31° S and south of 33° S can be unambiguously established because only pure populations of each species have been described beyond these limits and both species can be distinguished without difficulty by their coloration patterns. Adults of *R. atacamensis* have sexual dimorphism in background color (whitish in females and yellowish in males) and small reddish spots on the dorsum or on the eyelids, while the background

color of *R. arunco* varies from light grey to dark brown in both sexes (see more details in Correa et al. 2012). Postmetamorphics and juveniles of both species also can be distinguished for their color patterns. The permits for the capture and collection of the animals were provided by the Servicio Agrícola Ganadero (SAG) (resolutions 3085/2000, 2105/2004 and 13/2006). All collected individuals and tissues were deposited in the herpetological collection of the Departamento de Biología Celular y Genética of the Universidad de Chile (DBGUCH).

Obtaining DNA and mitochondrial sequences

We obtained sequences of the mitochondrial control region for 359 individuals from 43 localities (Table 1). The DNA was extracted principally from muscle tissue: from the thigh of adults, the tongue of juveniles and postmetamorphics, and from the tail of larvae. Occasionally we used liver, digit or interdigital membrane; the last only in those adults individuals which were returned to their habitat. DNA was extracted using a modification of the salt method of Jowett (1986). The mitochondrial fragment sequenced included the 3' extreme of the cytochrome

b gene and approximately 850 bases of the contiguous extreme of the control region (noncoding). The primers used to amplify this fragment were CytbA-L (5'-GAATYGGRGWCAACCAGTAGAAGACCC-3') and ControlP-H (5'-GTCCATAGATTCATTCCTCGTCAG-3'), designed by Goebel et al. (1999). The PCR protocol is the same used in Correa et al. (2012).

Obtaining AFLP markers

We obtained AFLPs for a representative subset of 205 individuals (among the 359 ones used for obtaining sequences) from 27 localities (Table 1). Details for obtaining (digestion, ligation, pre-selective and selective PCRs steps), genotyping and coding AFLPs are given in Correa et al. (2012). The only difference is that in the present study we used four combinations of selective primers, three of which were used in the earlier study. The combinations of selective primers were MseI-CAC/6FAM-EcoRI-ACT, MseI-CAA/VIC-EcoRI-ACC, MseI-CAT/NED-EcoRI-ACG and MseI-CAC/PET-EcoRI-ACA.

As a simple way of identifying hybrid individuals, a set of diagnostic markers (i.e., markers present in 100 % of the individuals of one species and absent in 100 % of

