Revista Chilena de Historia Natural
77: 559-567, 2004

Morphological and genetic differentiation among Chilean populations of *Bufo spinulosus* (Anura: Bufonidae)

Diferenciación morfológica y genética entre poblaciones chilenas de *Bufo spinulosus* (Anura: Bufonidae)

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ABSTRACT

Bufo spinulosus has a wide and fragmented distribution range in Chile (18° to 33° S) along altitudinal and latitudinal gradients. Genetic variation was estimated using RAPD (Random Amplified Polymorphic DNA) markers in 10 populations from northern and central Chile. Morphometric and genetic information was analyzed as a function of geographical origin. The correlation between genetic and morphometric differentiation was analyzed by the Mantel test. An increase in body size as a function of latitude was observed. Specimens from El Tatio had the smallest body size and the greatest morphometric divergence. The AMOVA applied to genetic data indicated that 57.85 % of the variance is explained by interregional differences and that 30.12 % of the variance is found within populations. Low levels of within-regions genetic differentiation was observed in northern populations while higher levels of genetic differentiation was found in populations from central Chile. Mantel tests revealed a significant, positive correlation between genetic variation and geographic distance. When we excluded El Tatio population, Mantel test analyses showed significant correlations between morphological distance and genetic and geographic distances. We discuss whether water temperature could explain the morphological divergence observed in individuals from El Tatio.

Key words: amphibians, geographic variation, morphometry, RAPDs, AMOVA.

RESUMEN

Bufo spinulosus presenta una amplia y fragmentada distribución en Chile (18º a 33º S) a lo largo de gradientes altitudinales y latitudinales. La variación genética fue estimada utilizando marcadores RAPD ("Random Amplified Polymorphic DNA") en diez poblaciones del norte y centro de Chile. La información morfométrica y genética fue analizada en función de la procedencia geográfica. La correlación entre diferenciación genética y morfométrica fue analizada utilizando la prueba de Mantel. Se observó un incremento en el tamaño corporal en función de la latitud. Los individuos de El Tatio mostraron el tamaño corporal más pequeño y la mayor divergencia morfométrica. El AMOVA aplicado a los datos genéticos indicó que el 57,85 % de la varianza es explicada por diferencias entre regiones y que el 30,12 % de la varianza se encuentra dentro de las poblaciones. Bajos niveles de diferenciación genética intrarregional fueron observados en las poblaciones del norte de Chile, mientras que las poblaciones de Chile central mostraron niveles más altos de diferenciación genética. Las pruebas de Mantel revelaron una correlación significativa y positiva entre la variación genética y la distancia geográfica. Cuando excluimos la población de El Tatio, las pruebas de Mantel mostraron correlaciones positivas y significativas entre las distancias morfológicas, genéticas y geográficas. Se discute si la temperatura del agua podría explicar la divergencia morfológica observada en los individuos de El Tatio.

Palabras clave: anfibios, variación geográfica, morfometría, RAPDs, AMOVA.

INTRODUCTION

Amphibians have limited dispersal capability and high philopatry (Seppä & Laurila 1999). These attributes allow the accumulation of genetic and morphological differences (Blouin & Brown 2000, Camp et al. 2000, Miaud & Merilä 2001) as well as in life-history traits (Berven & Gill 1983, Laurila et al. 2001, Bernardo & Reagan-Wallin 2002).

Bufo spinulosus (Wiegmann, 1835) has a wide geographic distribution, ranging from the Peruvian-Bolivian Altiplano to the eastern and western slopes of the Andes mountain range in Chile and Argentina (Cei 1962). In Chile this species is distributed between 18º and 33º S latitude, with populations ranging from sea level (in the case of the Azapa locality in the Region I of Chile) to 2,000-4,600 meters of altitude for all other localities from north and central Chile. There are no descriptions of *B. spinulosus* populations between 23º and 30º S latitude (Cei 1960, Veloso et al. 1982, Veloso & Navarro 1988, Cortés et al. 1995). Population studies have demonstrated geographic variation in morphological and ecological characters (Cei 1960, Nuñez et al. 1982). For instance, Nuñez et al. (1982) found differences in the length of the digestive tract in *B. spinulosus* specimens from populations in San Pedro de Atacama and El Tatio, two localities situated only 65 km apart.

