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PHYLOGENY AND PHYLOGEOGRAPHY OF THE *LIOLAEMUS DARWINII* COMPLEX (SQUAMATA: LIOLAEMIDAE): EVIDENCE FOR INTROGRESSION AND INCOMPLETE LINEAGE SORTING

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Abstract.—Although mitochondrial DNA markers have several properties that make them suitable for phylogeographic studies, they are not free of difficulties. Phylogeographic inferences within and between closely related species can be mislead by introgression and retention of ancestral polymorphism. Here we combine different phylogenetic, phylogeographic, and population genetic methods to extract the maximum information from the *Liolaemus darwinii* complex. We estimate the phylogeographic structure of *L. darwinii* across most of its distributional range, and we then estimate relationships between *L. darwinii* and the syntopic species *L. laurenti* and *L. grosseorum*. Our results suggest that range expansion of these lineages brought them into secondary contact in areas where they are presently in syntopy. Here we present the first evidence for introgression in lizards from temperate South America (of *L. darwinii* mitochondrial DNA into *L. laurenti* and *L. grosseorum*), and for incomplete lineage sorting (between *L. darwinii* and *L. laurenti*). We show that a combination of methods can provide additional support for inferences derived from any single method and thus provide more robust interpretations and narrow the range of plausible hypotheses about mechanisms and processes of divergence. Additional studies are needed in this group of lizards and in other codistributed groups to determine if Pleistocene climatic changes could be a general factor influencing the evolutionary history of a regional biota.

Key words.—Argentina, cytochrome *b*, introgression, lineage sorting, *Liolaemus*, phylogeography.

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For the past 20 years, the use of animal mitochondrial DNA (mtDNA) as a marker for phylogeographic studies has provided insights into population histories within the context of evolutionary and biogeographic models (Avise 2000). The traditional approach uses a haplotype tree overlain onto a current geographic distribution of populations, which can be particularly useful for lineages that have been separated for long periods of time. For more recent divergences, statistical parsimony (Templeton et al. 1992) in combination with nested clade analysis (NCA; Templeton et al. 1995) has been used to examine historical and current processes that shape the phylogeographic structure of species. The NCA approach has been criticized from a number of perspectives (Knowles and Maddison 2002; Petit and Grivet 2002), but some authors have offered suggestions for cross-validation of some NCA inferences in a context that should reduce uncertainty and false positives (Masta et al. 2003). It is becoming clear that maximizing inferences from any phylogeographic study requires a combination of approaches that examine haplotype relatedness and demographic history (Bernatchez 2001; Althoff and Pellmyr 2002; Pfenninger and Posada 2002), and these questions are being addressed with increasing statistical rigor (Knowles and Maddison 2002; Templeton 2002).

Although mtDNA markers have several properties that make them suitable for these studies (Wiens and Penkrot 2002; Morando et al. 2003), they are not free of difficulties. A gene tree will not necessarily reflect a species tree (Pamilo and Nei 1988; Nichols 2001), and phylogeographic inferences within and between closely related species can be influenced (misled) by introgression and retention of ancestral polymorphism (Harrison 1991; Redenbach and Taylor 2002; Funk and Omland 2003). Recent studies have documented secondary contact and subsequent hybridization and introgression in previously isolated taxa (Soltis et al. 1997; Pfenninger and Posada 2002), and others have shown that incomplete lineage sorting can produce gene trees that are incongruent with species trees (Moran and Kornfield 1993; Parker and Kornfield 1997). Despite these limitations, a phylogeographic perspective can yield insights into the historical and geographical contexts of these processes (Butlin 1998; Rieseberg 1998; Redenbach and Taylor 2002).

The *Liolaemus darwinii* complex (Etheridge 1993) belongs to the second most species-rich genus of New World lizards, with more than 160 described species. The genus is distributed over a wide geographic region and occupies a large range of latitudinal (14°30′–52°30′S), altitudinal (0–4500 m), and climatic regimes, from the extremely arid Atacama desert to the temperate *Nothofagus* rainforest (Morando et al. 2003 and references therein). This complex includes several closely related species with contiguous, sympatric, or syntopic distributions along western arid lands of Argentina (Etheridge 1993; L. J. Avila and M. Morando, pers. obs.). Earlier morphological studies show that the geographic variation in color pattern of the species *L. darwinii* (Cei 1986, 1993) is associated with specific geographic regions, but in fact several distinctive species were subsumed under the name *L. darwinii*