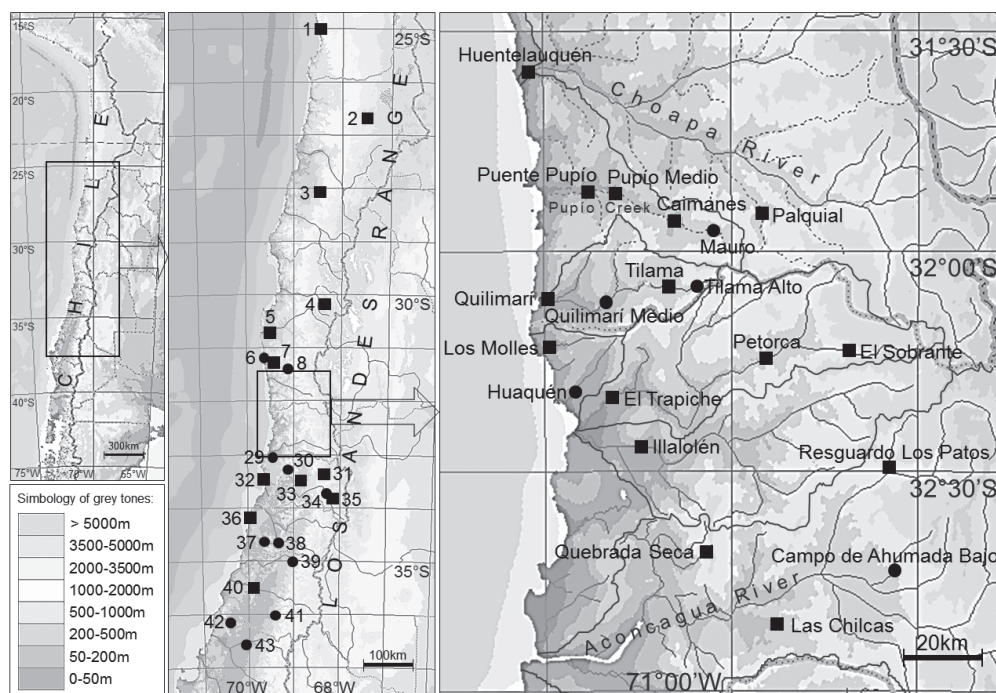


Fig. 1: Location of the 43 localities of *Rhinella* included in this study, numbered from north to south (see Table 1). Squares are localities from which both mitochondrial sequences and AFLP markers were obtained; only mitochondrial sequences were obtained from the localities with circles. The map at the extreme right is an amplification of the zone where sampling was more intense (31°30' - 33° S). Continuous thin lines in this map indicate the limits of the watersheds.

Ubicación de las 43 localidades de *Rhinella* incluidas en este estudio, numeradas de norte a sur (ver Tabla 1). Los cuadrados representan las localidades de las cuales se obtuvieron secuencias mitocondriales y marcadores AFLP, y los círculos aquellas de las cuales solo se obtuvieron secuencias. El mapa del extremo derecho es una ampliación de la zona donde el muestreo fue más intenso (31°30' - 33° S). Las líneas continuas delgadas de este mapa indican los límites de las cuencas hidrográficas.

TABLE 1

Coordinates and altitudes of the 43 localities of *Rhinella atacamensis*, *R. arunco* and the hybrid zone included in this study, ordered and numbered from north to south (see map in Fig. 1). The number of individuals of each locality used to obtain AFLP markers and mitochondrial sequences is also indicated.

Coordenadas y altitudes de las 43 localidades de *Rhinella atacamensis*, *R. arunco* y de la zona de hibridación incluidas en este estudio, ordenadas y numeradas de norte a sur (ver mapa de la Fig. 1). Se indica el número de individuos de cada localidad utilizado para obtener marcadores AFLP y secuencias mitocondriales.

Locality	Latitude (S)	Longitude (W)	Altitude (m)	AFLPs	Sequences
1. Paposo	25°01'33.2"	70°27'10.3"	332	18	20
2. Mostazal	26°40'53.9"	69°34'14.8"	2574	18	20
3. Quebrada Los Sapos	28°04'48.4"	70°24'35.2"	1002	15	20
4. Cochiguaz Alto	30°15'38.5"	70°19'33.8"	2277	12	12
5. Socos	30°43'52.4"	71°29'27.8"	96	20	20
6. Quebrada El Totoral	31°18'48.4"	71°35'36.4"	168	-	6
7. Canela Alta	31°23'20.4"	71°25'20.0"	307	2	6
8. Los Perales	31°28'58.5"	71°07'32.8"	653	-	6
9. Huentelauquén	31°35'16.6"	71°31'30.1"	19	6	6
10. Pupío Medio	31°51'55.0"	71°18'45.5"	160	15	15
11. Puente Pupío	31°52'14.1"	71°23'55.2"	70	9	19
12. Palquial	31°53'17.0"	70°56'42.5"	596	18	18
13. Caimanes	31°55'50.6"	71°08'39.8"	422	9	16
14. Mauro	31°56'59.5"	71°03'50.7"	764	-	4
15. Tilama Alto	32°05'04.5"	71°08'19.9"	555	-	4
16. Tilama	32°06'16.2"	71°10'22.7"	535	4	11
17. Quilimarí Medio	32°07'04.1"	71°19'25.3"	133	-	11
18. Quilimarí	32°07'12.6"	71°28'10.6"	13	16	14
19. Los Molles	32°13'28.4"	71°29'58.7"	10	3	3
20. El Sobrante	32°13'44.7"	70°44'11.3"	968	3	3
21. Petorca	32°15'05.6"	70°55'09.5"	513	3	3
22. El Trapiche	32°18'57.4"	71°16'48.0"	50	6	10
23. Huaquén	32°20'01.9"	71°25'01.2"	23	-	4
24. Illalolén	32°26'23.5"	71°14'10.0"	16	1	3
25. Resguardo Los Patos	32°29'54.0"	70°34'47.4"	1211	4	8
26. Quebrada Seca	32°40'03.2"	71°02'45.6"	1693	2	2
27. Campo de Ahumada Bajo	32°43'51.0"	70°34'01.5"	992	-	3
28. Las Chilcas	32°52'04.7"	70°50'35.1"	605	5	9
29. Marga Marga	33°03'20.9"	71°29'37.5"	32	-	6

