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ORIGINAL ARTICLE

Low genetic diversity of the successful invasive African clawed frog *Xenopus laevis* (Pipidae) in Chile

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In Africa, the genus *Xenopus* presents cryptic species and diverse hybrids between species. It has been assumed that the invasive populations of this genus correspond to *X. laevis* and that they are derived from the subspecies that inhabits the Mediterranean Cape region of South Africa. In part, this is supported by the successful establishment of this species in several Mediterranean regions of the world. In Mediterranean Chile, *Xenopus* has invaded an area of about 21,000 km^2, with scarce attention to genetic aspects underlying its invasion. Using mitochondrial DNA sequences we determined that *Xenopus laevis laevis* from the Cape region of South Africa is the subspecies that invaded Chile. The analysis indicated that the invaders have low genetic diversity (only two haplotypes, compared to 10 in two localities of their native range), and that probably the invasion in Chile occurred only once. Landscape genetics revealed that factors such as aridity and elevation have determined the spread of the species, both from the ecological and genetic points of view. Our results show that the invasion of the African clawed frog in Chile has been successful for at least 30 years, in spite of low genetic variability, few events of introduction, low propagule pressure, and bottlenecks in the founding population.

Keywords: biological invasions; mitochondrial DNA; *Xenopus laevis*; Chile

Introduction

Alien invasive species (AIS) are one of the main threats to biodiversity (Gamradt & Kats 1996; Hecnar & M'Closkey 1997; Alford & Richard 1999; Lawler et al. 1999; Kats & Ferrer 2003). Several authors referred to the harmful effects of exotic amphibians in the areas invaded (Kupferberg 1997; Kraus et al. 1999; Crossland 2000). The incorporation of genetic tools in the study of invasive species is considered a key element for understanding and managing them (Dlugosch & Parker 2008; Ficetola et al. 2008; Funk et al. 2011).

Xenopus laevis (African clawed frog) was introduced worldwide for its use in pregnancy tests in the 1950s (Shapiro & Zwarenstein 1934). The species is currently used as an animal model for developmental biology research (Cannatella & De Sá 1993), and has been incorporated into the exotic pet market (McCoid & Fritts 1989; Lobos & Jaksic 2005). Twenty-one species are currently recognized in the genus *Xenopus* (Frost 2014), all of which are native to Africa and are difficult to recognize morphologically (Kobel et al. 1996). According to Cannatella & De Sá (1993), *X. laevis* is distributed over a large part of sub-Saharan Africa, from South Africa to Zaire and from Cameroon to Uganda. In this range, Kobel et al. (1996) recognized several subspecies: *laevis*, *poweri*, *sudanensis* and *bunyonensis*. According to historical records, *X. laevis laevis* was the species exported from the Mediterranean Cape region to different parts of the world. Thus, it was assumed that the invasions of California in the USA (McCoid & Fritts 1980a, 1980b), South Wales in the UK (Measey 1998; Measey & Tinsley 1998), Portugal (Rebelo et al. 2010), Italy (Lillo et al. 2005, 2011), France (Fouquet & Measey 2006) and Chile (Lobos et al. 1999; Lobos & Measey 2002; Lobos & Jaksic 2005) were of *X. laevis laevis*. However, Measey & Channing (2003), using approximately half of the 12S gene and two fragments of 290 and 320 bp of the cytochrome b gene of mitochondrial DNA (mtDNA), concluded that in southern Africa there are two clades co-existing within the species, and noted that specimens exported from South Africa could belong to many different genetic forms.

One surprising aspect of the propagation of *X. laevis* around the world is the scarce attention to

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genetic aspects underlying its invasion, despite the fact that the species was established decades ago in the UK (1962), California, USA (1969) and in the 1980s in Chile and France (Tinsley & McCoid 1996; Fouquet & Measey 2006). Because the genetic relationships of these populations are unknown, the use of molecular markers may contribute significantly to understanding aspects such as the invasion origin center and introduction pathways (Bastrop et al. 1998), genetic variability (Barbaresi et al. 2003), possible bottleneck effects (Net et al. 1975), identification of cryptic species (Bastrop et al. 1998), and phylogeographic structure (Slade & Moritz 1998). It was shown in other species that knowing the source of an invader and the number of times it has been introduced (i.e. the propagule pressure) is an important step to understand its establishment success (see Dlugosch & Parker 2008 for a review). The use of such markers also led to the suggestion that the genetic diversity of the founder group has an important influence in its colonizing capability (Fenster & Dudash 1996; Kreiser et al. 2000; Kolbe et al. 2004, but see Ficetola et al. 2008).

In Chile, the presence of African clawed frogs in the wild beginning in the 1980s (Veloso & Navarro 1988) has caused alarm due to its potential impact on native aquatic environments (Glade 1988; Formas 1995; Lobos et al. 1999; Lobos 2002; Lobos & Garín 2002). *Xenopus* reached important densities in Chile (Lobos & Measey 2002), and is still in the expansion phase (Lobos & Jaksic 2005; Lobos et al. 2013). Because there are no historical data about its entry into the country, we do not know the origin of specimens and the number of introduction events. Veloso et al. (2004), based on karyotype analysis and cytochrome b sequences (866 bp) in three localities of Chile, concluded that the invading species is *X. laevis*. However, they used only a small number of animals from populations close to the city of Santiago, and ignored that other species of the genus are part of the pet trade in Chile (e.g. *X. tropicalis*).