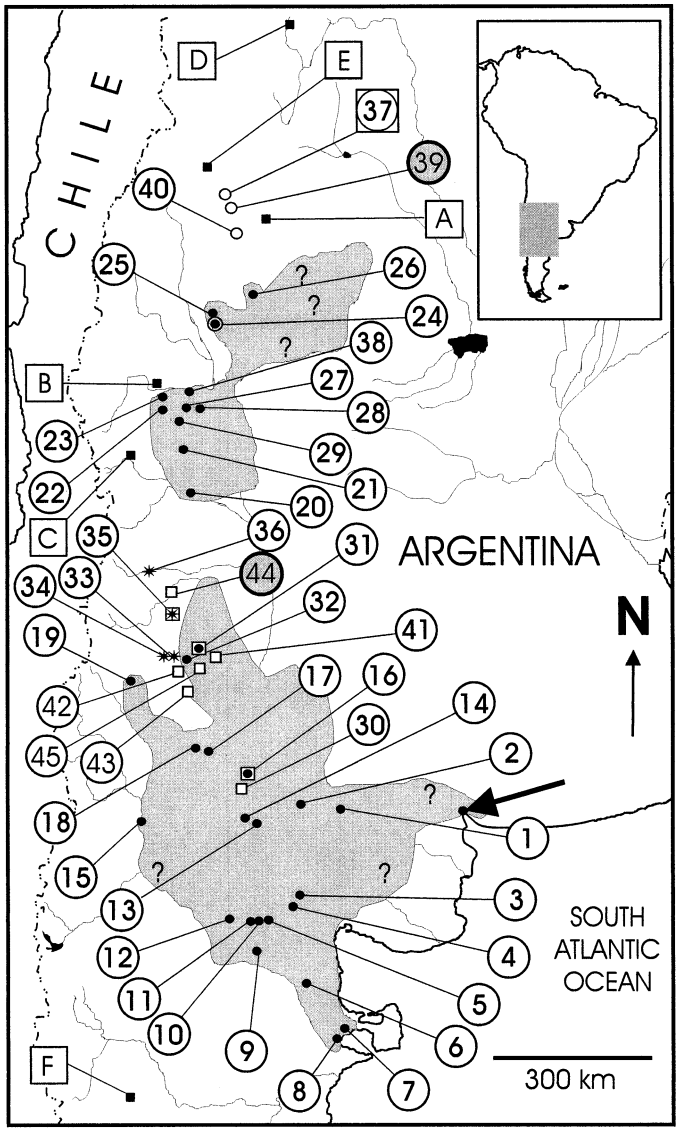

FIG. 1. Geographic distribution of the taxa included in this study. Shaded areas correspond to the approximate distribution of *Liolaemus darwinii* (sensu lato) according to literature and museum records (question marks denote estimate distribution based on historical records). Black dots, sampled localities for *L. darwinii* (sensu lato); open squares: sampled localities for *L. grosseorum*; stars, sampled localities for *Liolaemus* sp. nov.; open circles, sampled localities for *L. laurenti*; solid squares, localities for nonfocal and outgroup species; type localities: black arrow, *L. darwinii*; localities 39 (shaded circle), *L. laurenti*; localities 44 (shaded circle), *L. grosseorum*; upper-case letters in squares represent the nonfocal species. Locality numbers and letters correspond to those in Table 1.

(Etheridge 1992, 1993). More recently, additional species previously confused with *L. darwinii* have been described or discovered (Lobo and Kretschmar 1996; Cei and Scolaro 1999; Etheridge 2001; L. J. Avila and M. Morando, unpubl. data).

Current knowledge suggests that the nominal species *L. darwinii* consists of two geographically cohesive groups (Fig. 1): a northern group distributed in northern Mendoza, eastern San Juan, western San Luis, south-central La Rioja, and small areas of Catamarca, Córdoba, and Santiago del Estero provinces; and a southern group, found in southern Mendoza, western La Pampa, eastern Neuquén, northeastern Río Negro, northeastern Chubut, and southern Buenos Aires provinces. Both groups are apparently separated by a gap of almost 200 km, between 33°30′S and 34°30′S, and morphological differences are apparent between these two geographic groups (Etheridge 2001).