TABLE 1. Continuation

Locality	Latitude (S)	Longitude (W)	Altitude (m)	AFLPs	Sequences
30. Estero Puangue	33° 15'26.7"	71° 09'03.3"	320	-	10
31. Río Molina	33° 22'24.1"	70° 23'47.3"	1123	3	4
32. Quebrada de Córdova	33° 26'27.6"	71° 39'38.0"	40	4	11
33. Quebrada La Plata	33° 29'20.3"	70° 53'38.2"	600	1	2
34. Estero Coyanco	33° 41'26.4"	70° 20'58.5"	1051	-	12
35. Camino a El Yeso	33° 47'14.9"	70° 11'03.1"	1456	1	1
36. Topocalma	34° 06'54.1"	71° 55'40.0"	15	3	6
37. Pumanque	34° 37'20.9"	71° 36'21.6"	103	-	7
38. Los Cardos	34° 41'15.8"	71° 26'29.8"	145	-	3
39. Pumaitén	34° 58'00.3"	71° 07'29.3"	287	-	4
40. Linares de Perales	35° 28'09.2"	71° 51'54.0"	57	4	4
41. Vega de Salas	36° 00'13.6"	71° 26'35.5"	302	-	2
42. La Raya	36° 08'58.8"	72° 27'18.0"	207	-	10
43. Río Ñuble	36° 34'24.8"	72° 12'54.1"	84	-	1

the other) was defined using only individuals from the watersheds in which only haplotypes of one species were observed. The diagnostic markers of *R. atacamensis* were defined based on five localities north of the Choapa River watershed (83 specimens) and those of *R. arunco* from four localities south of the Aconcagua River watershed (16 specimens) (Fig. 1).

Phylogeographic and genetic analyses

Mitochondrial sequences were edited with BioEdit v7.0.7 (Hall 1999). We performed an initial alignment with ClustalX v2.0.12 (Larkin et al. 2007) of the first sequences obtained for the two species; the rest were then added manually. Haplotypes were generated with DnaSP v5.10.01 (Librado & Rozas 2009), including the sites with gaps. The phylogenetic relationships among the haplotypes of the two species were estimated by constructing a haplotype network with the method of median joining using the program Network v4.6.10 (Bandelt et al. 1999). Default parameters were used for obtaining the network, which was also used to visualize the mitochondrial genetic divergence at the intra- and interspecific levels (as mutational steps) and the relationships among the haplotypes present in the hybrid zone.

The presence of hybrids in *Rhinella* localities was investigated with NewHybrids v1.1beta3 (Anderson & Thompson 2002). This program uses a Bayesian framework to calculate the posterior probability that each individual of a sample belongs to one or more pre-defined categories of hybrids or to one of the parental species. We specified six categories: pure *R. atacamensis*; pure *R. arunco*; first generation (F1) hybrid; second generation (F2) hybrid; backcross between F1 and *R. atacamensis*; and backcross between F1 and *R. arunco*. We did not incorporate the species

as additional information for this analysis. The length of the Markov Chain Monte Carlo procedure was 1000000 iterations, with the first 100000 discarded as burn-in. We performed various replicas in order to judge the consistency of the results. The location of the hybrid zone was defined considering the geographic distribution of the haplotypes of the control region and the individuals classified by Newhybrids.

RESULTS

Variation of the mitochondrial sequences

We obtained an alignment of 921 sites, of which 170 were variable among the two species (12 sites with gaps). We found a total of 121 haplotypes for the two species (including those defined by the gaps): 58 corresponding to *R. atacamensis* and 63 to *R. arunco* (Fig. 2). Although the number of individuals with haplotypes of *R. atacamensis* was greater (185), the observed level of intraspecific divergence (estimated as mutational steps) was greater in *R. arunco* (22 steps vs. 19 in *R. atacamensis*). The intraspecific divergence was much less than that observed between the species (75 mutational steps).