RAPD-PCR is a useful technique for identifying polymorphism (Williams et al. 1990) and for studying the population genetic structure of vertebrates (Hadrys et al. 1992). Moreover, it is technically straightforward and applicable to any organism without previous knowledge of its genome (Parker et al. 1998). Although RAPD's dominant expression somehow biases population genetic parameters and some assumptions are needed (Lynch & Milligan 1994), cluster analysis is useful for detecting geographic patterns. In this sense, RAPD markers have the advantage of allowing screening a high number of polymorphic markers for population studies (Parker et al. 1998).

In this paper, we aimed to determine the extent of genetic differentiation of *B. spinulosus* in Chile using RAPD markers, and to evaluate whether correlations exist between morphological variation with abiotic factors and genetic differentiation.

MATERIAL AND METHODS

Specimens of *Bufo spinulosus* were collected along a latitudinal and an altitudinal gradient, from 10 localities of north and central Chile, between November 2000 and December 2001 (Fig.1): Parinacota (18º12' S, 69º16' W; 4,445 m of altitude; n = 23); Putre (18º11' S, 69º33' W; 3,507 m of altitude; n = 15); Azapa (18º30' S, 70º13' W; 164 m of altitude; n = 12); El Tatio (22º20' S, 68º01' W; 4,264 m of altitude; n = 22); Chita (22º25' S, 68º10' W; 3,741 m of altitude; n = 13); Vilama (22º52' S, 68º10' W; 2,579 m of altitude; n = 21); Jerez (23º11' S, 67º59' W, 2,513 m of altitude; n = 14); Portillo (32º51' S, 70º10' W; 2,119 m of altitude; n = 8); Farellones (33º21' S, 70º18' W; 2,331 m of altitude; n = 33); Lagunillas (33º36' S, 70º17' W; 2,242 m of altitude; n = 11). Specimens were deposited in the Herpetology Collection of the Departamento de Biología Celular y Genética de la Universidad de Chile (DBGUCH).

The following morphometric traits were measured in adult specimens: (1) snout-vent length; (2) head width; (3) head height; (4) mandible width; (5) forelimb length; (6) foot length; (7) tibia length; (8) femur length; (9) nostril-mouth distance; (10) nostril-eye distance; (11) interorbital width; (12) mouth-interorbital axis distance; (13) parotid diameter; (14) internostril distance; (15) eye diameter; (16) tympanum diameter; and (17) head length. Measurements were taken to the nearest 0.05 mm using a caliper. Each trait was $\log_{10}$ transformed to conduct parametric statistic analyses.

Geographic variation of morphological characters was assessed using ANOVA, Principal Components Analysis (PCA), and Stepwise Discriminant Analysis (DA). A matrix of misclassifications (Jackknife option) was estimated using locality as a discriminating factor. All morphological analyses were performed using SYSTAT 5.0 (Wilkinson 1996).

Eighty seven individuals were included in the RAPD analysis. Ten individuals were screened per locality, except for Azapa and Lagunillas where only three and four individuals were studied, respectively.

DNA was extracted from the toe tissues of each individual, using the phenol-chloroform (1:1) and chloroform-isoamyl alcohol (24:1) method (Sambrook et al. 1989). PCR was conducted using: 1.64 µL of H_20; 1.5 µL of buffer (10X); 0.75 µL of $MgCl_2$ (50 mM); 0.18 µL of dNTPs (10 mM of each one); 6.25 µL of primer (1.2 mM) (OPERON Technologies); 0.18 µL of Taq Gibco (5U/µL) and 4.2 µL of DNA (10ng μL^{-1}). The thermal profile for RAPD reactions was: 2 min at 95 ºC, followed by six cycles of 1 min at 94 ºC, 1 min at 35 ºC, and 2 min at 72 ºC,