Two other species from this complex, *L. laurenti* and *L. grosseorum*, are the only species known to be syntopic with *L. darwinii* in the northern and southern part of its distribution, respectively (Etheridge 1993; Avila et al. 2002). *Liolaemus grosseorum* was recently described by Etheridge (2001), and is clearly distinguished from *L. darwinii* by the absence of large, black, pre- and postscapular spots in adult males, smaller size, higher number of precloacal pores, and different dorsal and ventral pattern of coloration (Etheridge 2001; L. J. Avila, unpubl. data). It differs from *L. laurenti*, also described by Etheridge (1992), by the presence of prominent dorsolateral stripes (distinctly lighter than the background color), a significantly shorter tail, and the presence of a ventral bifurcation of the dark pigment of the antehumeral fold (Etheridge 2001). Dorsal color pattern is similar between *L. laurenti* and *L. grosseorum*, but the pattern is much bolder and sexual differences more marked in the latter. Unlike *L. darwinii*, *L. laurenti* exhibits no strong sexual dichromatism in the adult color pattern, and the number of midbody scales is fewer (Etheridge 1992). All of these species are known from moderate to large numbers of museum specimens ($n > 80$ in *L. laurenti* and $n > 40$ in *L. grosseorum*), and their taxonomic status has not been questioned; additional characters and comments about differences between the species are given in Etheridge (1993, 2001).

A previous phylogeographic study in the *Liolaemus elongatus-kriegi* complex revealed deep structure and cryptic species in widely distributed taxa (Morando et al. 2003), and these results motivated the present study that has two objectives. First, we estimate the phylogeographic structure of *L. darwinii* across most of its distributional range, and second, we estimate relationships between *L. darwinii* and the syntopic species *L. laurenti* and *L. grosseorum*. We are particularly interested in using a combination of methods to tease apart alternative explanations of observed phylogeographic patterns (Pfenninger and Posada 2002; Masta et al. 2003) as one means of addressing the criticisms that the NCA cannot distinguish statistically among alternative interpretations (Knowles and Maddison 2002).

MATERIALS AND METHODS

Taxon Sampling

Previous experience (Morando et al. 2003) suggested that the mtDNA cytochrome *b* (cyt *b*) gene was sufficiently variable for phylogeographic studies in *Liolaemus*. Sequence data from this gene were collected from a total of 126 lizards, of which 119 samples from 45 localities (Table 1) represented populations under the names *L. darwinii* (Bell 1843), *L. laurenti* Etheridge 1992, *L. grosseorum* Etheridge 2001, and an undescribed species suspected to be related to this complex (*Liolaemus* sp. nov.); these were the focal species (*sensu* Wiens and Penkrot 2002) of this study. *Liolaemus abaucan*

TABLE 1. Number of individuals of all ingroup and outgroup taxa, by locality, used in this study; locality numbers (in parentheses) match those in Figure 1 and Appendix 1 (which provides museum voucher numbers for all specimens). Numbers under the cytochrome *b* column give the number of lizards sequenced from each locality.