The objectives of this study are: (1) to clarify the identity and origin of the African clawed frog in Chile by means of the cytochrome b DNA sequence and comparison with species of this genus from southern Africa; (2) to describe the genetic patterns of the area invaded in Chile, using the control region of mtDNA.

Material and methods

Study area and sampling

We collected 166 animals from 23 localities, covering the whole area known to be invaded by *X. laevis* in Chile (a latitudinal range of approximately 210 km). We also sampled specimens in two localities in the Cape region of South Africa (Jonkershoek and Stellenbosch National Reserves; four and 18 individuals, respectively), from where the invaders in Chile are suspected to have been originally collected (Figure 1). The live traps described by Lobos & Measey (2002) were used for captures. Captured animals were euthanized by intraperitoneal injection of 10% lidocaine and deposited in Patricio Sanchez Collection at Catholic University of Chile in Santiago.

DNA analysis

The samples included adults, juveniles and larvae. Genomic DNA was extracted from toes, muscles and liver, using a modification of the salt extraction method of Jowett (1986). We amplified a 290 bp segment of the cytochrome b gene using the universal primers Cyt b I 5′ CCA TCC AAC ATC TCA GCA TGA TGA AA 3′ and Cyt b II 5′ CCC TCA GAA TGA TAT TTG TCC TCA 3′ (Measey & Channing 2003).

To analyze the genetic pattern of the invaded area, we amplified a 1300 bp fragment of the first segment of the control region of the mtDNA of *Xenopus laevis*. For this purpose, we designed the specific primers: dloopXenH 5′ ATC ACG GAG AYT GTT TAT TAAG 3′ and dloopXLS2 5′ TAT CTC CCG ACG TAT GCA CTAAG 3′. PCRs were performed in a total volume of 30 µl, with 3 mM 10× PCR buffer $MgCl_2$, 1.8 mM $MgCl_2$, 0.4 µl of dNtp (10 µM of each one), 0.6 µM of each primer, 0.25 U AmpliTaq Gold DNA polymerase (Applied Biosystems, Carlsbad, CA, USA) and 10–30 ng DNA. The protocol included an initial denaturation step of 2 min at 94°C, 36 cycles of denaturation at 94°C for 30 s, alignment at 56°C for 45 s and elongation at 72°C for 90 s, with a final extension at 72°C for 5 min. PCR products were sequenced in both directions in an ABI3730XL (Applied Biosystems) automatic sequencer by Macrogen (Seoul, Korea).

Genetic analyses

Sequence alignments were performed using BioEdit v.7.0.7.0 (Higgins et al. 1996; Hall 1999). To align the sequences of the cytochrome b gene we used two sequences of *X. epitropicalis* as reference (AY 217721 and AY 217722), while for the control region a sequence of *X. tropicalis* (AY 789013) was used. In both cases alignment optimization was done by eye (Bibb et al. 1981).

Haplotypes were extracted using the program DnaSP v.4.10.9, using the Network v.4.2.0.1 software

