SHORT NOTE

HERPETOLOGICAL JOURNAL, Vol. 16, pp. 93-96 (2006)

GENETIC DIVERGENCE IN THE ENDANGERED FROG *INSUETOPHRYNUS ACARPICUS* (ANURA: LEPTODACTYLIDAE)

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Insuetophrynus acarpicus is a poorly known frog restricted to the temperate forests of the coastal range of Chile (39º 25' S, 73º 10' W). Until recently, this species was known only from one type locality since its original description in 1970. However, in 2002 two new localities were reported, extending its distribution range to about 40 km^2. In order to evaluate genetic divergence, provide a preliminary evaluation of the genetic diversity of this species and the phylogenetic relationships among individuals from the three known populations, we analyzed the nucleotide variation of a fragment of the mitochondrial gene cytochrome *b*. We sampled just two or four individuals per population of this endangered frog. We found a low nucleotide divergence among populations suggesting a genetic homogeneity across the entire range. This highlights the need for further studies to define the conservation status of this endangered frog.

Key words: anuran, conservation, cytochrome *b,* population genetics

Insuetophrynus acarpicus Barrio 1970 is an endemic frog of the Chilean temperate forest, inhabiting the northern coast of Valdivia (39º 25' S, 73º 10' W), and is the only species of this monotypic genus. Studies on *I. acarpicus* have mainly focused on its natural history (Barrio, 1970; Formas *et al.*, 1980; Díaz *et al.*, 1983) and chromosomal characters (Barrio & Rinaldi, 1971; Díaz & Veloso, 1979; Díaz *et al.,* 1983). In ecological terms, this species is described as being strongly aquatic and restricted to coastal streams with slopes of 19º to 38º (Díaz *et al*., 1983). In this species larval development can take about 10 to 12 months, and recently metamorphosed individuals are found mainly in January. More details of life history and ecology of this species are provided by Díaz *et al*. (1983). Currently, *I. acarpicus* is considered to be endangered in Chile (Glade, 1993; Díaz-Páez & Ortiz, 2003), mainly due to low population abundances and its extremely narrow distribution range. In fact, for more than 30 years after its original description this species was known only from one locality, Mehuín (Barrio, 1970; Formas *et al*., 1980). Recently, two new localities have been described for this frog (Soto *et al*., 2002), extending its distribution range towards north-eastern Chile.

In this study, we conducted the first molecular analysis in this species, including individuals from all three localities where *I. acarpicus* has been reported. We made a preliminary evaluation of genetic variation of the cytochrome *b* gene within populations and inferred the phylogenetic relationships among the three populations. We also estimated the time elapsed since their divergence.

Specimens were obtained from the herpetological collections of the Departamento de Biología Celular y Genética of the Universidad de Chile (DBGUCH), the Instituto de Zoología of the Universidad Austral (IZUA) and the Museo de Zoología of the Universidad de Concepción (MZUC). We obtained samples of liver or toe from specimens preserved in ethanol (70%), representing the three currently known localities (Fig. 1): (1) Mehuín (39º 26' S, 73º 13' W - two specimens: DBGUCH 3133, IZUA 3249); (2) Queule (39º 24' S, 73º 13'W - four specimens: DBGUCH 3114, 3126, 3128, 3129) and (3) Colegual Alto (39º 24' S, 73º 06' W - two specimens: MZUC 26930, 26931). No other specimens were available for DNA extraction in these collections. We extracted total DNA using the phenol-chloroform (1:1) and chloroform isoamyl alcohol (24:1) method (Sambrook *et al*., 1989). We amplified a 1050 bp fragment of the mitochondrial gene cytochrome *b* (cyt *b*) via polymerase chain reaction (PCR) using Taq DNA Polymerase (GIBCO) with primers MVZ15-L (Moritz *et al.*, 1992) and Cyt*b*AR-H (Goebel *et al*., 1999). PCRs were performed using the following thermal profile: denaturation at 94 ºC (1 min 40 s), annealing at 50 ºC (1 min), extension at 72 ºC (2 min 30 s) for 42 cycles and 10 minutes of final extension at 72 ºC. All reactions were performed in a Biometra® Personal Cycler. Double-stranded PCR products were purified with the QIAquick kit (Qiagen) and sequenced using the Big Dye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems) in an ABI Prism 3100 automated sequencer. Sequences were aligned using BioEdit software (Hall, 1999) with the ClustalW option and compared by eye. We also checked the sequences using both DnaSP 3.53 (Rozas & Rozas, 1999) and BioEdit programs to translate them into amino acids. All sequences were submitted to

