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Evidence of hybridization in the Argentinean lizards *Liolaemus gracilis* and *Liolaemus bibronii* (IGUANIA: LIOLAEMINI): An integrative approach based on genes and morphology

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ABSTRACT

The lizard genus *Liolaemus* is endemic to temperate South America and includes more than 225 species. *Liolaemus gracilis* and *L. bibronii* are closely related species that have large and overlapping geographic distributions, and the objective of this work is to further investigate the *L. bibronii–L. gracilis* mtDNA paraphyletic pattern previously detected, using an integrative approach, based on mtDNA, nuclear DNA and morphological characters. We identified eight morphological *L. bibronii* introgressed with *L. gracilis* mtDNAs, and the reciprocal for one *L. gracilis*, from six localities in the region of sympatry overlap. The morphological identity of these introgressed individuals was confirmed by diagnostic nuclear markers, and this represents the first well-documented case of interspecific hybridization in the lizard genus *Liolaemus*. Of the three most likely hypotheses for these observed patterns, we suggest that asymmetrical mtDNA introgression as a result of recent or ongoing hybridization between *L. bibronii* and *L. gracilis* is the most likely. This may be due to size selection by *L. gracilis* female preference for the larger *L. bibroni* males in sympatry, but this requires experimental confirmation.

1. Introduction

Although mtDNA has been the workhorse of research in phylogeography for almost two decades, recent studies have summarized concerns about evolutionary interpretations based on mtDNA results alone (e.g. Edwards and Bensch, 2009). Mitochondrial genomes are thought to have a better chance of tracking species trees due to a higher mutation rate (this makes easier to estimate the gene tree) relative to nuclear genes, and alleles shared between incipient species will sort to reciprocal monophyly faster due to a smaller effective population size as a consequence of uniparetal inheritance and haploid status (Pamilo and Nei, 1988; Moore, 1995). However, this genome is a single locus and not necessarily representative of the multitude of evolutionary histories of the unlinked genes in the nuclear genome (Bossu and Near, 2009). Maddison (1997) suggested that phylogenetic analyses of multiple loci should be undertaken in an explicit coalescent framework, because all of the gene trees are part of the species tree, which can be visualized as a fuzzy statistical distribution; literally a "cloud" of gene histories. Thus analyses of multiple loci generally give a better signal for phylogenetic relationships, but also could represent massive incongruence among the evolutionary histories of loci (Than and Nakhleh, 2010).

Many instances of mtDNA paraphyly have been observed in animals (summarized in Funk and Omland (2003)), and particularly different levels of incongruence relative to nuclear gene genealogies. Many gene tree incongruence problems can, especially among recently diverged species, result from incomplete lineage sorting and/or gene flow (Belfiore et al., 2008; Brumfield et al., 2008; Carling and Brumfield, 2008; Eckert and Carstens, 2008). In this context, the mitochondrial genome is particularly useful to detect introgression, because a lack of recombination insures that all base positions introgress as a completely linked block (Smith et al., 1992). Thus, an introgressed mtDNA fragment will reflect the heterospecific origin of its mitochondrial genome, and recognizing this introgression requires evaluating a mitochondrial gene tree against a nuclear background that identifies the participating taxa (Funk and Omland, 2003).

In the particular case of a cytoplasmic genome, there are several mechanisms that could, independently and in combination, affect a single gene tree genealogy: sexual selection and asymmetric reproductive barriers (Chan and Levin, 2005), demographic effects (Rieseberg et al., 1996b), differences in the magnitude of selection on particular genes (Funk and Omland, 2003), and cyto-nuclear compatibilities (Rieseberg et al., 1996a). This biased cytoplasmic

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introgression can manifest itself without introgression of alleles from the nuclear genome, and because of the uniparental inheritance of the mitochondrial genome, it is possible to identify the directionality of introgression. Lastly, the phylogenetic pattern coupled with molecular branch lengths may also provide information on the relative timing of introgressive hybridization events (Bossu and Near, 2009).

The demographic processes that may influence gene genealogies are difficult to differentiate using topological information alone, because they may result in similar genealogical patterns (Funk and Omland, 2003). Integration of the genetic data with ancillary information, whether it is ecological, morphological, geographical, geological, or functional in nature, is key to maximizing evolutionary and ecological insights (Knowles, 2009). Spatial patterns of gene tree incongruence can aid in the differentiation of these processes, and the localization of discordance near phylogeographic boundaries may be a signature of current or historical interspecific gene flow (Leaché and McGuire, 2006; McGuire et al., 2007).

The South American lizard genus *Liolaemus* includes more than 225 described species (Avila et al., 2010; Lobo et al., 2010), and is distributed over a wide geographic area spanning a large range of latitudinal (14° ± 30′–52° ± 30′S), altitudinal (0–4500 m) and climatic regimes, from the extremely arid Atacama desert (southern Peru) to temperate *Nothofagus* rainforest (Tierra del Fuego, Argentina; Cei, 1986, 1993; Donoso-Barros, 1966; Etheridge, 1995; Etheridge and De Queiroz, 1988; Lobo, 2001). Two recent studies (Morando et al., 2003, 2007) suggest that the actual number of *Liolaemus* species could be double the recognized number. This reveals the poor state of taxonomic knowledge of *Liolaemus*, and indeed some studies have described new species from within taxa previously considered to be one widely distributed variable species (e.g. *L. darwinii*: Cei and Scolaro, 1999; Etheridge, 1992, 1993, 2001; Lobo and Kretzschmar, 1996; e.g. *L. boulengeri*: Abdala, 2003, 2005; e.g. *L. rothi*: Etheridge and Christie, 2003; Pincheira-Donoso et al., 2007).

Some of the recent molecular studies in *Liolaemus* have demonstrated mtDNA paraphyly, and this has been interpreted as either due to incomplete lineage sorting or as asymmetrical introgression for paraphyletic patterns in some haploclades of *L. darwinii–L. grosseorum* and *L. bibronii–L. gracilis* (Morando et al., 2004, 2007, respectively). In this second group, Morando et al. (2007) showed that the three individuals carrying introgressed haplotypes (in all cases *L. bibroni* phenotypes with *L. gracilis* mtDNA haplotypes) were collected from a zone of sympatry, located in an ecotone between Monte and Steppe habitats in Patagonia, Argentina. *Liolaemus gracilis* and *L. bibronii* are phenotypically distinct and easy to distinguish throughout their distributions, including sympatric localities.

The objective of this work is to further investigate the *L. bibronii–L. gracilis* mtDNA paraphyletic pattern using an integrative approach. We extend the work of Morando et al. (2007) by incorporating new terminal samples to the earlier dataset, adding additional mitochondrial (cyt-b and 12S) and new nuclear sequences (anonymous loci: LPB4g, LPA11e, and LPB9c), and including 10 morphometric and 10 meristic characters to quantify morphological variation in the *L. gracilis* and the *L. bibronii* populations. Here, we identified eight morphological *L. bibronii* individuals with introgressed *L. gracilis* mtDNA haplotypes, and the reciprocal pattern for one *L. gracilis* individual. These lizards were sampled from six localities in the area of sympatry and represent the first well-supported evidence of hybridization between *Liolaemus* species.