Province Department	Locality	Cytochrome *b*	Coordinates
Focal species			
Liolaemus darwinii			
La Pampa			
Lihué Calel	(1) Ruta Provincial 34, 0.5 km W junction Ruta Provincial 13 to Lihué Calel	3	38°41′S 65°20′W
Curacó	(2) Ruta Provincial 34, 0.5 km W Estancia San Eduardo	2	38°43′S 65°59′W
Curacó	(16) Ruta Provincial 23, 1 km junction road to 25 de Mayo	3	37°52′S 67°06′W
Chical Có	(31) Ruta Provincial 10, 4.6 km W Agua Escondida	1	36°09′S 68°14′W
Río Negro			
Valcheta	(3) Ruta Provincial 4, 84 km S junction Ruta Nacional 250, W edge Gran Bajo del Gualicho	3	40°01′S 66°00′W
Valcheta	(4) Ruta Provincial 4, N edge Laguna del Indio Muerto	3	40°24′S 66°02′W
Valcheta	(9) Ruta Provincial 60, 1 km N Chipauquil	3	40°56′S 66°38′W
Valcheta	(5) Ruta Nacional 23, Estación Nahuel Niyeu	1	40°30′S 66°32′W
Valcheta	(10) Ruta Nacional 23, 14 km W Nahuel Niyeu	3	40°29′S 66°43′W
Valcheta	(11) Ruta Nacional 23, Arroyo Yamihué, 23 km W Nahuel Niyeu	3	40°29′S 66°49′W
Valcheta	(6) Ruta Provincial 5 and Arroyo Verde	2	41°45′S 66°30′W
9 de Julio	(12) Ruta Nacional 23, 9 km E Sierra Colorada	3	40°33′S 67°39′W
General Roca	(17) Ruta Nacional 151, km 144, 5 km N Catriel	2	37°47′S, 67°44′W
General Roca	(18) Peñas Blancas	3	37°42′S, 67°52′W
General Roca	(13) Chichinales	2	39°06′S, 66°56′W
General Roca	(14) 18 km NE Villa Regina	5	39°02′S, 66°56′W
Neuquén			
Zapala	(15) 6 km W La Amarga	1	39°04′S 69°37′W
Mendoza			
Santa Rosa	(20) Ruta Provincial 153, 2 km S Las Catitas	1	33°19′S 68°04′W
Lavalle	(21) Ruta Provincial 142, 18.2 km N Costa de Araujo	3	32°36′S 68°20′W
Malargüe	(19) Ruta Nacional 40, Los Frisos, 5 km N El Zampal	3	36°28′S 69°38′W
Malargüe	(32) 5 km NE La Salinilla	2	36°13′S 68°31′W
Chubut			
Biedma	(7) Ruta Provincial 42, 11 km NE Puerto Madryn	2	42°39′S 64°59′W
Biedma	(8) Puerto Madryn	3	42°47′S 64°58′W
La Rioja			
Independencia	(25) Ruta Provincial 26, km 92, S edge Parque Nacional Talampaya	2	30°11′S 67°41′W
Chilecito	(26) Catinzaco	3	29°40′S 67°22′W
San Juan			
Caucete	(23) Medanos Grandes, near Caucete	1	31°40′S 68°16′W
Caucete	(27) Ruta Nacional 141, km 147, 7 km W Bermejo	2	31°36′S 67°42′W
Caucete	(28) Ruta Nacional 141, km 119, 21 km E Bermejo	1	31°35′S 67°41′W
Caucete	(22) Ruta Nacional 141, 15 km E Caucete	2	31°42′S 67°48′W
Caucete	(38) W slope Sierra de Pie de Palo	1	31°33′S 68°00′W
Valle Fértil	(24) Ruta Provincial 510, km 88, 2 km E Baldecitos	3	30°12′S 67°40′W
25 de Mayo	(29) Ruta Nacional 20, 2.8 km W Encón	2	32°11′S 67°49′W
L. grosseorum			
Mendoza			
Malargüe	(42) Ruta Provincial 180, 28.1 km N south entrance La Matancilla, near Puesto Loma Negra	4	36°37′S 68°36′W
Malargüe	(45) 4 km E Agua del Toro	2	36°38′S 68°25′W
San Rafael	(35) Ruta Provincial 180, 30 km S El Nihuil	4	35°17′S 68°41′W
San Rafael	(44) El Nihuil	2	35°03′S 68°39′W
La Pampa			
Chical Có	(41) Ruta Provincial 10, 5.1 km E La Humada	2	36°20′S 67°57′W
Chical Có	(43) Camino a Chos Malal, 54 km W junction Ruta Nacional 151	8	36°42′S 67°57′W
Chical Có	(31) Ruta Provincial 10, 4.6 km W Agua Escondida	2	36°09′S 68°14′W
Río Negro			
General Roca	(30) Ruta Provincial 6, between General Roca and Casa de Piedra (La Pampa)	1	38°32′S, 67°34′W
Curacó	(16) Ruta Provincial 23, 1 km junction road to 25 de Mayo	2	37°52′S 67°06′W

TABLE 1. Continued.