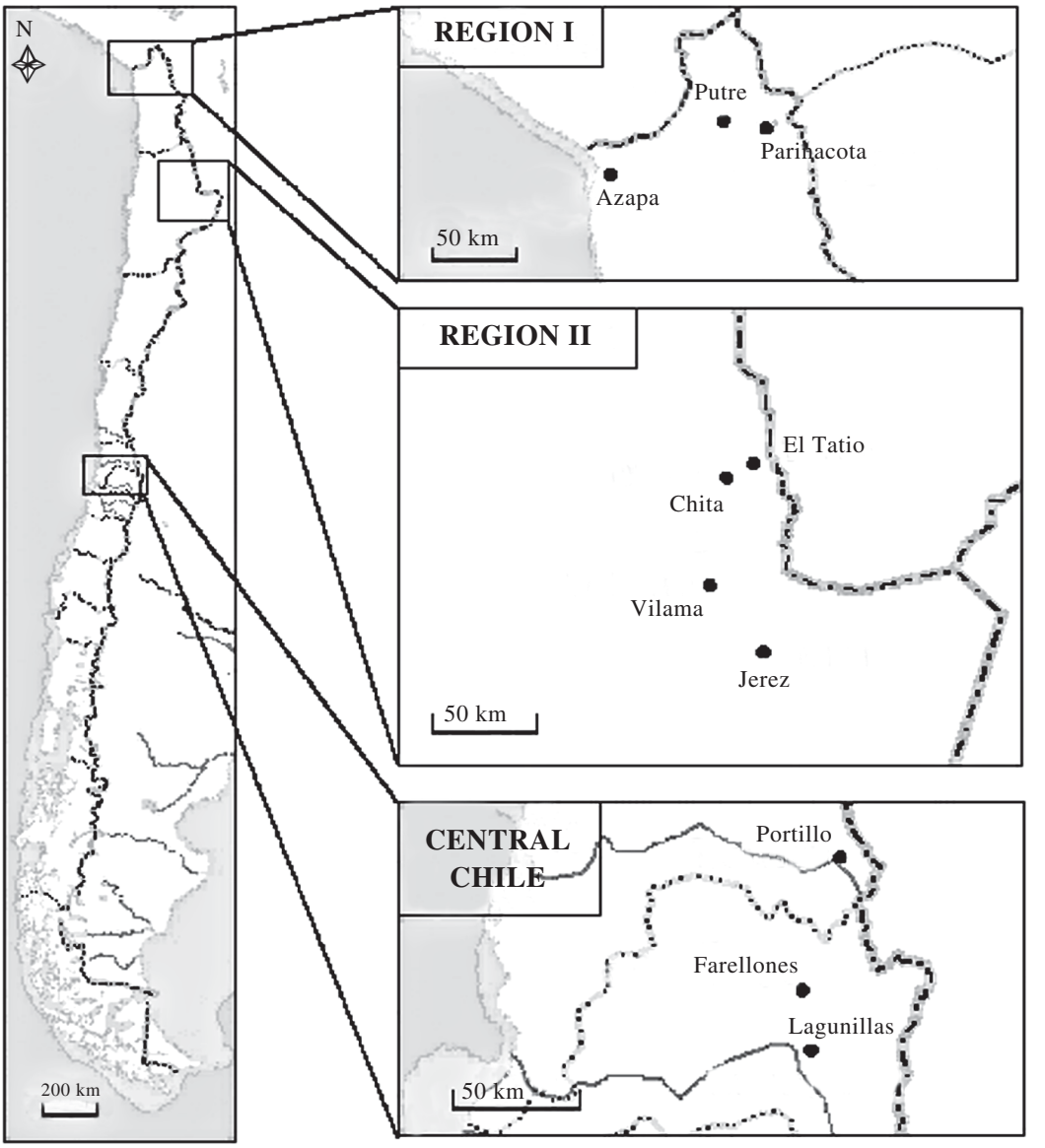

Fig. 1: Map of localities where individuals of *Bufo spinulosus* were collected.
Mapa de las localidades donde los individuos de *Bufo spinulosus* fueron recolectados.

followed by 30 additional cycles of 10 s at 94 ºC, 30 s at 35 ºC and 1 min at 72 ºC, and a final extension at 72 ºC for 5 min. Six primers out of 10 assayed were selected by their consistency and band pattern quality (Table 1). PCR products were run in agarose (1.5 %) at 7.5 V cm^{-1} in TBE buffer 0.5X (10 mM Tris, pH 7.5, 50 mM NaCl, 0.1 mM EDTA) with ethidium bromide. Resulting bands were UV-visualized and photographed.