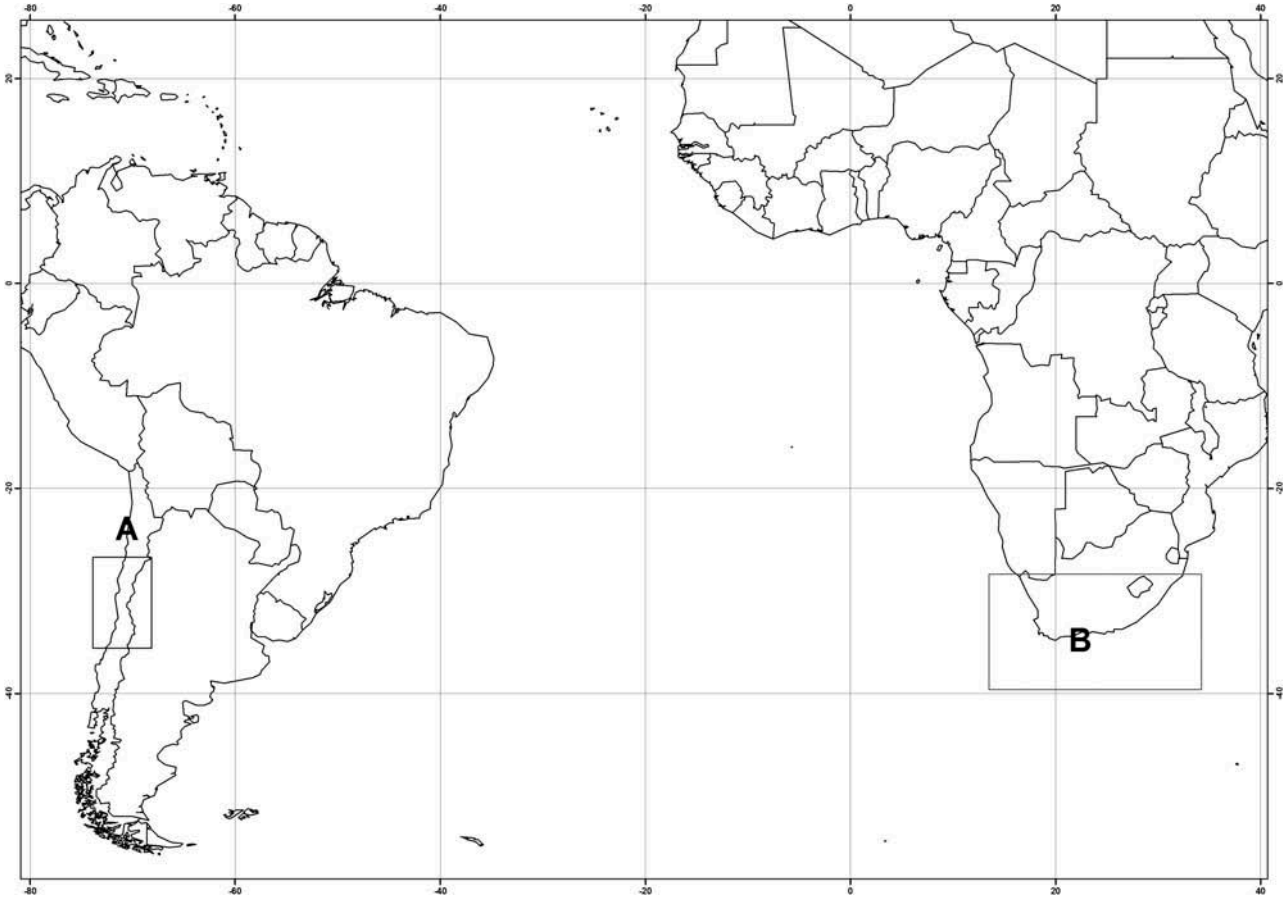

Figure 1. Origin of studied specimens of *Xenopus laevis*. A, Area invaded in central Chile; B, natural distribution range in the Cape region, South Africa.

to construct the haplotype network. The network was constructed with 95% statistical support (median-joining method). The ARLEQUIN 3.5.1.2 software was used to estimate nucleotide diversity (π) and haplotype diversity (H_d) for each of the native and introduced populations. The phylogenetic relations between the population samples were analyzed using maximum parsimony (MP) and maximum likelihood (ML), available in PAUP* 4.0 v10 (Swofford 2001). In both cases, the heuristic search option tree bisection reconnection was used. The confidence of the nodes was evaluated by non-parametric bootstrap with 1000 pseudoreplicates for ML and 5000 for MP (Felsenstein 1985). The best model of nucleotide substitution for ML was obtained using JMODELTEST 0.1.1 and the Akaike information criterion (Posada & Crandall 1998). In all analyses, *X. epitropicalis* was used as outgroup.

In order to visualize the spatial variation pattern between individuals of the invaded area, the software Alleles in Space (ALLIS) was used (Miller 2005). Using geographic data (projected in universal transverse mercator (UTM)) and with the results of the genetic distance obtained from ALLIS, a tri-dimensional landscape was created, with genetic distance in z-axis (x and y axes correspond to cardinal points). Due to the fact that ALLIS interpolates by a regression – that uses the residue of isolation by individuals' distance – a positive peak on the z-axis should be found in a geographic area where the genetic divergence is high. Finally, for the cartographic representation of the information, a geographic information system (ArcGIS 9.2, ESRI Company, Redlands, CA, USA) was used, with the information expressed in UTM coordinates, zone 19 and Datum WGS 84.

Results

Origin of the Xenopus *present in Chile*

The sequences obtained were submitted to Genbank and assigned accession numbers KJ 633829 and KJ633830 for Cyt b and KJ633831 to KJ634018 for Dloop inclusive. Analysis of the sequences of the cytochrome b gene of South African and Chilean samples of *Xenopus* are represented in the ML tree