Province Department	Locality	Cytochrome *b*	Coordinates
L. laurenti			
La Rioja			
Famatina	(40) Road to Antinaco, 3.8 km E Ruta Nacional 40	3	28°50′S 67°24′W
Famatina	(39) Ruta Nacional 40, Km 657, 9 km E Pituil	6	28°32′S 67°22′W
San Juan			
Valle Fértil	(24) Ruta Provincial 510, km 88, 2 km E Baldecitos	4	30°12′S 67°40′W
Catamarca			
Tinogasta	(37) Ruta Nacional 60, junction Río La Puerta, km 1298	1	28°14′S 67°27′W
Liolaemus sp. nov.			
Mendoza			
Malargüe	(33) Ruta Provincial 186, 10 km W Mina Ethel	1	35°59′S 68°55′W
Malargüe	(34) Ruta Provincial 186, 20 km W Mina Ethel	1	35°58′S 69°01′W
San Rafael	(35) Ruta Provincial 180, 30 km S El Nihuil	1	35°17′S 68°41′W
San Rafael	(36) Ruta Nacional 40, 4 km N Dique Agua del Toro	1	34°31′S 69°01′W
Nonfocal species			
L. chacoensis (A)	La Rioja, Capital Dep., Ruta Provincial 9, 37.3 km E Anillaco, Sierra de Mazan	1	28°52′S 66°38′W
L. olongasta (B)	San Juan, Ullum Dep., Matagusanos, on Ruta Nacional 40	1	31°14′S 68°38′W
L. uspallatensis (C)	Mendoza, Las Heras Dep., Ruta Nacional 7, 4 km W Uspallata	1	32°36′S 69°24′W
L. cf. *ornatus* (D)	Salta, La Poma Dep., Ruta Nacional 40, 2 km N La Poma	1	24°41′S 66°11′W
L. abaucan (E)	Catamarca, Tinogasta Dep., Ruta Provincial 36, 16 km S Palo Blanco	1	27°26′S 67°40′W
L. koslowskyi (39)	Catamarca, Tinogasta Dep., Ruta Nacional 40 y Río La Puerta, km 1298	1	28°14′S 67°27′W
Outgroup			
L. boulengeri (F)	Chubut, Río Senguer Dep, Ruta Provincial 20, 23 km W Los Manantiales	1	45°27′S 69°43′W

Etheridge 1993, *L. chacoensis* Shreve 1948, *L. koslowskyi* Etheridge 1993, *L. cf. ornatus* Koslowsky 1898, *L. olongasta* Etheridge 1993, and *L. uspallatensis* Macola and Castro 1982, also included in the *L. darwinii* complex (Etheridge 1993), were used as nonfocal species ($n = 1$ each, Table 1). *Liolaemus boulengeri*, from a related complex was used to root the trees, thus allowing the position of the nonfocal species to remain unconstrained with respect to the focal species.

Table 1 summarizes the number of individuals sequenced per locality and distributional information for all taxa used in this study. Material was sequenced from a sample of one to three individuals for most of the localities (Table 1, Fig. 1), and voucher specimens are deposited in the LJAMM herpetological collection (now housed in the Centro Nacional Patagónico [CENPAT], Puerto Madryn, Argentina), Fundación Miguel Lillo (FML, San Miguel de Tucumán, Argentina), Museo de Ciencias Naturales La Plata (MLPS, La Plata, Argentina) and M. L. Bean Museum (Brigham Young University [BYU], Provo, UT). Museum voucher numbers are listed by locality in Appendix 1, and museum acronyms follow Leviton et al. (1985).

Laboratory Procedures

Total genomic DNA was extracted from liver/muscle tissues preserved in 96% ethanol, following the protocol developed by Fetzner (1999). Three microliters of extraction product were electrophoresed on 1% agarose gel to estimate the quality and amount of genomic DNA, and sample dilutions were performed where necessary prior to polymerase chain reaction (PCR) amplification. The cyt *b* gene region was amplified via PCR following Morando et al. (2003). A 713-bp fragment was amplified using the light-strand primers GluDGL (Palumbi 1996) and cyt *b* 1 (Kocher et al. 1989) and the heavy-strand primer Cyt *b* 3 (Palumbi 1996). Cyt *b* 2 (Palumbi 1996) and F1 (A. Whiting, unpublished, available upon request from the authors) were used as internal sequencing primers. Double-stranded PCR amplified products were checked by electrophoresis on a 1% agarose gel (the size of the target region estimated using a molecular weight marker), purified using a GeneClean III kit (BIO101, Inc, Vista, CA), and directly sequenced using the Perkin Elmer ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction (PE Applied Biosystems, Foster City, CA). Excess of Dye Terminator was removed with CentriSep spin columns (Princeton Separations, Inc., Adelphia, NJ), and sequences were fractionated by polyacrylamide gel electrophoresis on a ABI PRISM 377 automated DNA sequencer (PE Applied Biosystems) at the DNA Sequencing Center at BYU. Sequences were deposited in GenBank under accession numbers AY389169–AY389294.