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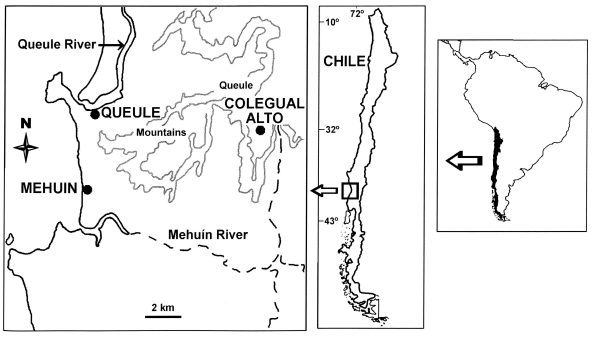

FIG. 1. Map showing the location of the three known localities of *Insuetophrynus acarpicus*. Dotted lines correspond to major rivers and continuous lines represent altitudinal contours.

GenBank (accession numbers AY386396-AY386401 and AY691185-AY691187).

We applied parsimony and maximum likelihood analyses; in both cases we used exhaustive search option. Support of the nodes was assessed by non-parametric bootstrap with 1000 pseudoreplicates. Phylogenetic analyses were performed using PAUP* 4*b*10 software (Swofford, 2001). In all analyses we used as outgroup one individual of *Eupsophus* sp. (DBGUCH 3273) from the Region VII, Chile. *Eupsophus* and *Insuetophrynus* are thought to be basal members of Leptodactylidae and related genera according to previous systematic studies (Lynch, 1978; Díaz, 1986).To calibrate a molecular clock for the sequences of *I. acarpicus* we evaluated the sequence neutrality using a hierarchical likelihood ratio test (hLRT) (Huelsenbeck & Crandall, 1997). Since there are no molecular clock calibrations available for Leptodactylidae, and the evolutionary relationships with other frog families remain unclear, we applied a standard nucleotide substitution rate of 0.5-1% per lineage per million years. This calibration value represents an estimate described for vertebrate mitochondrial genes (Moritz *et al*., 1987). We obtained an alignment of 707 bp with 170 variable sites among *Insuetophrynus* and *Eupsophus* sequences, and 14 variable sites among *Insuetophrynus* sequences, all of them parsimony informative sites. Only three haplotypes were observed for *I. acarpicus*, with no intrapopulation variation. Proportion of variable sites (p-distances) of haplotypes and geographic distances among *I. acarpicus* localities are shown in Table 1. Phylogenetic analyses recovered one tree for both reconstruction methods parsimony and maximum likelihood. Hence, we show the maximum likelihood tree (Fig. 2; -lnL = 1555.3914) constructed with the GTR+G substitution model which was obtained using Modeltest 3.06 and AIC criterion (Posada & Crandall, 1998).

Phylogenetic analyses separated *I. acarpicus* individuals in two groups, one including individuals from Queule and the other including those from Mehuín and Colegual Alto. At the population level, the Queule locality showed the same level of divergence (1.84%; Table 1) when was compared with localities of Mehuín and Colegual Alto. In contrast, between Colegual Alto and Mehuín populations we found a smaller amount of differentiation (0.28%; Table 1). These levels of divergence are similar than those reported in other amphibian species. For cytochrome *b*, Tan & Wake (1995) reported for *Taricha torosa* a population divergence between 0.6- 2.5 %. Instead, Shaffer *et al.* (2004) reported intraspecific divergence rates between 0-5 % for five species of genus *Rana*.

TABLE 1. Proportion of variable sites (p-distances) for *Insuetophrynus acarpicus* cytochrome *b* sequences (below diagonal) and geographic distances (km) among localities (above).