The consistency of the RAPD profiles was tested on each primer by reanalyzing them at least three times in a subsample. Genetic analyses were performed using AMOVA (Excoffier et al. 1992) and POPGENE (Yeh & Boyle 1997). POPGENE was used to create a

TABLE 1

Sequences of primers used in RAPD-PCR analysis and the number of informative bands (loci) utilized. The number of analyzed bands corresponds to the number of resulting bands following application of the Lynch & Milligan correction (1994)

Secuencias de los partidores utilizados en los análisis de RAPD-PCR y número de bandas informativas (loci) utilizadas. El número de bandas analizadas corresponde al número resultante siguiendo la corrección de Lynch & Milligan (1994)

Primer name	Sequence	Number of analyzed bands
OPB-08	5'-GTCCACACGG-3'	17
OPB-10	5'-CTGCTGGGAC-3'	15
OPB-11	5'-GTAGACCCGT-3'	11
OPG-05	5'-CTGAGACGGA-3'	15
OPG-06	5'-GTGCCTAACC-3'	14
OPG-14	5'-GGATGAGACC-3'	12

UPGMA cluster based on Nei distances (Nei 1972) and FREETREE (Pavlicek et al. 1999) to create a UPGMA cluster based on Rogers' distances. Bootstrap analysis (1,000 pseudoreplicates) was used to evaluate statistical nodal support.

Mantel Test 2.0 (Liedloff 1999) was used to determine the significance of correlations using matrices of pairwise distances between populations, with 2,000 randomizations. We used the following distance matrices in these analyses: (a) the morphological matrix of morphometric distances among populations (we used the canonical scores of group means of each population resulting from DA analysis); (b) the genetic matrix of Nei's genetic distances calculated by the POPGENE program, using the option for dominant markers; (c) the geographic matrix, calculated using the program "Surface distance between two points of latitude and longitude" (available at: www.wcrl.ars.usda.gov/cec/java/lat-long.htm); and (d) the altitude matrix, based on altitudinal measurements at each locality, using data obtained from a GPS (Garmin Ettrex). Original values of the different matrices were transformed to Euclidian distances.

RESULTS

No sex-linked differences were detected in body size. Nevertheless, significant differences exist in body size among populations as a function of locality (ANOVA: $F_{9,161} = 72.756$; $P < 0.0001$). In general, body size showed an increase as a function of latitude, with smaller sized individuals corresponding to the El Tatio locality, while individuals from central Chile were significantly larger than individuals from Regions I and II.

The first three axes of PCA conducted on 16 quantitative morphological characters explained 98.07 % of the variance. All characters had positive values and similar weights.

A graphic representation of the eigenvalues of the first two components revealed strong morphological differentiation in individuals from El Tatio (Fig. 2), which were clearly differentiated from all other individuals in the morphometric space. It was also possible to differentiate between populations from the north (Regions I and II) and populations from central Chile. DA of morphometric traits using locality as the discriminating variable, revealed significant differences among populations (Wilk's lambda = 0.139; $F_{18,320} = 29.877$; $P = 0.0001$). Classification matrices correctly distinguished specimens from El Tatio, Lagunillas and Farellones, with high values (95, 100 and 67 %, respectively). Low values (below 40 %) were found in other populations.

RAPD analysis included 84 polymorphic bands after the correction of Lynch & Milligan (1994). The partitioning of AMOVA indicated a 57.85 % differentiation at the regional level (Region I, Region II, and central Chile), 30.12 % within populations, and only 12.03 % was explained by variance among populations.

The greatest genetic differentiation detected in central Chile corresponded to Farellones and Lagunillas populations ($Ø_{st} = 0.4028$), while the lowest value was observed between Farellones and Portillo ($Ø_{st} = 0.3172$). The lowest value of population differentiation in Region II was found between Vilama and Chita populations ($Ø_{st} = 0.0977$), while Jerez and Chita populations registered the highest value ($Ø_{st} = 0.5024$). Values of $Ø_{st}$ ranged from 0.1352 to 0.4143 among the other populations from Region II. The greatest value of differentiation within Region I was recorded