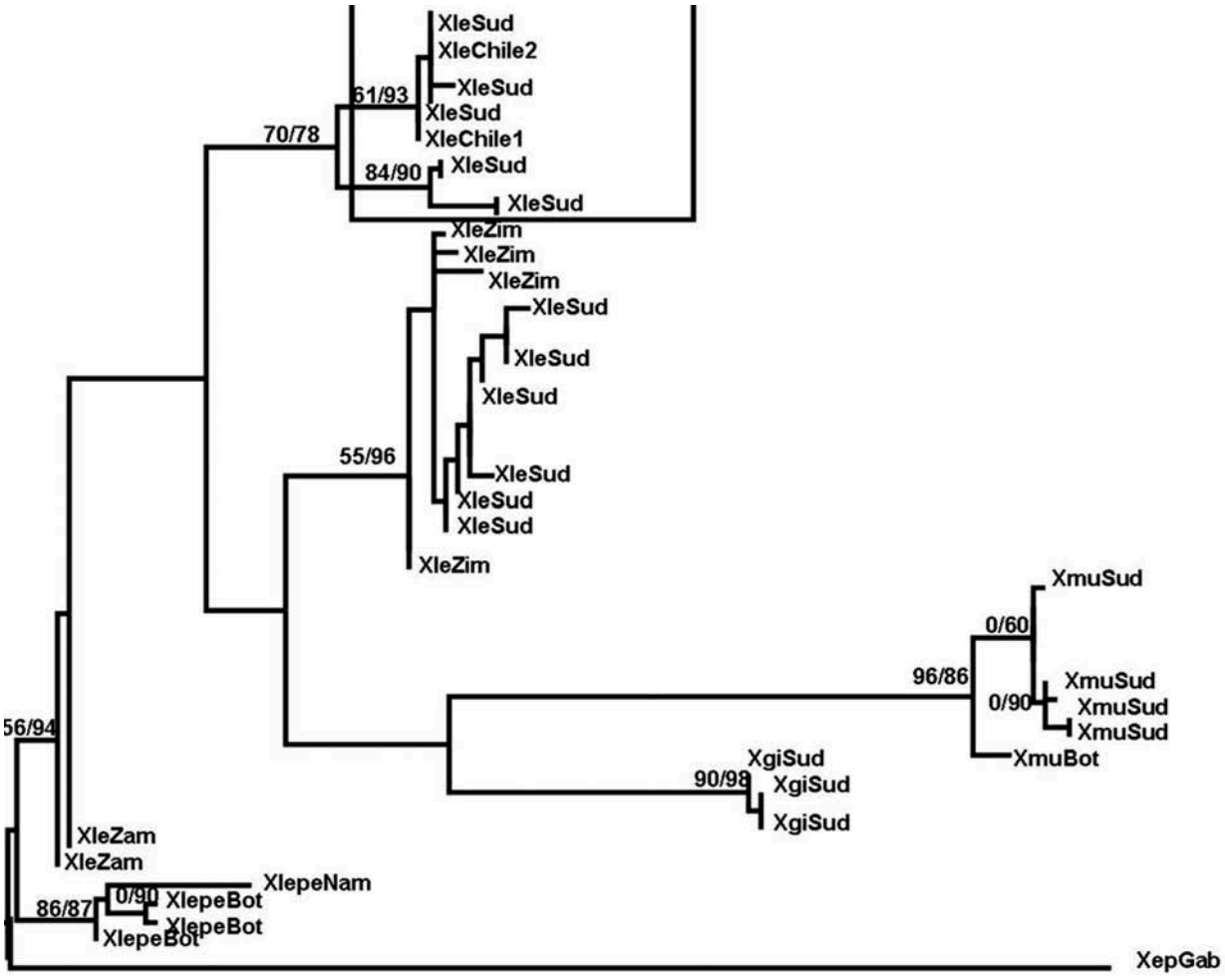

Figure 2. Maximum likelihood tree based on the cytochrome b gene, showing the phylogenetic relationships among species and subspecies of southern African and central Chilean populations of *Xenopus laevis*. Bootstrap values are given above the nodes of the tree, maximum likelihood and maximum parsimony, respectively (ML/MP). Only values above 50% are shown. Xep: *Xenopus epitropicalis*, Xle: *Xenopus laevis*, Xlepe: *Xenopus laevis petersi*, Xgi: *Xenopus gilli*, Xmu: *Xenopus mulleri*; Sud: South Africa, Zim: Zimbabwe, Zam: Zambia, Bot: Botswana, Gab: Gabon, Nam: Namibia. The box shows the clade of *Xenopus laevis laevis* of the Cape region, in which the two haplotypes present in Chile are located (XleChile1 and XleChile2).

(Figure 2; –lnL= 1382.66954). This tree was obtained using the substitution model TIM1+G. The MP analysis recovered a tree with a topology similar to that generated by ML (length 189, consistency index 0.55, retention index 0.84, corrected retention index 0.47). In both cases, the phylogenetic analysis showed that except for *X. laevis*, the rest of the African species form monophyletic groups. In spite of the low support of the nodes (bootstrap values of 55%) two subgroups are observed in *X. laevis*: the populations from the north of South Africa and those of the Cape region. Both analyses showed that the Chilean samples grouped with those of the Mediterranean Cape region.

Genetic pattern of the invaded area

The analysis of the mitochondrial control region of *X. laevis* showed the presence of two haplogroups (HAP1 and HAP2) in the 21,200 km^2 area invaded in central Chile (Table 1). By contrast, in the two localities from the Cape region of South Africa 10 haplotypes were found. Of the two haplotypes found in Chile, HAP1 has a wide distribution in the country, while HAP2 is more restricted (Figure 3). These two haplotypes differ by 13 mutations. The invaded area had lower haplotype and nucleotide diversity than the populations in its native range (Table 2).

Table 1. Distribution of the two haplotypes (HAP1, HAP2) of the mitochondrial control region of *Xenopus laevis* in specimens collected at 23 sites in Chile.

Locality	Site code	Specimens analyzed	Individuals with HAP1	Individuals with HAP2
Río Limarí	S1	9	9	—
Ocoa	S2	7	1	6
Las Chilcas	S3	10	1	9
El Yali, embalse los Molles	S4	9	9	—
Humedal Laguna El Peral	S5	2	2	—
Marga Marga	S6	10	10	—
Jardín Botánico	S7	6	6	—
Quebrada de Córdova	S8	6	6	—
Laguna de Batuco	S9	10	7	3
Antumapu	S10	11	11	—
Peñaflor	S11	1	1	—
Laguna Caren	S12	1	1	—
Chocalan	S13	13	11	2
Rinconada de Maipú	S14	11	9	2
Melipilla	S15	8	8	—
Lampa	S16	3	3	—
Parque del Recuerdo	S17	7	5	2
Laguna de Aculeo	S18	7	7	—
Estero Alhue	S19	10	10	—
Lago Rapel	S20	1	1	—
Yaquil	S21	10	10	—
Placilla	S22	9	9	—
Palmilla	S23	5	5	—
Total		166	142	24

One interesting aspect of the South African samples analyzed is that two groups of sequences were found, one of which has deletions in positions 420 (one adenine) and 730 (two adenines). Both of the haplotypes in the invaded area belong to one of these groups. This is clear in the Network analysis that separates the haplogroups (Figure 4).

In the samples from the Cape region, sequences identical to those present in Chile were not found. In the case of sequence HAP1, the closest sequence from the native range (SUD4) was different in six sites. The closest relative of HAP2 was sequence SUD6, which differs by seven nucleotides. The distribution of the African clawed frog is continuous in the central part of Chile except for the Limarí River population, which lies 250 km north of the core invasion area. In this locality only HAP1, the dominant haplotype in the invaded area, was found.