In four localities, Puente Pupío, Pupío Medio, El Sobrante and Las Chilcas (Fig. 1), we

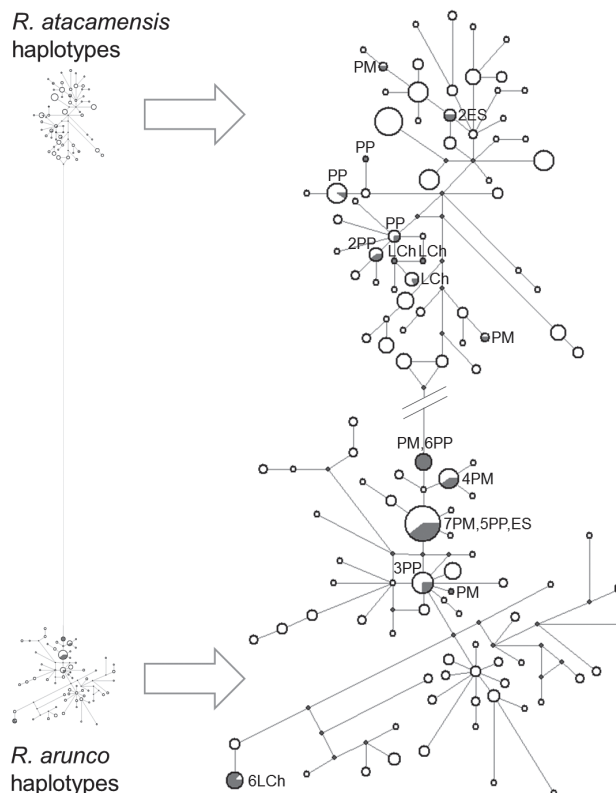


Fig. 2: Haplotype network of the mitochondrial control region of *Rhinella atacamensis* and *R. arunco*, including all observed haplotypes (121). The amplifications of the networks for each species are shown on the right; the haplotypes of both species found mixed in four localities of the hybrid zone are indicated in gray: Pupio Medio (PM), Puente Pupio (PP), El Sobrante (ES) and Las Chilcas (LCh). The size of the circles is proportional to the sampling frequency of each haplotype and the length of connecting lines is proportional to the mutational steps that separate them.

Red de haplotipos de la región control mitocondrial de *Rhinella atacamensis* y *R. arunco* que incluye la totalidad de los haplotipos observados (121). A la derecha se muestran las ampliaciones de las redes correspondientes a cada especie, donde se marcan en gris los haplotipos de ambas especies que se encuentran mezclados en cuatro localidades de la zona de hibridación: Pupio Medio (PM), Puente Pupio (PP), El Sobrante (ES) y Las Chilcas (LCh). El tamaño de los círculos es proporcional a la frecuencia de muestreo de cada haplotipo y las líneas que los unen son proporcionales a los pasos mutacionales que los separan.

detected mixtures of haplotypes of both species. Figure 2 illustrates the relationships among the haplotypes of these localities. The haplotypes are not directly related, but are dispersed within the networks of each of the species. This pattern was more accentuated in the haplotypes of *R. atacamensis*.

The sequences were deposited in GenBank with accession numbers AY818062, AY818063, HQ132482-HQ132670 and KC778198-KC778365.

Variation of the AFLP markers

Among the 224 markers coded (all of them variable considering both species), we found

13 diagnostic for *R. atacamensis* and 13 diagnostic for *R. arunco*. The diagnostic markers per primer combination are (size in base pairs and species in parenthesis): eight with MseI-CAC/6FAM-EcoRI-ACT (96, 140 and 161, *R. arunco*; 107, 129, 134, 143 and 299, *R. atacamensis*), six with MseI-CAA/VIC-EcoRI-ACC (77 and 370, *R. arunco*; 143, 302, 325 and 369, *R. atacamensis*), six with MseI-CAT/NED-EcoRI-ACG (81, 133, 154 and 249, *R. arunco*; 190 and 373, *R. atacamensis*) and six with MseI-CAC/PET-EcoRI-ACA (89, 90, 201 and 241, *R. arunco*; 192 and 264, *R. atacamensis*).

In eight localities between the Choapa and Aconcagua River watersheds (31°30' - 33° S)

we found individuals with a mixture of the diagnostic markers of the two species, including the four in which there were mixed haplotypes. In seven other localities between those latitudinal limits we found only individuals with markers of one or the other species (see below and Fig. 3).