	1	2	3
1. Queule		4	11.5
2. Mehuín	0.0184		10.7
3. Colegual Alto	0.0184	0.0028	

The hLRT did not show a significant difference ($P > 0.05$) between topologies with enforced vs. non-enforced molecular clock, therefore we cannot reject the neutral evolution of sequences. Using divergence rates of 0.5 and 1% per lineage per million years, we postulated that divergence times between Queule and Mehuín-Colegual Alto populations occurred between 1840000 and 920000 years ago, respectively. Instead, divergence between Mehuín and Colegual Alto populations could have occurred around 280000 and 140000 years ago. These divergence times suggest that separations among these populations occurred in the Pleistocene. Although there is a considerable literature about paleoclimatic reconstructions of the south of Chile, most of them are related to Holocene period (Clapperton, 1994; Veit, 1994; Villagrán, 1994, 2001; Potts & Behrensmeyer, 1992; among others) and there is no paleoclimatological evidence from this region about Pliocene-middle Pleistocene (Hinojosa & Villagrán, 1997). This fact does not allow us to discuss

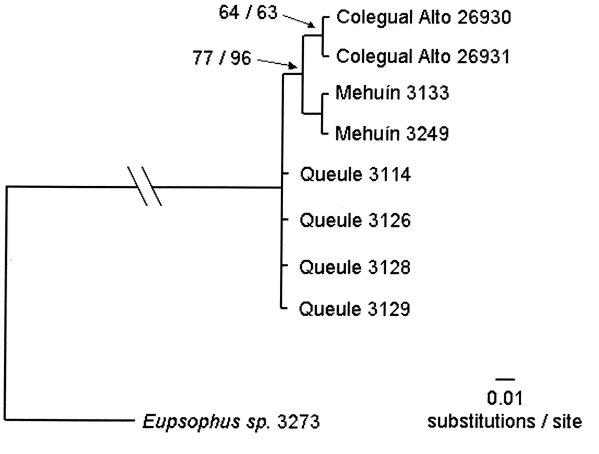

FIG. 2. Maximum likelihood (ML) tree for cytochrome *b* sequences of eight *Insuetophrynus acarpicus* individuals from the three known localities. Bootstrap values are indicated above each node of the tree, maximum likelihood (ML) and maximum parsimony (MP), respectively (ML/MP). Only values over 50% are shown.

in detail the observed population divergence in an historical context. In this respect, we only can say that during glacial events, processes of expansion and contraction of habitat could have influenced the differentiation of *I. acarpicus*. Our phylogenetic analyses show that Mehuín and Colegual Alto diverged after than Queule population, however, because our knowledge of distribution of *I. acarpicus* is fragmentary, we cannot say that Queule population gave rise to others two. In this sense we propose that Queule population belong to an ancestral lineage in comparison to other two populations. On the other hand, the existence of a common drainage area for Colegual Alto and Mehuín populations could explain why these populations appear separated from Queule population which belongs to another watershed. Accordingly, it is possible that Colegual Alto and Mehuín were connected by streams and gorges in this region and, because this species is strongly aquatic and restricted in its distribution to streams (Díaz *et al,* 1983), the mechanism of colonization could be related to passive dispersion via stream running. Finally, given that this genus inhabits a narrow geographic range (about 40 km^2; Fig. 1) without naturally protected areas, and where the human population is currently in expansion and strongly modifying the environment, it is important to continue studying this species, focusing on the development of a more extensive survey programme to find new localities inhabited by this frog. In fact, the historic absence of records for this species is a strong indicator of low population abundances, especially since other rare frog species (e.g. *Eupsophus migueli*) have been collected in the same areas (Méndez *et al.*, *in press*). This information will be highly relevant for understanding the genetic diversity and conservation status of *I. acarpicus*.

Acknowledgments. We thank R. Eduardo Palma, Cecilia Smith and Paula Neill for comments on previous drafts. Financial support was provided by FONDECYT grant 3000048/2000 to MAM, and FC49 "Biodiversity Assessment and Systematization of Existing Biological Knowledge on the Coastal Range of the Lakes, X Region, Chile" and Contract ICA 4-CT-2001-10095 BIOCORES PROJECT funded by EC under the INCO IV programme. We also thank ExpEdiciones al Conocimiento for support in collecting the Colegual Alto samples. We are also grateful to A. Crawford and an anonymous referee for useful suggestions and comments that improved remarkably the earlier version of this paper.

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Accepted: 3.1.05