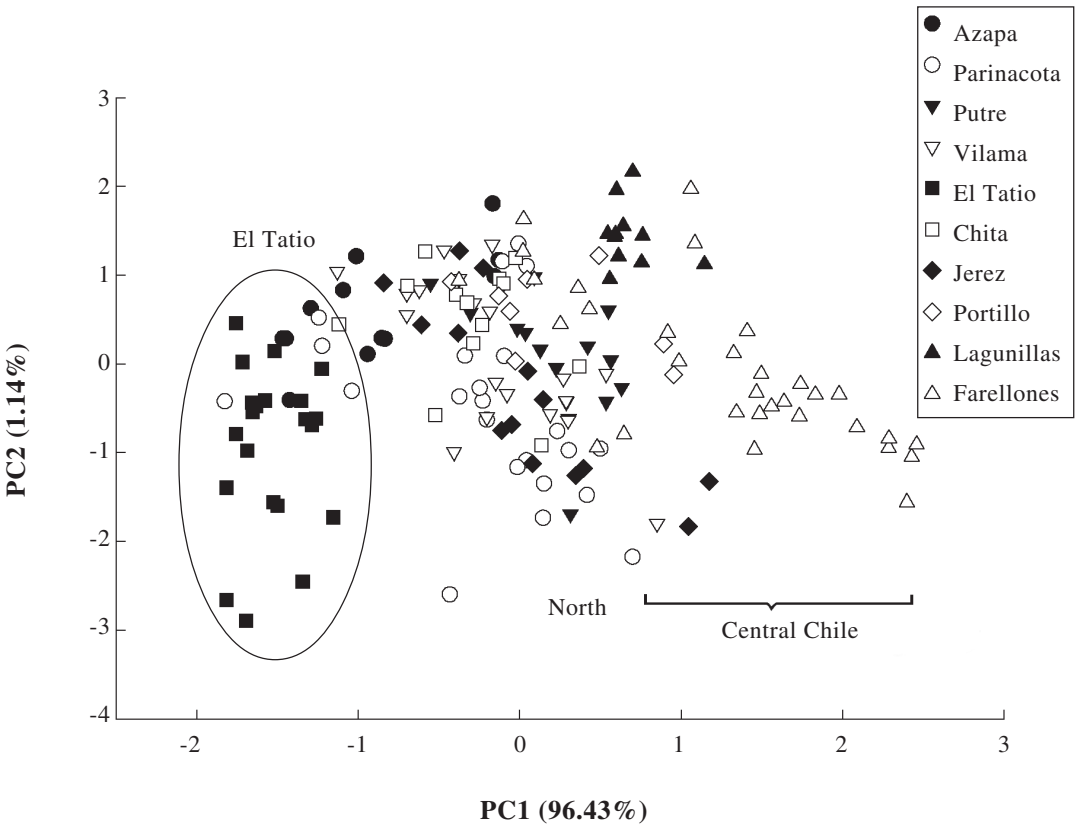

Fig. 2: Principal Components Analysis (PCA) of 16 morphometric characters measured in 10 populations of *Bufo spinulosus*. Values in parentheses correspond to percentages of explained variance.

Análisis de Componentes Principales (PCA) de 16 caracteres morfométricos medidos en 10 poblaciones de *Bufo spinulosus*. Los valores entre paréntesis corresponden a los porcentajes de varianza explicada.

for Azapa and Putre populations ($Ø_{st}$ = 0.3186), whereas the lowest value ($Ø_{st}$ = 0.1313) was found between Parinacota and Putre, suggesting limited genetic differentiation.

Cluster analyses based on Rogers distances confirmed the genetic differentiation observed among the three main regions as indicated by its bootstrap support (bootstrap values: Region I, 99 %; Region II, 100 %; and central Chile, 100 %; Fig. 3). However, when populations were analyzed, genetic differentiation was found only for the Azapa population in Region I (58 %), the Jerez population in Region II (63 %), and the Lagunillas population in central Chile (62 %). Bootstrap values for all of the remaining populations were below 50 %.

Correlation values for Mantel tests of genetic distances, morphological data, geographic distances and altitude are given in Table 2. Significant correlations were found only between matrices of genetic distances and geographic distances (r = 0.65, P < 0.005, Table 2). When the morphologically divergent population of El Tatio was excluded, the correlation between the genetic and geographic distance matrices increased (r = 0.76, P < 0.005). Significant correlations between morphology and both genetic and geographic distances was also found when the El Tatio population was excluded (morphological-genetic, r = 0.47, P < 0.005; morphological-geographic, r = 0.32, P < 0.005).