Sequence Alignments

Sequences were edited and aligned using the program Sequencher 3.1.1 (Gene Codes Corp., Inc., Ann Arbor, MI), and translated into amino acids for confirmation of alignment. No indels were present, and missing data were coded with a question mark.

Phylogenetic Analyses

Only nonredundant haplotypes (selected with the program Collapse ver. 1.1, available from http://bioag.byu.edu/zoology/crandall_lab/programs.htm), were used for the tree based phylogenetic methods. Maximum parsimony (MP) and maximum likelihood (ML) criteria were implemented using PAUP* 4b5 (Swofford 2002). For MP analyses, all characters were equally weighted, and two searches were performed with 1000 replicates and maxtrees set to 100, followed by tree-bisection reconnection (TBR) branch swapping. The same score was obtained for all saved trees in both searches. Another search was then performed with 1000 replicates (maxtrees = 5000) but saving up to five trees with greater scores than the tree obtained in the first round search, resulting in 2775 equally parsimonious trees. Another round of branch swapping was then performed on the 2775 saved trees, but no shorter trees were found. For ML analyses, the model of evolution GTR + Γ (Yang 1994), selected using ModelTest (ver. 3.04; Posada and Crandall 1998), was implemented in two searches of 10 replicates each, with maxtrees = 100 and swapping with the TBR algorithm. Support for the clades was estimated with nonparametric bootstrapping (Felsenstein 1985) using 2000 pseudoreplicates for MP and 400 pseudoreplicates for ML. Bootstrap values $\geq$ 70% are considered strong support for a clade (Hillis and Bull 1993). A Sp2 IBM supercomputer was used for the majority of the MP and ML analyses.

Bayesian analyses was performed using MrBayes 2.0 (Huelsenbeck and Ronquist 2001), based on the same model of evolution used for the ML searches. A priori the specific parameter values were uniform and were estimated as part of the analysis. To more thoroughly explore the parameter space we ran Metropolis-coupled Markov chain Monte Carlo simulations (MCMCMC) with four incrementally heated chains, using the default values. From a random starting tree we ran 1.0×10^6 generations, and sampled the Markov chains at intervals of 100 generations to obtain 10,000 sample points. We determined when stationarity was reached (to discard the burn-in samples) by plotting the log likelihood scores of sample points against generation time; when the values reached a stable equilibrium, before 100,000 generations, stationarity was assumed. The equilibrium samples (the 19,000 trees retained after burn-in) were used to generate a 50% majority rule consensus tree. The percentage of samples that recover any particular clade on this tree represents that clade's posterior probability; these are the *P*-values, and we consider $P \geq 95\%$ as evidence of significant support for a clade (Huelsenbeck and Ronquist 2001). Recent simulation analyses indicate that although Bayesian support values are usually higher than those from nonparametric bootstrap, they provide a much closer estimate of the phylogenetic accuracy (Wilcox et al. 2002).

Nested Clade Analyses

The complete set (no missing data) of short cyt *b* sequences (584 bp) was used for these analyses. NCA (Templeton et al. 1995; Templeton 1998) was used to infer the population history of *L. darwinii*. The program TCS ver. 1.13 (Clement et al. 2000; available from http://bioag.byu.edu/zoology/crandall_lab/programs.htm) was used to construct the haplotype network and nesting categories were assigned following Templeton et al. (1995) and Templeton and Sing (1993). The networks were then used for NCA, which was implemented with the GeoDis program (ver. 2.0, Posada et al. 2000) available from the same website. All the statistical analyses were performed using 10,000 Monte Carlo replications. Ambiguous connections (loops) in the network were resolved using predictions from coalescent theory (reviewed in Nordborg 2001; Rosenberg and Nordborg 2002) validated with empirical datasets (Crandall and Templeton 1993). These predictions can be summarized as three criteria that can be used to break the ambiguous connections: (1) the frequency criterion: haplotypes are more likely to be connected to haplotypes with higher frequency than to singletons; (2) the topological criterion: haplotypes are more likely to be connected to interior haplotypes than to tip haplotypes; and (3) the geographical criterion: haplotypes are more likely to be connected to haplotypes from the same population or region than to haplotypes in distant populations (Pfenninger and Posada 2002). Statistically significant results were interpreted following the inference key of Templeton (2001, available from the same website).