The Newhybrids analysis agreed completely with the distribution of the diagnostic markers. As expected by their phenotype (color patterns) and region control haplotypes, all individuals from localities north of $31^{\circ}30'$ S and south of 33° S were correctly identified as belonging to their respective species. Also, according

to their color patterns, the individuals of the localities of the upper zone of the Choapa River (Canela Alta, Los Perales, Palquial) and the two from Quebrada Seca were classified as *R. atacamensis* (Fig. 3), whereas the individuals from the lower sectors of the watersheds south of the Pupío watershed (Quilimari, Los Molles, El Trapiche, Illalolén) were classified as *R. arunco*. Only in three localities individuals were found which could be considered first or second generation hybrids, while in another five only some individuals classified as backcrosses were present (Fig. 3).

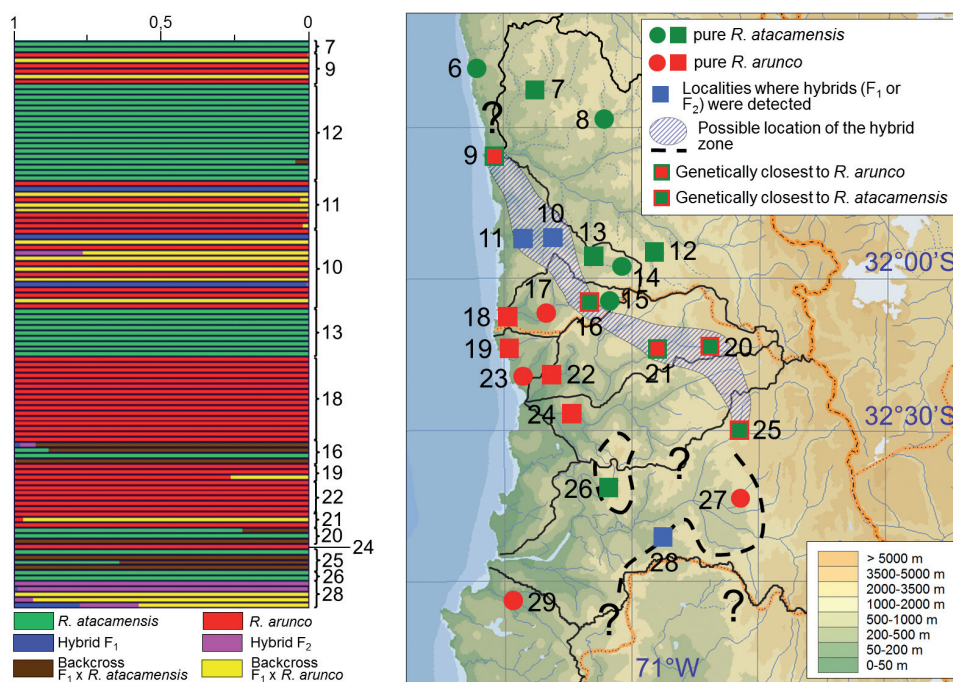


Fig. 3: Result from Newhybrids (left) and map of the hybrid zone of *Rhinella atacamensis* and *R. arunco*. Each colored bar represents one individual and the extension of the color indicates the probability of belonging to one of the six categories specified below the bars according to the AFLPs analysis performed in Newhybrids. Numbers at the right of the bars indicate the localities of the individuals (locality numbers are the same as Figure 1 and Table 1). The probable location and extension of the hybrid zone is indicated with a hatched area and discontinuous lines on the map, which also has question marks in zones which need to be explored. The localities indicated with squares are those included in the Newhybrids analysis; circles represent the localities with only haplotype data. As in Fig. 1, continuous thin lines in the map indicate the limits of the watersheds.

Fig. 3: Resultado de Newhybrids (izquierda) y mapa de la zona de hibridación de *Rhinella atacamensis* y *R. arunco*. Cada barra coloreada representa un individuo y la extensión del color indica la probabilidad de pertenecer a una de las seis categorías especificadas bajo las barras según el análisis de los AFLPs realizado en Newhybrids. La numeración a la derecha de las barras indica las localidades de los individuos (los números de las localidades son los mismos de la Figura 1 y Tabla 1). En el mapa se trazó la posible ubicación y extensión de la zona de hibridación, donde también se señalan con signos de interrogación algunas zonas que requieren exploración. Las localidades señaladas con cuadrados son aquellas incluidas en el análisis de Newhybrids; los círculos representan las localidades con datos solo de haplotipos. Al igual que en la Fig. 1, las líneas continuas delgadas en el mapa indican los límites de las cuencas hidrográficas.