2. Materials and methods

2.1. Field sampling

We collected a total of 193 samples of *L. gracilis* from 68 different localities, 63 of *L. bibronii* from 31 localities, three of *L. saxatilis* from two localities, and one each of *L. ramirezae* and *L. robertertmertensi*, closely related species to the focal species (Morando et al., 2007), and *L. punmahuida* (Fig. 1). Specimens were collected by hand, sacrificed by a pericardic injection of sodium pentothal Abbot®, dissected slightly to extract a sample of liver for molecular study, fixed in 10–20% formalin, and later transferred to 70% ethanol. Lizards are deposited in the Herpetological Collection L.J. Avila/M. Morando (LJAMM-CNP) of the Centro Nacional Patagónico, Puerto Madryn, Argentina (CENPAT–CONICET, http://www.cenpat.edu.ar/nuevo/colecciones03.html), and the herpetological collection of Bean Life Science Museum, Brigham Young University (BYU) (Appendix A).

2.2. Laboratory procedures

Genomic DNA was extracted using the Qiagen® DNeasy® 96 Tissue Kit following the protocol provided by the manufacturer. PCR and sequencing protocols follow Morando et al. (2003, 2004) for the mitochondrial genes (cyt b [725 bp] and 12S [883 bp]), and for the ANL (LPA11e [785 bp], LPB4g [661 bp] and LPB9c [740 bp]) we used the touchdown cycle described by Noonan and Yoder (2009), with standard reaction conditions (per sample: 2 μl dNTPs (1.25 mM), 2 μl 5× Taq buffer, 1 μl each primer (10 μM), 1 μl MgCl (25 mM), and 0.1 μl Taq DNA polymerase (5 U/μl; Promega Corp., Madison, WI); 14 ml total reaction volume). All sequences (ANL and mitochondrial) were edited using the program Sequencher v4.8. (™Gene Codes Corporation Inc. 2007), and aligned sequences with ClustalX (Higgins and Sharp, 1988; Thompson et al., 1997); alignments were checked by eye and manually adjusted if necessary to maximize blocks of sequence identity. Missing data in all cases were coded as "?", and sequences are deposited in GenBank (Accession Nos. JN410363–JN410558). For each gene we selected the best-fitting model using JModelTest v0.1.1 (Guindon and Gascuel, 2003; Posada, 2008) using the Bayesian information criterion (BIC) (Table 1). In all nuclear genes, recombination was tested using RDP: Recombination Detection Program v3.44 (Heath et al., 2006; Martin and Rybicki, 2000).

2.3. Phylogenetics analysis

As a first approximation, we reconstructed a Bayesian tree using partial sequences of cyt-b, from 64 samples of *L. gracilis* representing its complete distribution. From this analysis we selected representatives from the most distinct clades, and made further analyses of all mt and nuclear sequence data collected from a total of 47 lizards, including a subsample of 22 *L. gracilis* from 16 localities, and 18 individuals from 16 localities representing all *L. bibronii* clades. These were the "focal species" (Wiens and Penkrot, 2002) of this study, and three samples of *L. saxatilis* (Avila et al., 1992), and one each of *L. robertmertensi* (Hellmich, 1964) and *L. ramirezae* (Lobo and Espinoza, 1999) (also recovered within this clade by Morando et al. (2007)) were included as non-focal species. *Liolaemus punmahuida* (Avila et al., 2003), a member of the *chiliensis* subgenus (Lobo et al., 2010), was used to root the trees. Appendix A summarizes the number of individuals sequenced per locality and distributional information for all taxa used in this study.

All further analyses of the subsamples of lizards were based on the two mitochondrial and three ANL. We conducted separate Bayesian analyses for each nuclear and mitochondrial region separately and repeated these analyses for all mtDNA and nuclear regions combined, using MrBayes v3.1.2 (Ronquist and Huelsenbeck, 2003). Each analysis used four heated Markov chains (using default heating values) and was run for 10 million generations, with Markov chains sampled at intervals of 1000 generations. The equilibrium samples (after discarding 10% as

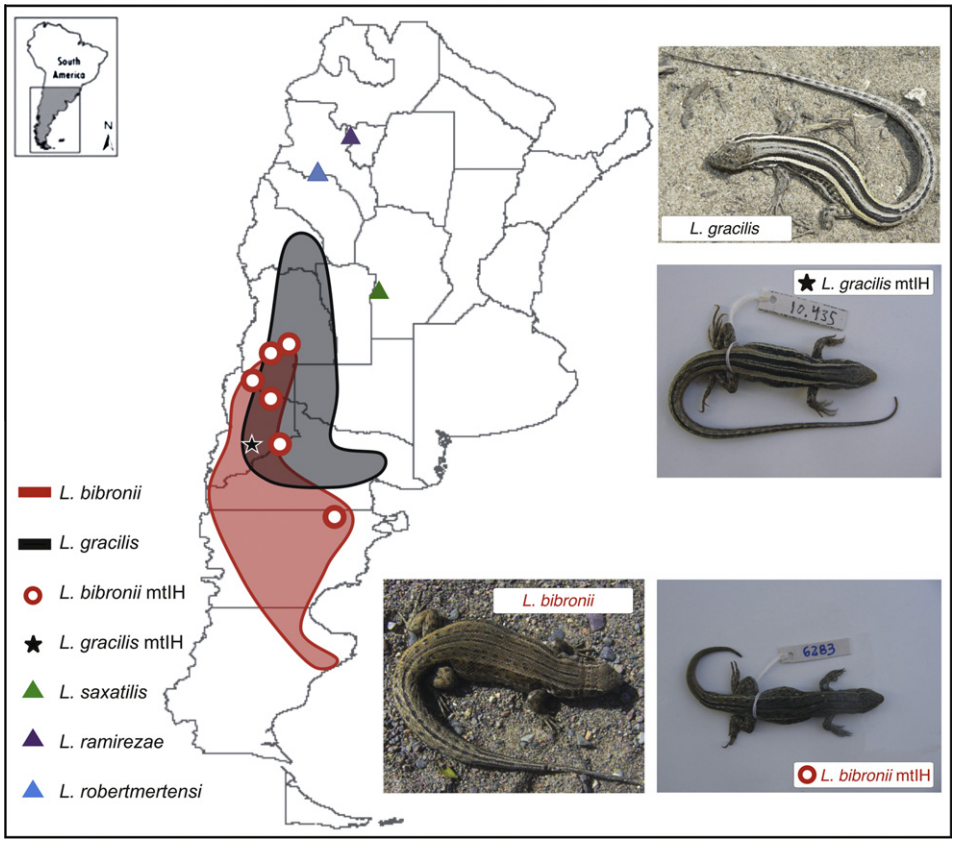

Fig. 1. Distribution of the focal and outgroup taxa used in this study. *Liolaemus gracilis* and *L. bibronii* are identified in black and red, respectively; the black star shows the location of the *L. gracilis* individual with an introgressed *L. bibronii* mtDNA haplotype, and red circles show the reciprocal for *L. bibronii* samples with *L. gracilis* mtDNA introgressed haplotypes. Other non-focal taxa are shown with different colors (*L. saxatilis*, green; *L. ramirezae*, purple; and *L. robertmertensi*, blue). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Gene regions, primer sequences, lengths, nucleotide substitution models and genome used in this study.