The spatial distribution of *X. laevis* in Chile shows an area of high genetic discontinuity in the northeastern part of the invaded area, where HAP2 is dominant (Figures 5 and 6).

Discussion

Origin of the Xenopus *present in Chile*

The comparative study of the species and subspecies of *X. laevis* in southern Africa (Measey & Channing 2003) confirms that the two haplotypes present in Chile are assignable to the subspecies *laevis* of the Cape region, and that there are no haplotypes of the northern subspecies, confirming the historical information. Also, the genetic analysis confirmed that the isolated population of Limarí River in northern Chile (250 km north of the core invaded area) is due to translocation of individuals from central Chile. This long-distance movement implies a high risk of propagation of the African clawed frog and has been suggested as one of the factors involved in its successful invasion of the USA (McCoid & Fritts 1980a).

Genetic pattern of the invaded area

In South Africa, the mutation level within and between groups is similar, thus they cannot be considered as different populations, especially since two Chilean haplotypes were shared between localities Sud1 and Sud3. It is nevertheless interesting that each haplogroup contributed one sequence to the invaded area.

The haplotype network indicates the presence of a large and diverse population in the area sampled in South Africa (open network, haplotypes in low frequency, high number of inferred haplotypes), thus it is likely that sampling from other localities of the subspecies *laevis* would allow greater precision in determining the origin of the two haplotypes present in Chile. The genetic analysis of this invader revealed very low haplotype and nucleotide diversity. However, the high genetic diversity of the invasive source (native range) suggests the scenario of a single invasion (p-distance 0.00989 for South African populations and 0.003542 for Chile).

The low genetic variability of the *X. laevis* in the invaded area should be a negative factor for the establishment of a feral population. Nevertheless, studies on other taxa (insects, Simberloff 1989; beetles, Grevstad 1999; bees, Zayed et al. 2007) show that populations with low genetic diversity are able to establish and perdure, suggesting that invasion success may depend on factors such as chance events and favorable ecological traits More specifically among

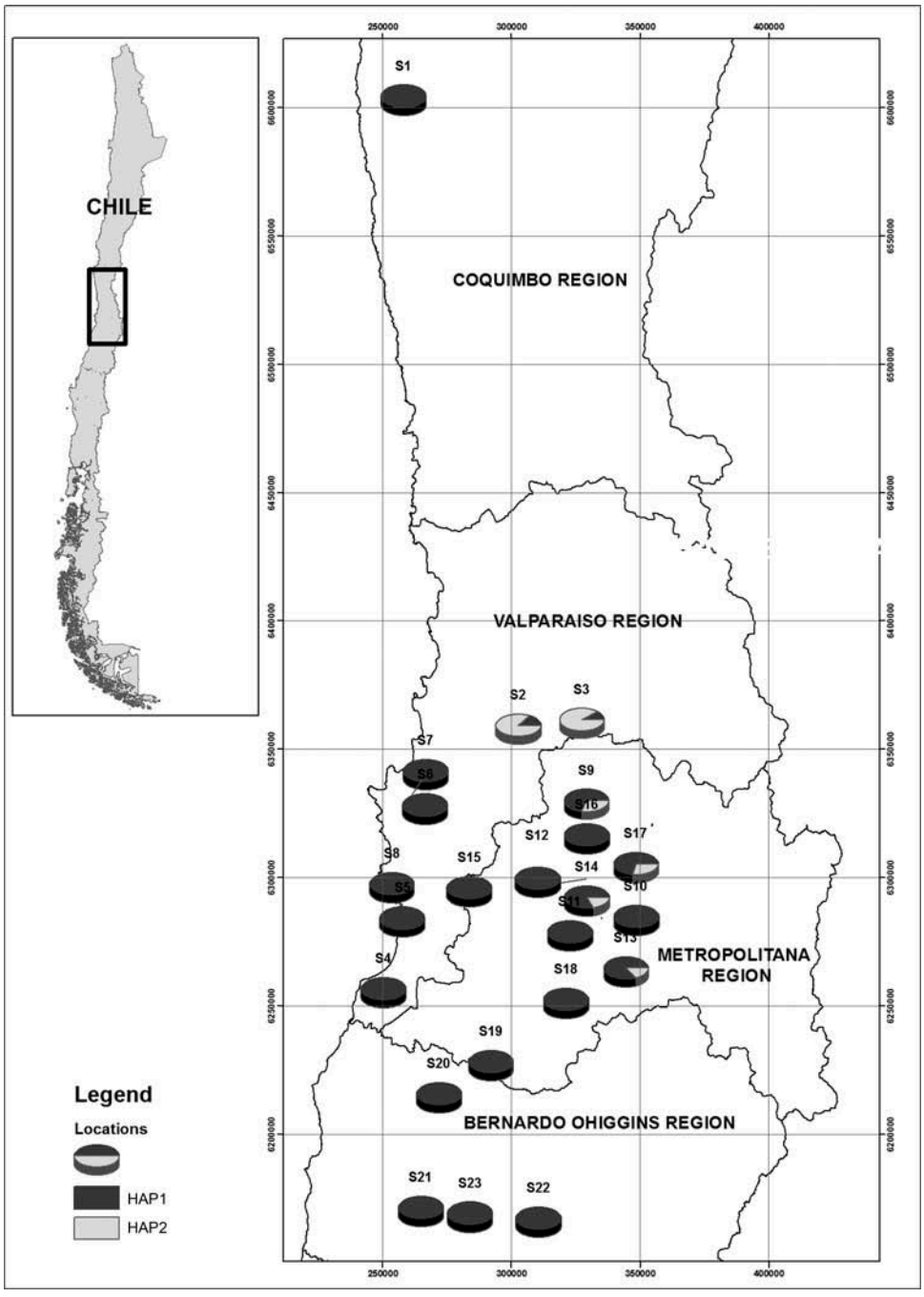

Figure 3. Distribution and proportion in studied specimens of haplotypes for the mitochondrial control region, HAP1 and HAP2, of *Xenopus laevis* in the invaded area of central Chile.