Neutrality Tests and Molecular Diversity Analysis

Using molecular variation to answer a broad range of evolutionary questions, such as population history and structure, involves the critical assumption that the variation is evolving neutrally. To test the validity of this assumption we used the McDonald-Kreitman (1991) test (hereafter M-K) of neutrality, which compares the ratio of polymorphic nonsynonymous to synonymous substitutions within species relative to this same ratio of substitutions that are fixed in relation to one or more closely related outgroup species. The M-K and similar tests of neutrality are based on statistics with distributions that are either independent of genealogy or only dependent on it through a nuisance parameter that can be eliminated (Nielsen 2001). To assess population equilibrium independent of the NCA inferences, we implemented Tajima's (1989) *D*-test and Fu's (1997) *Fs*-test. These tests differ in their statistical power (Wayne and Simonsen 1998; Ford 2002), but both assume that populations are in mutation-drift and migration-drift equilibrium. Significant values for either may indicate that the populations are not evolving in a neutral manner (i.e., they are not in mutation-drift equilibrium) or that they were previously subdivided and/or have experienced past population growth (i.e., they are not in migration-drift equilibrium). In this study, if the M-K test fails to reject the null hypothesis of neutral evolution for our cyt *b* sequences, then we interpret results of the *D*-test or the *Fs*-test in the context of migration-drift equilibrium. The *Fs*-test appears to be especially sensitive to detection of population expansion (Fu 1997), and we evaluated significance of this test by comparing the *Fs*-statistic against a distribution generated from 1000 random samples under the hypotheses of selective neutrality and population equilibrium.

For the main clades identified in the phylogenetic analyses and NCA (see below), we calculated the corrected average pairwise genetic distances (taking into account the intrapop-

ulation mean divergences of the two groups being compared) using the Tamura and Nei (1994) model of evolution. We also estimated gene diversity (Nei 1987, p. 180) and nucleotide diversity (π, the mean of pairwise sequence differences; Nei 1987, p. 257) for these same clades. The parameter θ can be estimated using π or the number of segregating sites (*S*). If evolution is neutral then both estimates give the same value of θ, and this can be compared via the Tajima's test (Tajima 1989) to assess whether impacts of selection or population change can be detected (see below). As a final assessment of population demographic histories, we performed mismatch analyses (with pairwise distances) and calculated the raggedness index (Harpending 1994) for clades 2-1, 2-2, and *L. grosseorum* (see below). This index takes larger values for multimodal distributions expected in stationary populations relative to unimodal and smoother distributions typical of expanding populations. We calculated the probability of observing by chance a higher value of the raggedness index than the observed one $P(Rag_{obs})$, under the hypothesis of population expansion.

The nucleotide diversity, population structure, and Tajima and Fu tests of neutrality were performed with the software ARLEQUIN ver. 2.000 (Schneider et al. 2000). The M-K test was performed in the program DNASP (Rozas and Rozas 1999). This combination of methods permits us to independently evaluate specific NCA inferences tied to population growth—including dispersal or range expansions—by statistical tests based on completely different assumptions.

Results

Phylogenetic Relationships of Cytochrome b Haplotypes

The MP searches recovered 2775 equally parsimonious trees ($L = 490$, CI = 0.551, RI = 0.842), and a strict consensus tree was generated to compare with the ML and Bayesian trees. The two independent Bayesian analyses reached stationarity before 100,000 generations; the 19,000 remaining trees yield almost identical consensus topologies in both cases and both were very similar to the strict consensus MP tree. One of the ML analyses recovered two trees and the other a single tree ($\ln L = -3350.4503$), and because all analyses produced very similar results, the single ML tree is the only one presented here (Fig. 2). Although there is no support for definitive relationships among most of the nonfocal species from the *L. darwinii* group, there is weak evidence that either *L. chacoensis* (posterior probability [PP] = 0.53) or *L. olongasta* (parsimony bootstrap [PB] = 73) is the sister species of the strongly supported (PP = 1.0, PB = 96, likelihood bootstrap [LB] = 70) monophyletic [*L. darwinii* complex (clade A) + *L. grosseorum*] group. Within this group two main clades are recovered with strong support (Fig. 2), *L. grosseorum* (PP = 1.0, PB = 100, LB = 79), and clade A (PP = 1.0, PB = 100, LB = 73). Clade A includes haplotypes from an undescribed species with a very distinctive morphology (previously assigned to *L. boulengeri* by Schulte et al. 2000), and is hereafter referred to as *Liolaemus* sp. nov., *L. laurenti*, several populations under the name *L. darwinii*, and also four haplotypes from lizards corresponding to *L. grosseorum* (open squares, localities 16, 30, and 41; Fig. 2).