Locus	Primer sequences	Length (bp)	Evolution model	Nst/rates	Genome
cyt-b	Morando et al. (2003)	725	TIM3 + I	6/gamma	Mitochondrial
12S	Morando et al. (2003)	883	TPM2uf + G	6/equal	Mitochondrial
LPB9c	F 5′ TGACTTGTGAGTAGTTAGGGTATGC 3′ R 5′ TTTGGTGTGGCATGTGCATGTGAAAT 3′	740	HKY + I	2/equal	Nuclear (ANL)
LPB4g	F 5′ TCGAAACTCCTTCAGGGCTA 3′ R 5′ TTTCCTACCTCGGTCACCAC 3′	661	K80 + G	2/gamma	Nuclear (ANL)
LPA11e	F 5′ CAAGGATCCATAGCACAGCA 3′ R 5′ CACCTTCTGAGGCAATCCAT 3′	785	HKY + G	2/gamma	Nuclear (ANL)

burn-in) were used to generate a 50% majority rule consensus tree, and posterior probabilities (Pp) were considered significant when $\geqslant$0.95 (Huelsenbeck and Ronquist, 2001).

We also obtained a species tree from the nuclear genes by minimizing deep coalescences (MDC), using the dynamic programming (DP) algorithm (Than and Nakhleh, 2009) implemented in Phylonet software package (Than et al., 2008). This method takes gene trees as input and seeks the species tree that requires the fewest deep coalescence events to explain, and therefore provides the most parsimonious explanation for the observed gene trees (Maddison, 1997). Although this approach assumes that all discordance is a consequence of incomplete lineage sorting, both simulation (Eckert and Carstens, 2008) and empirical (Knowles and Carstens, 2007) studies have corroborated that this approach performs well even when the "no gene flow" assumption is violated.

2.4. Population genetic and demographic analyses

We implemented DNAsp (Rozas and Rozas, 1999) to estimate genetic (Nei, 1978) and nucleotide (Nei, 1987) diversity indexes, and using a concatenated matrix of the mitochondrial genes we calculated Tajima's D (Tajima, 1989) and Fu's Fs (Fu, 1997) to test the hypothesis of neutral evolution. We also performed two gene flow tests: γ_{st} based on haplotype data (Nei, 1973) and F_{st} based on sequence data (Hudson et al., 1992) using 10,000 replicates; in both cases gaps were taken as fifth states. These tests were performed between clades of *L. gracilis* recovered in the mitochondrial phylogenetic analysis (see below) and between *L. gracilis* and *L. bibronii*. We also used the clades recovered from the nuclear genes analyses to further test gene flow with the same concatenated mitochondrial matrix. Finally, we estimated past population dynamics for *L. gracilis* from Bayesian skyline plots of the cyt-b

sequences, using BEAST (Drummond and Rambaut, 2006), with a MCMC run of 20 million generations and a mutation rate of 0.0223 per site per Ma (Fontanella et al., in review).

2.5. Morphological analysis

We examined 179 individuals of *L. gracilis* (Appendix A) sampled from throughout the entire distribution, and 39 specimens of *L. bibronii* from different localities within the region of sympatry.

2.5.1. Standard morphometric characters

We used a Schwyz electronic digital caliper of 0.1 mm precision to measure 10 biometric variables: head length (**HL** – from posterior edge of auricular opening to anterior of the rostral scale), head width (**HW** – between corners of the mouth), head height (**HH** – distance between the snout and the parietal scales), snout–vent length (**SVL** – distance from the tip of the snout and the posterior margin of the precloacal scales.), axilla-groin distance (**AGD** – distance from the armpit of the right front leg to the anterior insertion of the hind limb), hand length (**HaL** – distance between the base of the wrist and base of the nail of the third digit**;** measured ventrally), foot length (**FoL** – distance between the base of the heel to the base of the nail of the fourth digit; measured ventrally), tibio-fibula length **(TFL** – from knee to the internal angle with the foot), knee-knee distance (**KKD** – distance between knees bent at right angles to the abdomen, measured ventrally), and inter-nostil distance (**IND** – dorsally measured distance between nostrils). We implemented a bilateral *t* test to compare sample means and set the significance level to 0.05. Because the morphometric variables are highly correlated with the SVL of individuals, in the cases where we detected differences in any variable, we then performed an ANCOVA using SVL as covariable (Vega and Bellagamba, 2005; Vega et al., 2008). This analysis adjusts morphometric measures to individual body sizes and permits tests of differences after removal of size as a confounding variable.

2.5.2. Meristic characters (scale counts)

We recorded 10 different scale count variables: scales a around midbody (**SAM** – around the midbody measured at the trunk), dorsal scales between occiput and thigh (**DSOT** – from the superciliary scales down to the ring of scales anterior to the vent), ventral scales (**VS** – from first gular scale to preclocal scales), right enlarged suparalabials (**RESL** – scales on the upper right corner of the mouth, with the exception of the rostral), left enlarged suparalabials (**LESL** – **s**cales on the upper left corner of the mouth, with the exception of the rostral), right enlarged infralabials (**REIL** – scales on the lower right corner of the mouth, with the exception of the rostral), left enlarged infralabials (**LEIL** – scales on the lower left corner of the mouth, with the exception of the rostral), infradigital lamellae of 3rd toe of the hand (**IL3H** – under the third digit of the forelimb from the edge of the palm to the nail), infradigital lamellae of 4th toe of the pes (**IL4P** – under the fourth digit of the hind limb from the edge of the heel to the nail), and number of scales with keels (**NSK** – up to the front legs). We implemented a bilateral *t* test to compare sample means with a significance level of 0.05.

2.5.3. Statistical analyses

We used the INFOSTAT® software for all uni- and multivariate analyses. We first tested for sexual dimorphism within *L. gracilis* using both data sets, and then tested for interspecific differences between *L. gracilis* and *L. bibronii*. We then included samples with mixed mitochondria haplotypes (hypothetized to result from hybridization and introgression of the mtDNA locus, here designated as: mtIH, mitochondria introgressed haplotype). We performed Student *t* tests for all of these analyses. Given that for morphological variables we have $n = 3$ in *L. bibronii* mtIH, we used the morphometric and meristic characters in a Principal Component Analysis (PCA), and used the three first principal components (PC) to reduce the number of variables in the analysis (so they are not higher than the number of samples). Then we performed a Discriminant Analysis (DA) from the PCA to graph the differences between *L. gracilis*, *L. bibronii*, and the mtIH samples.