DISCUSSION

The high levels of genetic differentiation among Regions I, II, and central Chile indicated by AMOVA and cluster analyses of

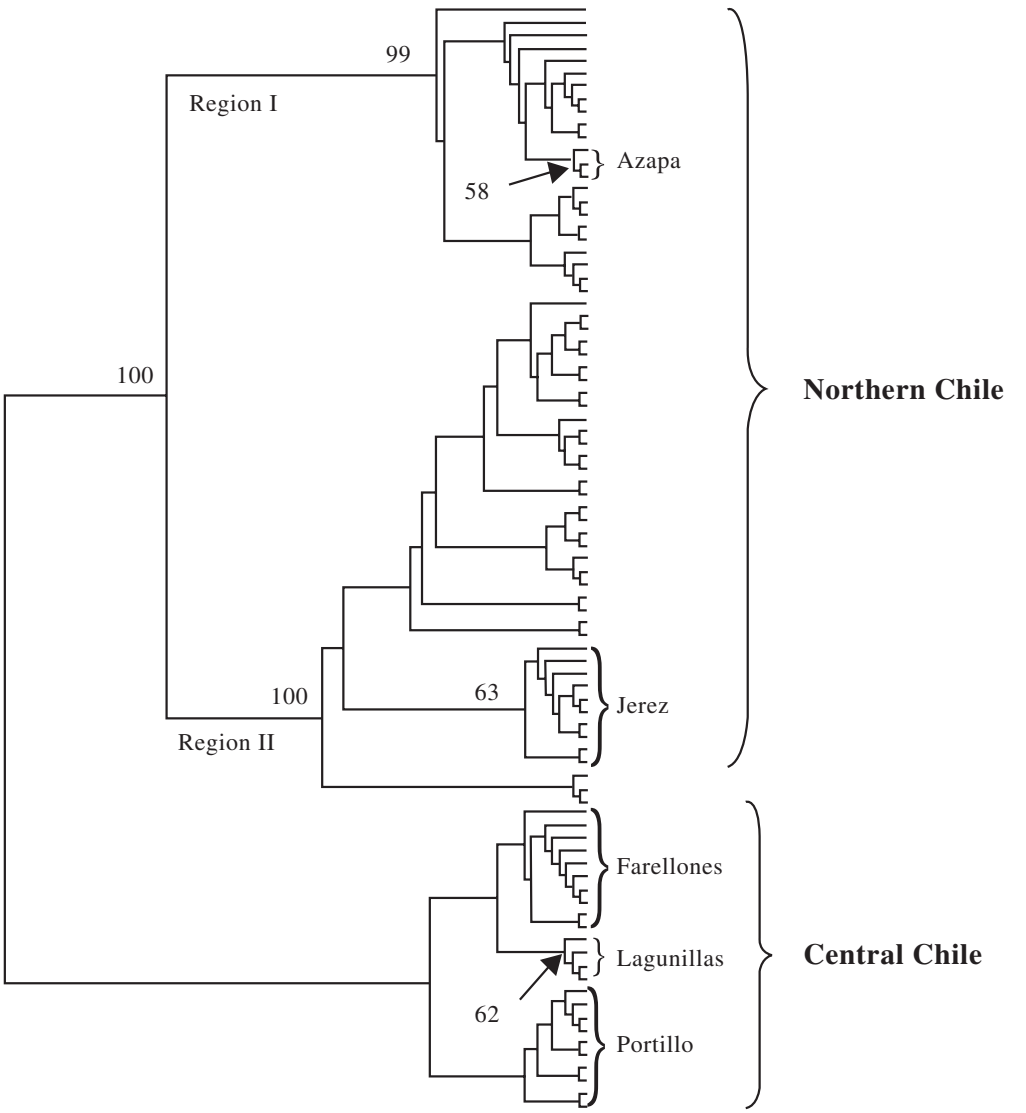

Fig. 3: UPGMA cluster analysis based on Rogers distances for 85 individuals belonging to 10 populations of *Bufo spinulosus*. Numbers above nodes represent bootstrap values obtained from 1,000 pseudoreplicates.

Análisis de UPGMA basado en las distancias de Rogers para 85 individuos pertenecientes a 10 poblaciones de *Bufo spinulosus*. Números sobre los nodos representan los valores de bootstrap obtenidos de 1.000 pseudorréplicas.

RAPD markers, suggest a strong geographic structure. This was expected considering the evaluated scale (1,677 km between the most distant localities) and the restricted mobility of amphibians (Berven & Grudzien 1990, Beebee 1996). Rowe et al. (2000) described a similar pattern of genetic divergence as a function of distance in *Bufo calamita*.