Table 2. Genetic characterization of the populations of *Xenopus laevis*, according to the analysis of the mitochondrial control region.

Area	Individuals	Number of haplotypes	π (nucleotide diversity ± SD)	H_d (haplotype diversity ± SD)
Invaded area in Chile	166	2	0.237 ± 0.0019	0.249 ± 0.1100
Native area in South Africa	22	10	0.510 ± 0.2533	0.857 ± 0.0500

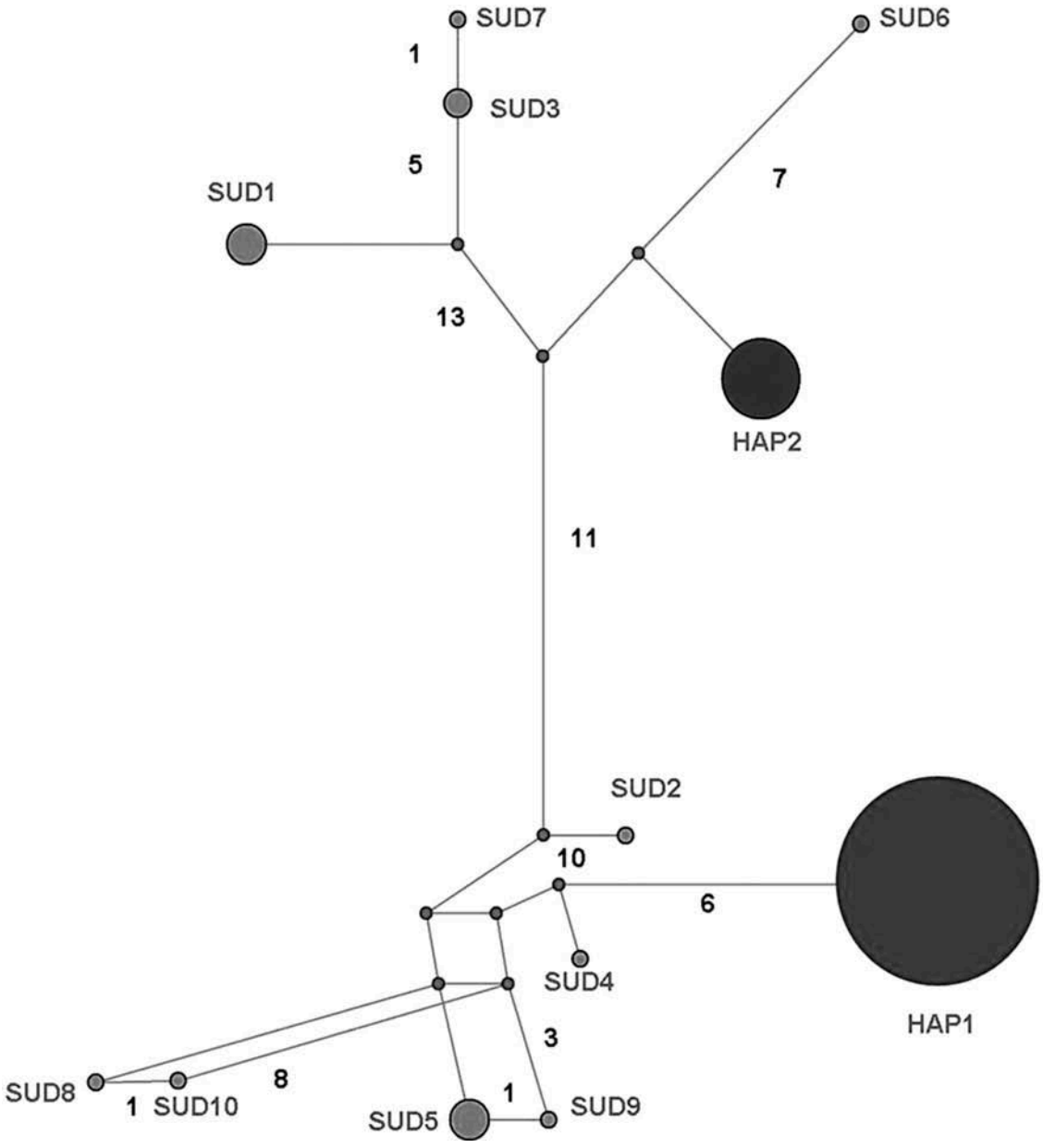

Figure 4. Haplotype network of the mitochondrial control region (median-joining network) of 166 individuals from Chile (HAP1 and HAP2, black circles) and 22 from South Africa (SUD 1 to SUD 10, grey circles) of *Xenopus laevis*. Numbers above the nodes are the number of mutations between pairs of haplotypes.