3. Results

3.1. Phylogenetics analysis

Table 1 summarizes alignment lengths and models of evolution for all sampled genes. The Bayesian tree obtained from the cyt-b+12S mtDNA concatenated matrix is depicted in Fig. 2a. *Liolaemus gracilis*, *L. bibronii* and *L. saxatilis* are not recovered as clades. Three well-supported (pp = 1.0) major clades are recovered: the most nested clade (A) includes most of the *L. gracilis* haplotypes (21 terminals) + *L. bibronii* (8 red terminals, haplotypes from northern-most distribution) + *L. saxatilis* (3 green terminals); clade (B) recovers *L. bibronii* (3 red terminals, northern distribution) and one *L. gracilis* from Neuquén province (star in Fig. 2); and the basal clade (C) including 7 red terminals of *L. bibronii* (southern distribution). We sequenced nuclear genes for five of the eight *L. bibronii* samples recovered in clade (A), and we identify these samples as mitochondrial introgressed haplotypes (mtIH; red circles in Fig. 2). We recovered a single *L. bibronii* haplotype (northern distribution area, Mendoza province) as sister to (A); and *L. robertmertensi* as a sister to this clade. The relationship between (A), (B), and *L. ramirezae* is unresolved.

Within clade A (Fig. 2a) we recovered three clades (mC1, mC2, mC3), although only mC3 is well supported (pp = 0.99) and phylogenetic relationships among these are unresolved. We performed phylogeographic analysis based on these clades as well as for the entire tree. There is no clear correlation between the clades and their geographic distribution, as they present high levels of overlap.

The Bayesian tree based on the concatenated nuclear dataset is presented in Fig 2b. We recovered *L. gracilis* as paraphyletic, one well-supported clade is unresolved (nC1), and a second well-supported clade (nC2) as sister to the (*L. robertmertensi* + (*L. ramirezae* + *L. saxatilis*)) clade, but with low statistical support. Also *L. bibronii* was recovered as paraphyletic; one major clade (pp = 0.82) including individuals from its southern distribution was the most basal clade in the tree (pp = 1.0). The other clade (pp = 1.0) includes individuals from the northern distribution, and is recovered as sister (pp = 0.54) to (*L. gracilis* + (*L. robermertensi* + (*L. ramirezae* + *L. saxatilis*))). A single *L. bibronii* individual from Neuquen province was recovered as sister to all others.

The mitochondrial gene tree (Fig. 2a) is clearly discordant with the nuclear gene tree (Fig. 2b). All *L. gracilis* ($n = 1$) and *L. bibronii* ($n = 5$) mtIH haplotypes that were recovered within the other species mtDNA gene tree, with nuclear data are recovered in their "correct" clade based on phenotype identification. The nuclear gene tree also recovers a well-supported group (*L. robermertensi* + (*L. saxatilis* + *L. ramirezae*)), while in the mt gene tree these species are recovered in different clades, with *L. saxatilis* within *L. gracilis*. The MDC tree shows high concordance with the Bayesian tree (results not shown).

3.2. Population genetic and demographic analyses

As part of an exploratory analysis we present results based on the three *L. gracilis* mitochondrial clades (mC1, mC2, mC3; Fig. 2) that summarize the main phylogeographic patterns. We implemented the Tajima's *D* and Fu's Fs tests for these three *L. gracilis*

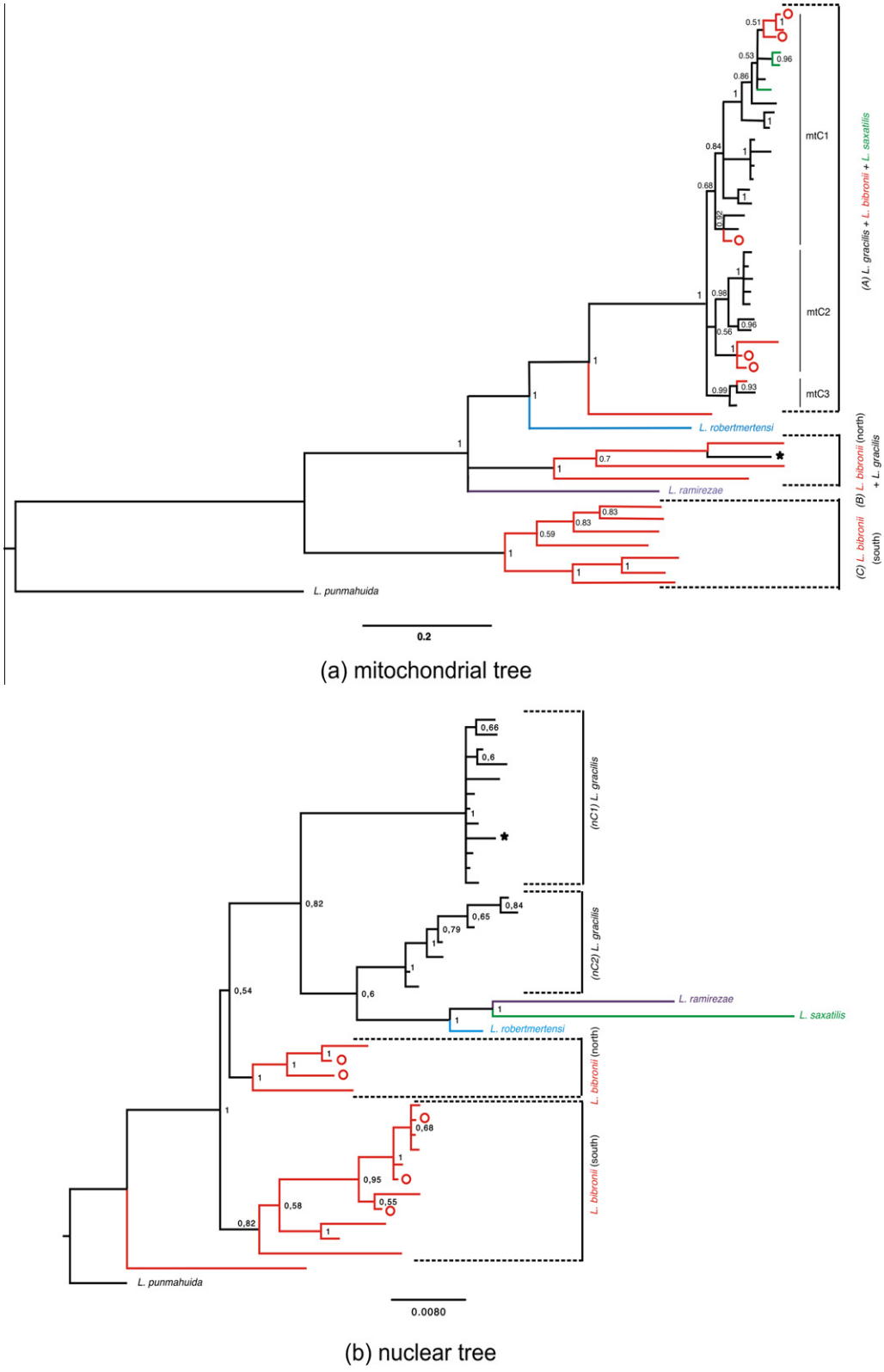

Fig. 2. Mitochondrial and nuclear trees. Bayesian trees for: (a) concatenated mitochondrial; and (b) concatenated nuclear sequences. Colored branches represent nominal species: black, *L. gracilis*, red, *L. bibronii*, green *L., saxatilis*, purple, *L. ramiraezae* and blue, *L. robertmertensi*. Red circles and black star correspond to *L. bibronii* and *L. gracilis* mitochondrial introgressed haplotypes (also depicted in Fig. 1). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

clades, and then for all samples; results are summarized in Table 2. Results for each clade separately showed non-significant results in both tests, in agreement with a neutral evolution hypothesis. However, for all *L. gracilis* samples we obtained significant results, suggesting a deviation from neutral equilibrium in the direction of more haplotypes and lower number of segregating sites than