The smallest specimens were found in El Tatio population whereas individuals from central Chile were larger than northern ones. The multivariate morphological divergence was high among regions while low levels within regions were found. No significant correlations between morphometric differentiation and latitudinal or genetic differentiation were found by analyzing matrix data of all populations. However, when El Tatio population was excluded, positive correlations were found (Table 2). Although correlation does not imply causality, it suggests that among-region morphological differentiation as well as in

TABLE 2

Summary of Mantel tests performed between matrices of geographic (GeogDist), morphological (MorpDist) altitude (Altit Dist) and genetic (GenDist) distances. All analyses were carried out using the program Mantel 2.0 (Liedloff 1999) with 1,000 permutations. The analyses were performed first comparing all populations and then excluding the El Tatio population, which presented the greatest morphological divergence

Resumen de las pruebas de Mantel realizadas entre las matrices de distancias geográficas (GeogDist), morfológicas (MorpDist) de altitud (Altit Dist) y genéticas (GenDist). Todos los análisis fueron llevados a cabo usando el programa Mantel 2,0 (Liedloff 1999) con 1.000 permutaciones. Los análisis fueron realizados primero comparando todas las poblaciones y luego sin la población de El Tatio, la cual presentó la mayor divergencia morfológica

Matrix	All populations			Excluding El Tatio		
	g	R	P	g	r	P
MorpDist x AltitDist	0.494	0.112	> 0.05	1.2	0.29	> 0.05
MorpDist x GenDist	-2.325	-0.2558	> 0.05	3.15	0.47	0.005
MorpDist x GeogDist	-0.353	-0.0483	> 0.05	4.514	0.32	0.005
GenDist x AltitDist	0.235	0.0288	> 0.05	-0.321	-0.04	> 0.05
GenDist x GeogDist	4.241	0.65	0.005	4.40	0.76	0.005

genetic composition may result from restrictions to gene flow. Cei (1962) proposed that there is geographic isolation between populations from north and central Chile, caused by climatic barriers (principally xeric conditions), which could affect the distribution range of this species. Our data are in agree with this proposition. However, high levels of gene flow were detected among populations within regions as indicated by the $Ø_{st}$ values for northern populations. Such a pattern could be associated to larval or adult dispersal due to sporadic flooding caused by the El Niño Southern Oscillation (Messerli et al. 1993, Vargas et al. 2000, Garreaud et al. 2003). The distance between rivers fluctuates between 3-9 km for Region I and 3-13 km for Region II, thus making possible the connection of populations during flooding events. An alternative explanation for the low levels of genetic differentiation in northern populations emphasizes recent colonization events in the area. Thus, the distribution of RAPD markers in Regions I and II could result from incomplete or recent isolation among populations. Data at hand makes impossible to discriminate between these alternatives or to identify a general mechanism to explain the observed pattern. The use of mitochondrial markers (e.g., control region) could shed some light in this regard.

The morphometric divergence and genetic homogeneity of El Tatio population can be addressed by considering abiotic factors, since larval development occurs in streams having water temperatures between 25 ºC and 30 °C during all day long. At other localities, temperatures fluctuate between 15 °C and 25 °C during the day and remain around 7 °C at night (Benavides 2003). Although pH differences could also account for differences in larval development, information is scarce and has only been described in *Rana arvalis* (Räsänen et al. 2003). We favor the probable effect of water temperature on morphology and larval development, since it is a relevant factor affecting body size in ectotherms (Atkinson 1996). Warmer waters induce a smaller size at metamorphosis, which, in turn, determines a smaller adult size as described in *Rana sylvatica* (Berven 1990), *Rana cascadea* (Blouin & Brown 2000), *Desmognathus quadramaculatus* (Camp et al. 2000), and *Discoglossus galganoi* (Alvarez & Nicieza 2002), among others (but see Laugen et al. 2003). Ongoing experimental evidence will shed light on water temperature as a causative factor affecting body size in *Bufo spinulosus*.

ACKNOWLEDGMENTS

Financial support was provided by FONDECYT grants 3000048/2000 to MAM. SAG (Servicio Agrícola Ganadero) supplied collecting permits (Resolution number 3085/2000). ERS thanks CONICYT for fellowship support. We are also grateful to Paula Neill and two referees for useful suggestions and comments on an earlier version of the manuscript.

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Associate Editor: Milton Gallardo
Received March 4, 2004; accepted June 30, 2004