Delimitation of the hybrid zone

The limits of the hybrid zone were drawn to include all localities in which mitochondrial haplotypes of both species were found and/or individuals were classified in one of the last four hybrid categories of Newhybrids mentioned above. In its northern part, the zone appears to have a regular form, crossing several watersheds in a NW-NE orientation (Fig. 3). However, in the southern part, two points located in the Aconcagua River watershed, Quebrada Seca and Las Chilcas, suggest that the geography of the zone is more complex. For this reason, this part of the hybrid zone was drawn with discontinuous lines, one which surrounds Quebrada Seca, which would be an isolated locality of *R. atacamensis*, and the other extends in Los Andes foothills on the border of the Aconcagua River watershed and includes Las Chilcas (Fig. 3). We also added question marks in the Fig. 3 to indicate those areas which should be sampled to define the limits of the hybrid zone with greater precision.

The zone described in this study extends from the locality of Huentelauquén to Las Chilcas, a latitudinal distance of about 150 km (Fig. 3). Examining the distributions of the two species, *R. atacamensis* was found at increasingly higher altitudes towards the south, although the locality that until now defined the southern limit of its distribution (Quebrada Seca) appears to be isolated from the rest of the populations of Los Andes range. Inversely, *R. arunco* was found only in the lower part of the watersheds to the north of the Aconcagua River watershed, reaching the coastal strip at the mouth of the Choapa River (Fig. 3). The locality of Las Chilcas is an exception to this pattern, since there *R. atacamensis* was found at less than 1000 m in the Aconcagua River watershed; in the other two localities of this watershed, Resguardo Los Patos and Quebrada Seca, it was found at 1211 m and 1693 m, respectively.

DISCUSSION

This study revealed two important biogeographic aspects of the hybrid zone of *R. atacamensis* and *R. arunco*: first, the zone has a relatively reduced extension compared to the distribution of both species, and second, there is a consistent altitudinal segregation

between them. The estimated extension of the zone (150 km in latitude) is wide in comparison with the distribution of each species, but is reduced in relation to the combined distribution (about 1450 km length). Additionally, the two species are not homogeneously distributed in the watersheds; thus the hybrid zone occupies a reduced fraction of each and may be represented as a narrow strip which crosses them. The second aspect, the altitudinal segregation, was observed by Correa et al. (2012) in one of the watersheds of the zone (Pupío creek). However, the present study shows that it is a consistent pattern in all the area of contact of the two species. In animals, this kind of hybrid zone, where the parental species are consistently separated along elevational gradients, has been scarcely reported in the literature (Culumber et al. 2010).

A factor that limits the spatial extension of hybrid zones is the extent to which the species are mixed and are permeable to the gene introgression. If there exist strong reproductive barriers (prezygotic and/or postzygotic), hybrids are scarce and the hybrid zone is relatively narrow or almost nonexistent, when the surrounding areas where introgression has been detected are not considered (e.g. Colliard et al. 2010, , Taylor et al. 2012, Miraldo et al. 2013). Correa et al. (2012) provided genetic and reproductive evidence showing that the populations of *R. atacamensis* and *R. arunco* of the Pupío creek (32° S) conform a possible hybrid swarm, suggesting the absence of reproductive barriers. This study reinforces and spatially extends that initial observation, showing also no evidence of mitochondrial and nuclear introgression outside of the defined hybrid zone. This apparent absence of reproductive barriers between *R. atacamensis* and *R. arunco* (which allow us to dismiss a priori a process of parapatric speciation), the large genetic divergence compared to the intraspecific variation (which is evident in the region control network) and the reduced extension of the hybrid zone detected so far suggest that the hybrid zone originated by secondary contact, which implies that one or both species extended its distribution. The paleoclimatic and orographic information given below allows us to hypothesize that one species expanded its range (*R. arunco*), displacing the

other, which would explain the geographic conformation of this hybrid zone.