Table 2
Summary statistics for the mitochondrial clades recovered in Fig. 2; where *n*: number of samples; *h*: number of haplotypes; *S*: number of segregating sites; $\theta \pm 1$ SD: gene diversity estimated (±standard deviation); $\pi \pm 1$ SD: average pairwise distance (±standard deviation); Prob. ($|D_t|$) > 0: Probability of $D_t \neq 0$; PCS.: Probability of $D_t \neq 0$ based on coalescent simulations (5000 replicates), R_2: R_2 de Ramos-Onsins & Rozas ($\pi L/2$ vs. $\eta 1$). All tests were calculated from the same concatenated mtDNA matrix.

Mitochondrial clade (mC)	*n*	*h*	*S*	$\theta \pm 1$ SD	$\pi \pm 1$ SD	Prob. ($\|D_t\|$) > 0	PCS	F_s test	R_2
mC1	11	9	37	0.00813 ± 0.00341	0.00714 ± 0.00052	>0.10	0.29780	>0.10	0.1155
mC2	7	6	11	0.00282 ± 0.00147	0.00245 ± 0.0007	>0.10	0.30108	>0.10	0.1207
mC3	2	2	5	0.00314 ± 0.00243	0.00314 ± 0.00157				0.5
All	22	19	108	0.04306 ± 0.01464	0.02278 ± 0.00799	<0.05*	0.01300	<0.05*	0.1296

Table 3
Two different gene flow tests made for *L. gracilis* populations recognized based on mitochondrial clades and nuclear clades taken from Fig. 2; and between *L. gracilis* and *L. bibronii* species. All tests were calculated from a concatenated mitochondrial matrix (cyt-b + 12S).

Population		Haplotype data (Nei, 1973)		Sequence data (Hudson et al. 1992)	
		γ_{st}	Nm	F_{st}	Nm
mC1	mC2	0.21945	1.78	0.33638	0.99
mC1	mC3	0.11072	4.02	0.17727	2.32
mC2	mC3	0.23007	1.67	0.17460	2.36
nC1	nC2	0.09856	4.57	0.09087	5.0
L. gracilis	*L. bibronii*	0.15922	2.64	0.22731	1.7

expected. This pattern represents a common result when a population experienced a recent range expansion.

Wright (1931) determined that Nm > 1 is sufficient to overcome the effects of genetic drift and that Nm > 4 indicates that there has been general mixing of the populations. Migration tests (Table 3) applied to different clades of *L. gracilis* revealed gene flow among them (Nm > 1), with a single exception for mC1 × mC2 (Nm = 0.99; sequence data, but this value is not likely significantly different from 1). In *L. gracilis*, the highest index of gene flow was detected for mC1 × mC3 (Nm = 4.02; haplotype data), while with sequence data the highest value was estimated for mC2 × mC3 (Nm = 2.36). However, this last case had the lowest value based on haplotype data (Nm = 1.67). From the nuclear gene topology, we obtained high values in both gene flow tests performed (γ_{st}, Nm = 4.57; F_{st}, Nm = 5.0), and we recovered gene flow signal between *L. gracilis* and *L. bibronii* in both migration tests (γ_{st}, Nm = 2.64; F_{st}, Nm = 1.7).

We used BEAST to explore the demographic history of *L. gracilis*, and recovered a signal of population expansion ~50 ka yr ago (Fig. 3). Our estimates suggest that ~100 ka yr ago the effective population size per generation length ($N_e * t$) was ≈1.25, and after 50 ka $N_e * t$ doubled, and today the population has increased about threefold ($N_e * t = 4$) compared to its size 100 ka ago. Before that time, the population size apparently was constant.

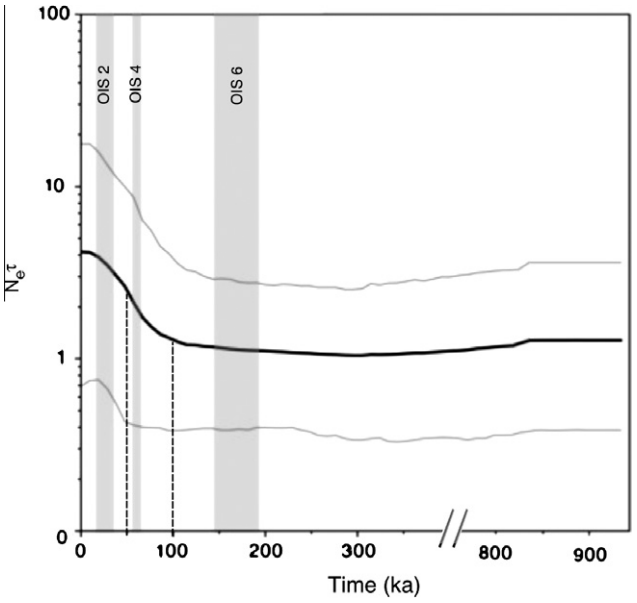

Fig. 3. Bayesian skyline plot. Bayesian skyline plot based on a mutation rate of 0.0223 per site per Ma. The *y* axis represents the product of effective population sizes and the generation length ($N_e * t_g$) on a log scale, and the *x* axis represents the time (ka). The bold black line is the mean estimate, and areas indicate 95% highest posterior density (HPD) regions. The most significant glaciations of the last 180 ka in the southern Andes are shaded in gray (OIS 2, 4 and 6), and dotted lines show inflection points in population growth between 100 ka and 50 ka yr ago.

3.3. Morphological analysis

3.3.1. Sexual dimorphism

We present means, standard errors and ranges of the variables in Table 4. We obtained significant differences for SLV [*p* value = 0.0093**]; thus, we used it as covariable in ANCOVA. Our tests show a pronounced sexual dimorphism in *L. gracilis*; where six of the other 9 variables are significantly different (*M* > *F* in all cases for HL [*p* values <0.0001***], HW [*p* value = 0.0032**], HH [*p* value = 0.0005***], FoL [*p* value = 0.0022**], TFL [$N_e * t$ value = 0.0438*], KKD [*p* value = 0.0061**]). However, we did not find any differences between sexes based in meristic characters in the bilateral *t* test.

3.3.2. Interspecific comparisons

We found significant differences for SLV [*p* value < 0.0001***] between *L. gracilis* and *L. bibronii*, and the ANCOVA revealed significant differences in six of seven morphometric variables tested (*L. gracilis* < *L. bibronii* in all cases: HL [*p* value = 0.0021**], HW [*p* value = 0.0011**], AGD [*p* value = 0.0021**], HaL [*p* value < 0.0001***], FoL [*p* value = 0.0053**], TFL [*p* values = 0.0141*]). Six of the meristic variables were significantly higher in *L. gracilis* than in *L. bibronii* (SAM, DSOT, VS, RESL, LESL, [*p* values < 0.0001***] and IL4P [*p* value = 0.0374*]); and for one where *L. gracilis* > *L. bibronii* (NSK [*p* value < 0.0001***]).