amphibians, for the ranid *Lithobates catesbeianus* the low variability recorded in its area of invasion in Europe (usually one haplotype per population, five haplotypes in all of Europe) does not appear related to its success as invader (Ficetola et al. 2008). Another example is that of the invasion of *Rhinella marina* (Bufonidae) in Australia, Indonesia and Hawaii. In its native range (USA to Peru) 14 haplotypes have been recognized, while in all invaded areas only one haplotype has been found (Slade & Moritz 1998). In spite of this low genetic diversity, *R. marina* has expanded into a range of about 1.2×10^6 km since it was introduced in 1935, with negative consequences for 12 native species (Urban et al. 2007). Species that have explosive reproductive episodes that generate a large number of descendants (e.g. adult females of *Xenopus* may lay up to 11,000 eggs) are capable of establishing large populations quickly, which increases the potential rate of successful mutations in a new area, even allowing the appearance of genotypes not found in their original range (Facon et al. 2006). Thus, species with high fertility and short

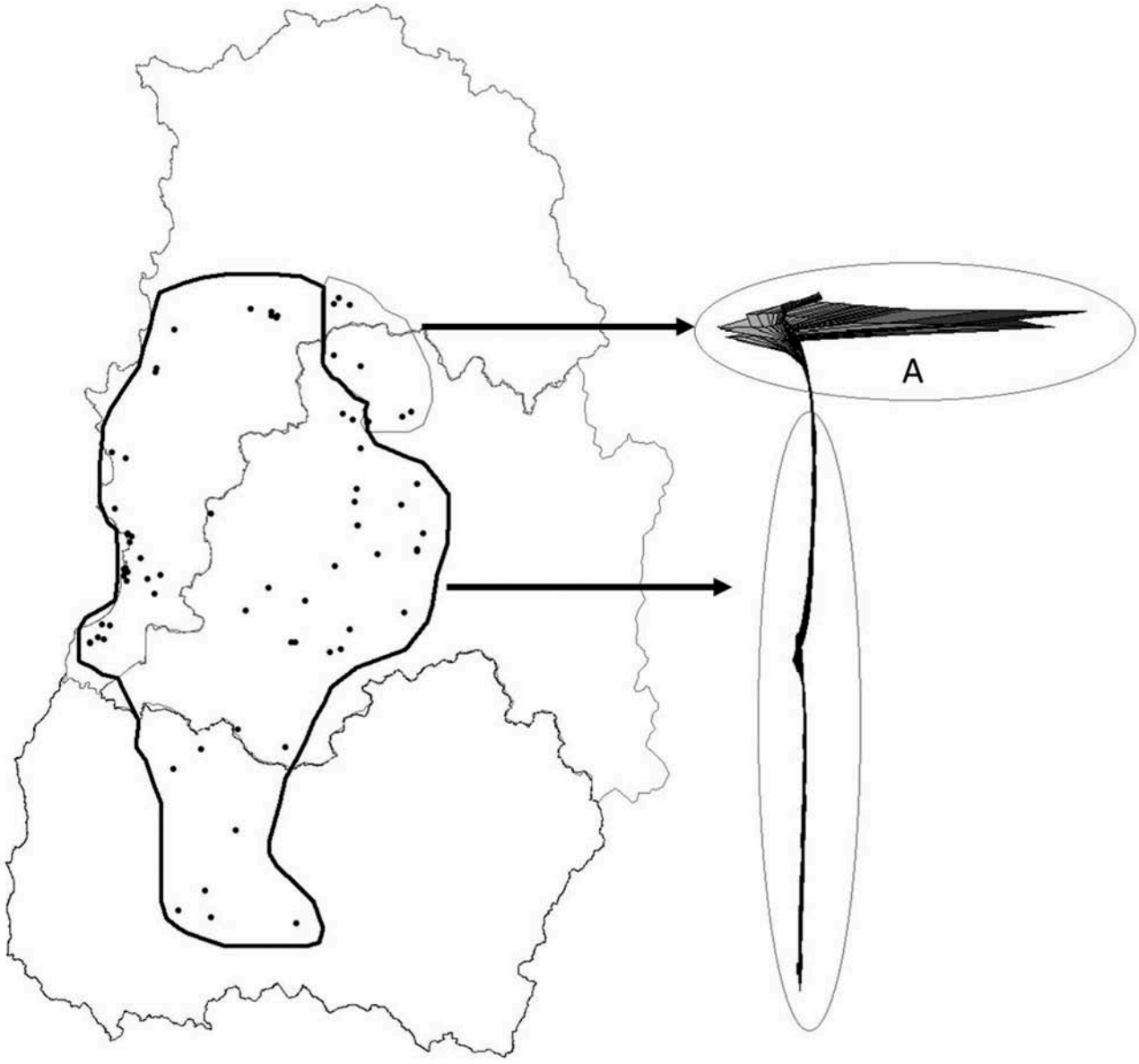

Figure 5. Interpolation of alleles in the space, for data from the mitochondrial control region of *X. laevis* from the invaded area in Chile. A, The positive peak along the *z*-axis (residue of Nei genetic distance) indicates a high genetic discontinuity.

generation times would have a greater probability of counteracting inbreeding depression (Wang 2000; Ficetola et al. 2007). However, it has also been suggested that an increased spreading of a species (usually mediated by humans) may be sufficient to generate a successful invasion, especially if the species is pre-adapted to the new environment (Facon et al. 2006). This may be the case for *Xenopus* in central Chile, since its origin was clearly in the Cape region, which has a similar, Mediterranean-type climate.

The spatial distribution of *X. laevis* in Chile shows an area in the northeastern part of the invaded area, wherein there has been an effect of fixation or drift towards the HAP2. This fixation effect might be explained by the greater aridity (fewer irrigation canals and thus less connectivity) and the presence of a mountain barrier (the Chacabuco Range, which cuts the longitudinal valley east–west), which may limit the colonization by the HAP1 haplotype dominant in central Chile. The frequency of the second haplotype decreases from the northernmost populations toward the Chacabuco Range (which separates the localities of Las Chilcas and Batuco) and towards the center of the core range.