In Chile, the changes in the distribution of the flora due to the climatic fluctuations produced by the Pleistocene glaciers and during the Holocene are well known (Villagrán & Hinojosa 1997, Villagrán et al. 1998, Villagrán 2001). Due to the geographic conformation of the country, vegetation displacements have mostly been north and south. However, these changes were modified by the geographical relief, thus these displacements have reached different latitudes depending upon their altitude. Thus the Mediterranean vegetation (mainly sclerophyllous shrubland) reached a lower latitude in the last glacial maximum, mainly in the coastal zone (Villagrán 1995, 2001, Villagrán & Armesto 2005). Considering that *R. arunco* is an endemic species of the Mediterranean ecoregion (central Chile), the northward expansions of the Mediterranean vegetation during the glacial cycles of the Pleistocene may explain the presence of this species mainly in the coast between 31°30' and 32°30' S (Fig. 3). If in this area there were glacial refugia for this or other species, they have not been described. Moreover, although the coastal plain is currently narrow, it was wider during the last glacial maximum and could have served as a corridor which allowed the northward expansion across the successive watersheds. These same mechanisms have been suggested to explain the colonization of south-central Chile by a sigmodontine mouse and a continental fish (Unmack et al. 2009, Palma et al. 2012). A range expansion of *R. arunco* through areas of low altitude and slight slopes (i.e., the valleys) would also explain the presence of *R. atacamensis* at increasingly higher altitudes towards the south, including a high locality, Quebrada Seca, apparently isolated south of 32°30' S (Fig. 3). A possible explanation for the formation of this distributional pattern is a competitive displacement of the lowland populations of *R. atacamensis* by *R. arunco*, although if this or other ecological processes are involved need to be evaluated.

There are reports of hybrids zones closely associated to environmental transition areas or ecotones, where the hybridization is frequent, but limited by extrinsic and/or intrinsic factors (e.g. Yanchucov et al. 2006, Hapke et al. 2011,

Chavez et al. 2011, Culumber et al. 2012). The altitudinal segregation which defines the hybrid zone of *R. atacamensis* and *R. arunco* suggests different environmental preferences, but this does not agree with the fact that both species occupy both lower and higher zones of the watersheds, in the areas of their distributions where only one species occurs. Moreover, there are no abrupt changes in environmental parameters along the courses of the rivers in which the hybridization occurs, or at least they are not apparent. In a wider climatic context, the zone is located in the xeric-oceanic Mediterranean bioclimate, which borders on the north with the oceanic-desert Mediterranean bioclimate and on the south with the seasonal rain-oceanic. The transition between these bioclimates is gradual and is closely associated with a north-south precipitation gradient (Luebert & Plischoff 2006). Also this zone is within the area in which the transverse valleys (which are more or less perpendicular to Los Andes range) disappear and the Coastal range begins to appear; these are low, older mountains found from 33° to 41° S and are parallel to Los Andes range. In both cases, the transitions are gradual and do not coincide with the location of the hybrid zone, so we discount that the climatic conditions and/or geographic relief might be more relevant than a possible historic event (expansion of *R. arunco*) to explain its origin and current conformation.

Finally, we would like to consider briefly the consequences of the existence of a hybrid zone of *R. atacamensis* and *R. arunco* for the taxonomy and conservation of *Rhinella* in north-central Chile. The great majority of populations between 25° and 38° S may be assigned unequivocally to one or other species, by phenotype, mitochondrial and nuclear markers (diagnostic AFLP markers). However, populations of the hybrid zone may be considered as a fusion of both species (Fig. 3). The geographic location of the zone implies the presence of pure populations of both species in the main watersheds between 31°30' and 33° S, but they are separated by mixed populations whose exact extension is difficult to determine without estimations of gene introgression in these systems. Moreover, Correa et al. (2012) and the present study demonstrate that the majority of individuals from hybrid populations have different proportions of the nuclear

markers of both species, thus they cannot be assigned to one or another. Therefore, we suggest expanding the taxonomic definition of each species, including all the respective pure populations and those composed of a mixture of both. Thus, now the distribution range of *R. atacamensis* would be defined between 25° and 32° 52' S (Las Chilcas), whereas that of *R. arunco* would be defined between 31° 35' (Huentelauquén) and 38° S. In a conservation context, Allendorf et al. (2001) suggest this type of natural hybrid zone, where apparently the reproductive success of hybrids is similar to that of parental species (Correa et al. 2012), constitutes a eligible conservation unit, although in this case their exact geographic limits remain to be defined. These proposals would allow us to formalize the discovery of this hybrid zone, which also adds an interesting evolutionary dimension to the study of the biogeography of the amphibians of Chile.

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