Results comparing the mtIH samples with averages for *L. gracilis* and *L. bibronii* individuals showed significant differences in several variables (Table 5). *Liolaemus gracilis* samples with mtIH (presumably introgressed with *L. bibronii* mtDNA) showed more similarity

Table 4
Summary measurements obtained from the standard morphometric characters (left) and the meristic characters (right) partitioned in *L. bibronii* mtIH, *L. gracilis* and *L. birbonii*. The first value (left) indicates the sample size and to the right we show the mean ± standard deviation; below the mean, the range is shown in brackets (min–max). nd: no data. The measurements are presented in mm.

Standard morphometric characters							Meristic characters						
Variable	*L. bibronii* mtIH		*L. gracilis*		*L. bibronii*		Variable	*L. bibronii* mtIH		*L. gracilis*		*L. bibronii*	
SVL	$n = 4$	50,60 ± 3,85 (46,10–54)	$n = 171$	44,62 ± 5,38 (29–58,6)	$n = 38$	50,88 ± 4,51 (40,1–60,6)	SAM	$n = 4$	47,75 ± 2,22 (45–50)	$n = 168$	39,15 ± 2,73 (32–47)	$n = 39$	50,62 ± 2,16 (47–56)
HL	$n = 4$	10,59 ± 0,70 (9,9–11,55)	$n = 171$	10,27 ± 1,07 (7,2–12,5)	$n = 38$	10,28 ± 0,89 (9–12,9)	DSOT	$n = 4$	61,50 ± 2,89 (58–65)	$n = 171$	51,35 ± 4,37 (32–60)	$n = 39$	61,74 ± 3,44 (55–70)
HW	$n = 4$	8,56 ± 0,68 (8–9,54)	$n = 168$	7,01 ± 0,88 (4,5–9,7)	$n = 38$	8,28 ± 0,78 (9–12,9)	VS	$n = 4$	81.50 ± 4,04 (78–85)	$n = 168$	69,57 ± 5,44 (55–83)	$n = 37$	85,51 ± 3,07 (77–94)
HH	$n = 3$	5,83 ± 0,21 (5,6–6,0)	$n = 170$	5,5 ± 0,77 (3,3–7,3)	$n = 38$	6,03 ± 0,64 (4,7–7,3)	RESL	$n = 3$	6 ± 0	$n = 171$	3,98 ± 0,23 (3–5)	$n = 38$	5,97 ± 0,37 (5–7)
AGD	$n = 4$	22,96 ± 2,54 (20,80–26,6)	$n = 171$	20,97 ± 2,9 (13,3–27,6)	$n = 39$	24,69 ± 2,76 (19–31,2)	LESL	$n = 3$	6 ± 0	$n = 171$	4 ± 0,24 (3–5)	$n = 38$	6,03 ± 0,37 (5–7)
HaL	$n = 4$	9,69 ± 0,49 (9,10–10,30)	$n = 169$	5,97 ± 0,81 (12,15–1,47)	$n = 39$	13,34 ± 1,06 (11,12–15,7)	REIL	$n = 3$	4,67 ± 0,58 (4–5)	$n = 171$	3,92 ± 0,33 (3–5)	$n = 38$	4,03 ± 0,68 (3–5)
FoL	$n = 1$	6,86	$n = 168$	12,15 ± 1,47 (7,2–19,3)	$n = 39$	14,15 ± 1,18 (11,3–16,6)	LEIL	$n = 3$	4,67 ± 0,58 (4–5)	$n = 171$	3,9 ± 0,32 (3–5)	$n = 38$	3,89 ± 0,65 (3–5)
TFL	nd		$n = 170$	8,6 ± 1,06 (5,1–10,5)	$n = 39$	9,96 ± 0,88 (7,5–11,7)	IL3H	$n = 4$	16,25 ± 1,26 (15–18)	$n = 170$	16,56 ± 1,62 (11–19)	$n = 38$	16,53 ± 1,54 (12–21)
KKD	nd		$n = 169$	18,15 ± 2,19 (10,7–22,1)	nd		IL4P	$n = 4$	21,75 ± 1,71 (20–24)	$n = 171$	22,09 ± 1,91 (17–27)	$n = 39$	22,77 ± 1,53 (20–26)
IND	nd		$n = 171$	1,92 ± 0,28 (1,2–1,3)	nd		NSK	$n = 3$	15,67 ± 0,58 (15–16)	$n = 171$	17,4 ± 1,79 (14–23)	$n = 39$	16,28 ± 0,83 (15–18)

Table 5
Statistical tests of comparisons for mitochondrial introgressed haplotype (mtIH) samples and *L. gracilis* and *L. bibronii*. Mophometric characters: (HL) head length (HW) head width, (HH) head height, (SVL) snout–vent length, (AGD) axilla-groin distance, (HAL) hand length, (FoL) foot length, (TFL) tibio-fibula length, (AL) arm length, (KKD) knee-knee distance, (IND) inter-nose distance. *Meristics characters*: (SAM) scales around midbody, (DSOT) dorsal scales between occiput and thigh, (VS) ventral scales, (Pores) precloacal pores, (IL3H) infradigital lamellae of 3th toe of the hand, (IL4P) infradigital lamellae of 4th toe of the foot, (NSK) number of scales with keels.

Variable	*L. gracilis* mtIH ($n = 1$)		*L. bibronii* mtIH ($n = 4$)		*L. gracilis* mtIH ($n = 1$) *vs. L. bibronii* mtIH ($n = 4$)
	L. gracilis	*L. bibronii*	*L. gracilis*	*L. bibronii*	
n	178	39	178	39	
SVL	<0.0001***	0.3616	0.0483*	0.9069	0.8486
HL	<0.0001***	<0.0001***	0.4594	0.9898	0.0506
HW	0.8003	<0.0001***	0.0108*	0.4992	0.0198*
HH	<0.0001***	0.2191	0.4697	0.6024	0.6349
AGD	<0.0001***	<0.0001***	0.1835	0.2335	0.6418
HaL	<0.0001***	<0.0001***	nd	nd	nd
FoL	0.1647	<0.0001***	nd	nd	nd
TFL	<0.0001***	<0.0001***	nd	nd	nd
KKD	<0.0001***	nd	nd	nd	nd
IND	<0.0001***	nd	nd	nd	nd
SAM	0.3737	<0.0001***	<0.0001***	0.0156*	0.0042**
DSOT	<0.0001***	<0.0001***	<0.0001***	0.8922	0.0138*
VS	0.2619	<0.0001***	<0.0001***	0.0204*	0.0085**
IL3H	<0.0001***	0.0417*	0.6626	0.7310	0.7177
IL4P	<0.0001***	<0.0001***	0.7007	0.2156	0.1328
NSK	<0.0001***	<0.0001***	0.0989	0.2148	0.0198*
Proportion of similitude	4/16 = 0.3	2/14 = 0.15	6/11 = 0.54	9/11 = 0.82	6/11 = 0.54

with *L. bibronii* mtIH than to the other groups (equal means for 6/11 variables: SVL, HL, HH, AGD, IL3H and IL4P). However, *L. bibronii* mtIH (presumably introgressed with *L. gracilis* mtDNA) showed significant differences from all other groups, but the smallest number of differences were with *L. bibronii* (equal means for 9/11 variables SVL, HL, HW, HH, AGD, DSOT, IL3H, IL4P and NSK).