The first report of *Xenopus* in Chile was from the Caren Lagoon, near the Santiago International

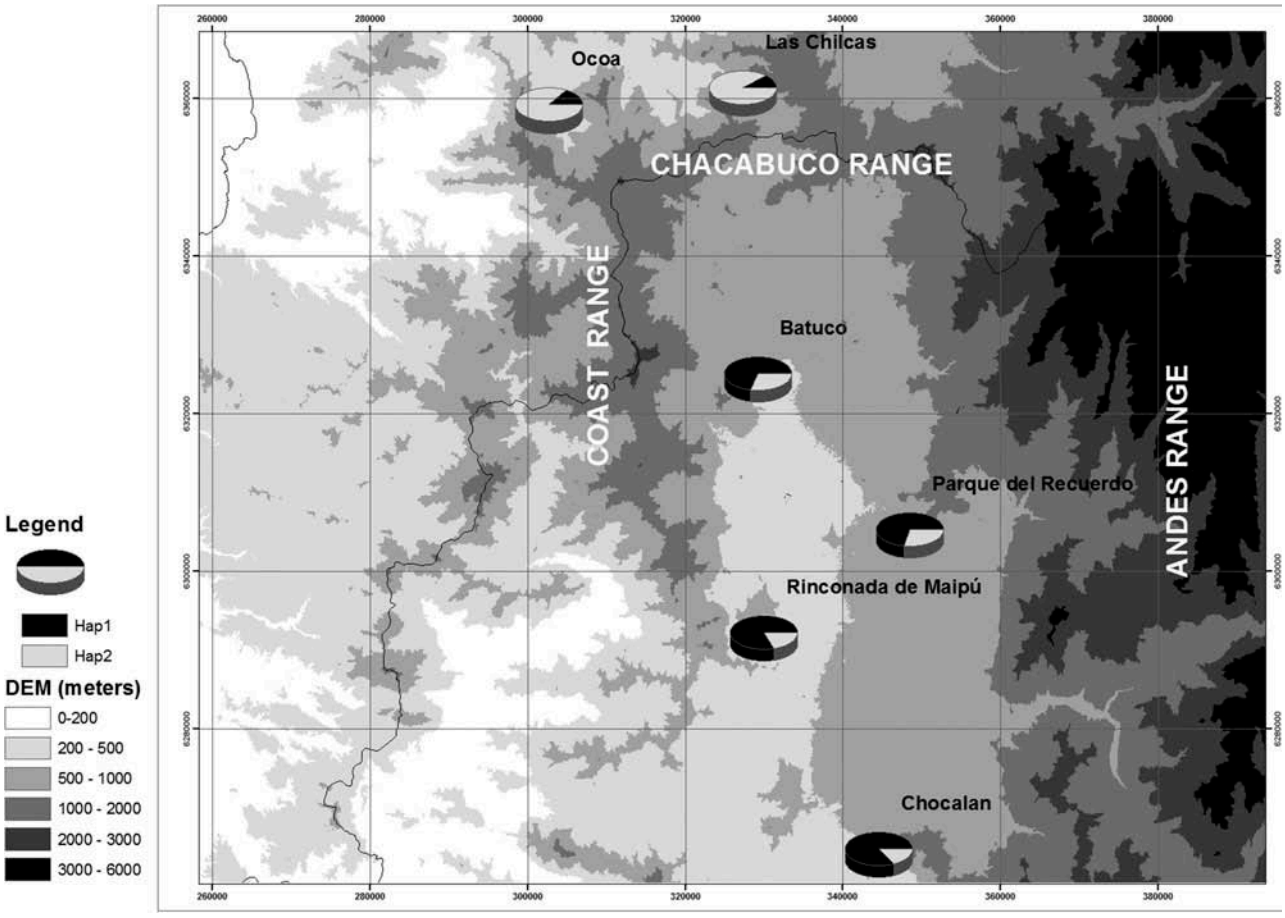

Figure 6. Area invaded by the two of *Xenopus laevis* haplotypes present in Chile (HAP1 and HAP2).

Airport (Glade 1988). Under a scenario of a single invasion event, the two haplotypes might have been released, with HP2 in lower frequency. Then, HAP2 might have spread toward the watersheds of Lampa and Colina and crossed the Chacabuco range. During rainy winters there are temporary watercourses that flow on both sides of the Chacabuco range and whose waters are collected in irrigation ponds. This chain of ponds may have provided a likely dispersal route. However, we should not ignore the possibility of human-mediated transport, since the main highway of the country crosses this area. The isolation of *X. laevis* in this area concurs with previous studies in Chile indicating that the species is incapable of invading sites above 600 m elevation (Lobos 2002; Lobos & Jaksic 2005). Chile's high longitudinal elevation gradient from sea level to mountains is the reason for high runoff rates of watercourses (Vila et al. 2006), which, reinforced by the aridity of the surrounding land (di Castri 1968), are a physical barrier for *Xenopus*.

The invasion of *X. laevis* in Chile suggests that this species may be successful even with the low genetic variability documented here, few introduction events, low numbers of founders, and strong population bottlenecks. This imposes important challenges for the control of the species in invaded areas and the prevention of its expansion to others, especially since a recent study (Measey et al. 2012) showed that the invasion of this frog at a global scale has been continuous over the last 50 years.

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