The Principal Component Analyses (PCA) showed that the first three principal component variables (PCv) explained 69.00% of the observed variance (data not shown). These PCv were used to perform a discriminant analysis (Fig. 4) in which we *a priori* defined three classes: *L. gracilis*, *L. bibronii* and *L. bibronii* mtIH (homogeneity of covariance matrix test: *p*-value >0.99). Canonical axis 1 explains 99.98% of the variance, and cross-validation obtained from the discriminant function (Table 6) shows that two of 172 samples of *L. gracilis* and five of 35 samples of *L. bibronii* should be clustered with *L. bibronii* with mtIH. Fig. 4 shows that the *L. bibronii* mtIH individuals are positioned more proximal to *L. bibronii*, but they do not overlap along either of the canonical axes.

4. Discussion

4.1. Liolaemus gracilis genetic structure

Our mtDNA tree recovered three clades within *L. gracilis*, (mC1, mC2 and mC3, Fig. 2a), two with weak statistical support (mC1 pp = 0.68 and mC2 pp = 0.56), and these geographically overlap each other. Our nuclear data recovered two well-supported clades in *L. gracilis* (nC1 and nC2), also with considerable distributional

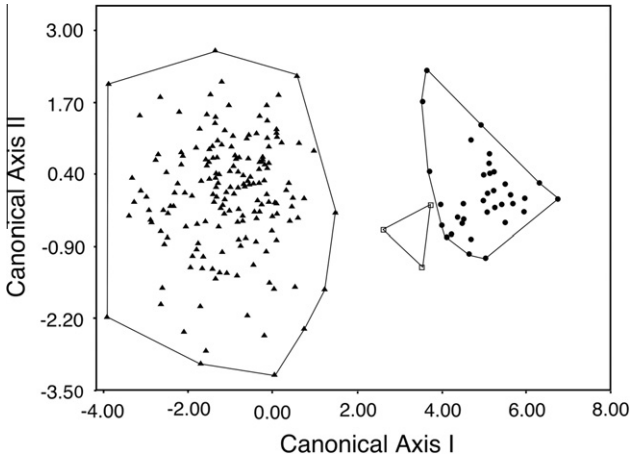

Fig. 4. Discriminant analysis. Discriminant analysis obtained from the first three principal components based on morphological data (including morphometric and meristic data). Samples are identified as follows: black triangles = *L. gracilis*, black circles = *L. bibronii*, and open squares = *L. bibronii* mtIH. Autovalues and centroids are presented in Tables B.1 and B.2 of Appendix B.

Table 6
Cross-validation summary: in rows the groups assigned *a priori* from the observations, and in columns by the discriminant function (DF); the error (%) represents estimates the mis-classification of the *a priori* groups.

Group	*L. gracilis*	*L. bibronii* (mtIH)	*L. bibronii*	Total (*a priori*)	Error (%)
L. gracilis	170	2	0	172	1.16
L. bibronii mtIH	0	3	0	3	0.00
L. bibronii	0	5	30	35	14.29
Total (DF)	170	10	30	210	3.33

overlap. We did not find clear correspondence between these nuclear and mitochondrial clades.

Neutrality tests (Tajima's *D* and Fu's *F*) for mitochondrial clades within *L. gracilis* were not significant, but combined together both tests were significant. These tests compare the number of polymorphic sites and the number of different haplotypes, showing in this case the existence of more haplotypes than expected, which suggests a recent range expansion for the species (Fu, 1997). This inferred range expansion is also supported by Bayesian Skyline Plot (BSP) analysis (Fig. 3).

During the glacial cycles of the Quaternary, ice expanded east and west from the Andean divide, with lobes first advancing along existing valleys (Clapperton, 1993). The very large expansion during the most extensive glaciation of the past half million years, known as the Oxygen Isotope Stage 6 (OIS 6) glaciation, occurred 180–140 ka years ago (Rabassa and Clapperton, 1990; Singer et al., 2004). Our results suggest that *L. gracilis* population size remained relatively constant during the OIS 6. However, after it concluded, this species experienced a population expansion beginning about 100 ka ago, and continuing until the Holocene. More recent glacial advances, OIS 4 (70–60 ka years ago and OIS 2 (35–15 ka - years ago) had no effect on the *L. gracilis* population size.

These results are similar to those found by Ruzzante et al. (2008) for the fish *Percichthys trucha*, distributed in Patagonian river basins east and west of the Andes. Similar analyses based on mtDNA data showed large population growth at the end of OIS 6, followed by much less effect of the OIS 4 and OIS 2 events. Similar patterns found in different unrelated co-distributed groups are suggestive of shared historical responses to some drivers of climate changes in Patagonia.

If changing environments favored population expansion of *L. gracilis* at the end of OIS 6, this may have promoted secondary contact and gene flow between previously isolated populations, thus over-riding earlier phylogeographic signal of isolation and contributing to the low-support for some clades (Fig. 2). Further, our gene flow tests results revealed Nm > 1 for combinations of *L. gracilis* samples. When mutation rates are high, the sequence-based statistics (F_{st}) are more powerful gene flow tests (Hudson, 1992) and should be given preference, assuming that the range expansion hypothesis is the process underlying a higher number of haplotypes. In this case the haplotype frequency tests also returned significant results for gene flow.

Based on these results, we consider *L. gracilis* a widely distributed genetically cohesive species throughout its distribution. Other phylogeographic studies of widely distributed Patagonian *Liolaemus* species (Avila et al., 2006; Morando et al., 2003, 2004, 2007), have revealed a general pattern of more genetically structured clades in northern regions that were ice-free during glaciation cycles, and larger ranges characterized by lower genetic diversity in southern regions. The main distribution area of *L. gracilis* is further north than most of the taxa considered in these earlier studies, and its genetic structure is different from those previously reported, possibly as a result of different levels of influence from glacial cycles over this different and more northern geographic area.

4.2. Interspecific analysis

We have recovered *L. gracilis* and *L. bibronii* as reciprocally paraphyletic in mitochondrial and nuclear gene trees (Fig. 2). In the mitochondrial gene tree (Fig. 2a), three *L. saxatilis* terminals are nested within *L. gracilis*, while eight *L. bibronii* terminals are nested within *L. gracilis* (clade A). We also recovered both *L. robertmertensi* and *L. ramirezae* in the "wrong" clades in the mitochondrial gene tree. In all of these instances, the nuclear genes do not recover any of these terminals in heterospecific clades (Fig. 2b), although *L. bibronii* is not recovered as monophyletic.

Paraphyly in the mtDNA gene trees could be explained by at least three hypotheses, including: (1) poor taxonomic resolution of species boundaries (Funk and Omland, 2003); (2) incomplete lineage sorting (Harrison, 1991; Knowles, 2001; Maddison, 1997; Sullivan et al., 2002); or (3) introgression of the mtDNA locus across species (or strongly delineated intraspecific haploclades) via historical or ongoing hybridization and gene flow (Ferris et al., 1983; Rieseberg and Wendel, 1993). All of these processes have been commonly documented in mtDNA studies in animals (Funk and Omland, 2003).

The lineage sorting process eliminates ancestral polymorphisms in time. Accordingly, sister taxa eventually would be reciprocally monophyletic (four times faster for mtDNA than nDNA). In those cases, a single gene tree could differ from the species tree, due to stochastic sorting processes and the mutation rate (Degnan and Rosenberg, 2009; Knowles, 2009; Moritz et al., 1992; Redenbach and Taylor, 2002; Rosenberg, 2002).

Hybridization and introgression between two species or distinct intraspecific clades, is frequently characterized by extensive and often asymmetrical mitochondrial introgression, perhaps because persistence of the mtDNA locus on the "wrong" background is less constrained by linkage to selected loci than are the alleles of the nuclear genome (Funk and Omland, 2003). When the divergence among closely related species is low, introgression may be difficult to discriminate from ancestral polymorphism (Avise and Ball, 1990), but these processes have different geographically spatial expectations, and a phylogenetic analysis can be used to distinguish between them (Goodman et al., 1999). If for example recent hybridization has occurred, we would expect that the common alleles would be detected in contacts zones, whereas ancestral

polymorphisms would be distributed in equal frequency throughout the distribution range (Barbujani et al., 1994).

For the species included in this study, we do not have the sample sizes needed to evaluate the cases of paraphyly in *L. robertmertensi*, *L. ramirezae* and *L. saxatilis*, but because our samples represent disjunct areas, incomplete lineage sorting may be a better provisional explanation for these patterns. However, in the case of the eight samples of *L. bibronii* nested within the *L. gracilis* clade, and one sample of *L. gracilis* nested within the *L. bibronii* northern clade, we have additional relevant data. Our nuclear sequences, for example, recover these samples in their "correct" place; i.e., in agreement with their general morphological characteristics. All these individuals were collected from localities in the region of sympatric overlap (Fig. 1). The gene flow tests reflected high values (Nm = 1.7), in support of a "recent hybridization" hypothesis, over incomplete lineage sorting between *L. gracilis* and *L. bibronii*.

4.3. Sexual dimorphism and sexual selection

Our morphological comparison between *L. gracilis* and *L. bibronii* revealed several significantly different characters, including SLV and other six of seven traditional morphometric and seven of 10 of meristic variables studied. All cases of different means of morphometric variables are significantly smaller for *L. gracilis* relative to *L. bibronii*. The single sample of *L gracilis* mtIH is more similar to *L. bibronii* mtIH than to the other tested groups (Table 5), but we found differences in almost half of the morphometric variables between them. The *L. bibronii* mtIH samples have a clearer signal, and are more similar to "pure" *L. bibronii* relative to other samples tested (Table 5). This is easily visualized graphically (Fig. 4); the *L. bibronii* mtIH samples group proximal to the reference *L. bibronii* cloud of points in bivariate space, relative to *L. gracilis*; they do not overlap along canonical axes. The cross-validation summary also shows the existence of three groups (Table 6), but with errors in the a *priori* classification, recognizing two *L. gracilis* and five *L. bibronii* samples that should be clustered with *L. bibronii* mtIH. This reveals that there are phenotypic similarities between *L. bibronii* mtIH and both parentals.

The morphological phenotypes of the mtIH samples reflect a common signature of hybrid offspring. The morphometric variation in this species is characteristic of that predicted by transgressive segregation (Bell and Travis, 2005; Chiba, 2005; Renaud et al., 2009; Rieseberg et al., 1999; Seehausen, 2004), in which hybrid offspring display a range of phenotypic variability outside that of the parental taxa (Rieseberg et al., 1999). Here we recognized a third phenotype for these purported hybrid samples (Fig. 4), which is consistent with expectations of transgressive segregation.

On the other hand, we found marked sexual dimorphism in *L. gracilis*, with males larger than females (Section 3.3.1). Several hypotheses have been proposed to explain sexual dimorphism in animals (Fairbairn, 1997; Hedrick and Temeles, 1989); among the most relevant for lizards are those related to natural (Fairbairn, 1997) and sexual selection (Andersson, 1994). There is a close relationship between body size and competitive efficiency (Andersson, 1994), with larger males having larger and/or better territories. Sexual selection often favors males with larger body and mandible sizes, which are characteristics linked to success in fighting (Carothers, 1984; Carpenter and Ferguson, 1977). The sexual selection hypothesis predicts that females should prefer larger males (Heisig, 1993; Manzur and Fuentes, 1979; Vitt and Cooper, 1985), and given the pronounced sexual dimorphism documented here in *L. gracilis*, we predict that mate choice experiments would show that female *L. gracilis* would select larger males.

The maternal inheritance of mtDNA gives us a clue about the directionality of introgression. In most of our introgressed individuals (eight individuals of *L. bibronii* mtIH), most probably *L. gracilis* females preferentially mated with *L. bibronii* males (except one case), this reveals an asymmetric mating pattern. If sexual selection is responsible for the observed sexual dimorphism in *L. gracilis*, then females prefer larger males. Interestingly, *L. bibronii* has higher means in all morphometric variables that are significantly different. Thus we can hypothesize that where they live in sympatry, *L. gracilis* females could be selecting *L. bibronii* males because of their larger size. Further, *L. bibronii* males may compete with *L. gracilis* males, and because of their larger sizes, the *L. bibronii* males may out-perform heterospecifics when the two are in sympatry, but specific experimental designs are needed to further test these hypotheses.

There is no consensus among evolutionary biologists regarding the definition of "species". Traditionally the concept of a species is envisioned as a "closed system" with discrete beginning and end (Rieppel, 2009). In this case, it is clear that a closed system concept is not a good one for *L. gracilis*, and evidence of mtDNA introgression in other species of *Liolaemus* (Avila et al., 2006; Morando et al., 2004) suggests that this may be a widespread phenomenon. While some previous studies detected mtDNA introgression between species as consequence of recent or ancient hybridization in other Iguanian lizard genera, including *Sceloporus* (Marshall and Sites, 2001; Leaché and Cole, 2007; Leaché, 2009), *Crotaphytus* (McGuire et al., 2007), and *Phrynosoma* (Leaché and McGuire, 2006), this study is the first in *Liolaemus* to integrate nuclear sequence and morphological data into a previously hypothesized case of mtDNA introgression, and now provides a much clearer picture on the direction and extent of introgression between closely related species in a region of sympatry. Given the high species diversity of this genus, the still very limited taxonomic knowledge, and the importance of hybridization in evolution (Arnold, 1997), future studies in other sympatry areas should consider the importance of hybridization process as a relevant diversification mechanism in *Liolaemus*.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2011.07.006.

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