For the results presented here, we use the name ''*darwinii*'' group for all the closely related species previously referred as *L. darwinii* complex by Etheridge (1993, 2001), which includes *L. abaucan*, *L. albiceps*, *L. calchaqui*, *L. darwinii*, *L. grosseorum*, *L. irregularis*, *L. koslowskyi*, *L. laurenti*, *L. olongasta*, *L. ornatus*, *L. quilmes*, *L. telsen*, and *L. uspallatensis*; we restrict the use of ''*L. darwinii* complex'' to clade A (Fig. 2). We also restrict the use of the name ''*L. darwinii*'' to the southern populations because the nominal species was originally described from this area (Fig. 1, locality identified by arrow); and we use ''*L. darwinii* N1'' and ''*L. darwinii* N2'' to refer to the geographically separated populations in the northern part of the distribution (Fig. 1, localities 20–23, 27–29, 38, and 24–26, respectively). Within clade A, clade 4-1 (Fig. 2, PP = 0.86, PB = 53) includes a weakly supported monophyletic group (clade 2-1, PP = 0.9, PB = 51, LB = 60), which includes all haplotypes corresponding to *L. darwinii*, and three haplotypes (Fig. 2, localities 16, 30, represented by open squares) from *L. grosseorum*. Another monophyletic group is in clade 4-1 (Fig. 2, clade 2-6, PP = 1.0, PB = 86, LB = 82) includes all haplotypes from *Liolaemus* sp. nov.. The remaining haplotypes from clade 4-1 are unresolved; one corresponds to *L. grosseorum* (Fig. 2, clade 3-2, localities 41, square), and the others to *L. darwinii* N1 (Fig. 2, clade 2-2), including one haplotype from *L. laurenti* (Fig. 2, localities 39, open circle).

Haplotypes from *L. darwinii* N2 (Fig. 2, clades 2-7, 2-8, and 3-3) do not form a monophyletic group, and four haplotypes from *L. laurenti* (three from localities 24, and one from 39) are recovered interdigitated with other clades within clade 4-2 (Fig. 2). Most of the other haplotypes ($n = 9$) from *L. laurenti* are recovered as monophyletic (Fig. 2, clade 2-4, PP = 0.93, PB = 99, LB = 97). For clarification in our discussion, haplotypes from *L. laurenti* that are interdigitated with *L. darwinii* N2 in clades 3-3 and 3-4 and with *L. darwinii* N1 in clade 2-2 (Fig. 2) are referred to as ''*L. laurenti* A.''

Nested Clade Analyses

Application of the Templeton et al. (1992) algorithm (as implemented in TCS) to the 49 cyt *b* haplotypes in the *L. darwini* complex (Fig. 2, clade A, Appendix 2) showed that sequences differing by up to 10 substitutions have at least a 0.95 probability of being parsimoniously connected. A single network (Fig. 3) linked all haplotypes from *L. darwinii*, *L. darwinii* N1, *L. darwinii* N2, *L. laurenti*, and *Liolaemus* sp. nov., and four from *L. grosseorum*, but the most parsimonious network was not fully resolved; five loops were needed to depict alternative haplotype connections (Fig. 3). For the resolution of loop A, we applied the geographic criterion, but this still left two ambiguous options: one connecting haplotypes 23 and 24 and the other connecting haplotypes 2 and 25. Both these connections link haplotypes from *L. darwinii* N1 with *L. darwinii*, and the loop was broken between the two more geographically distant haplotypes (Fig. 3, arrow A). For resolution of loop B (Fig. 3, arrow B), the geographic and frequency criteria were applied, whereas the geographic criterion alone allowed resolution for loops C and D. For loop E, we applied the geographic criterion, and two ambiguous options remained (Fig. 3, E1 and E2). Resolving the cladogram in these two places leads to different nested de-

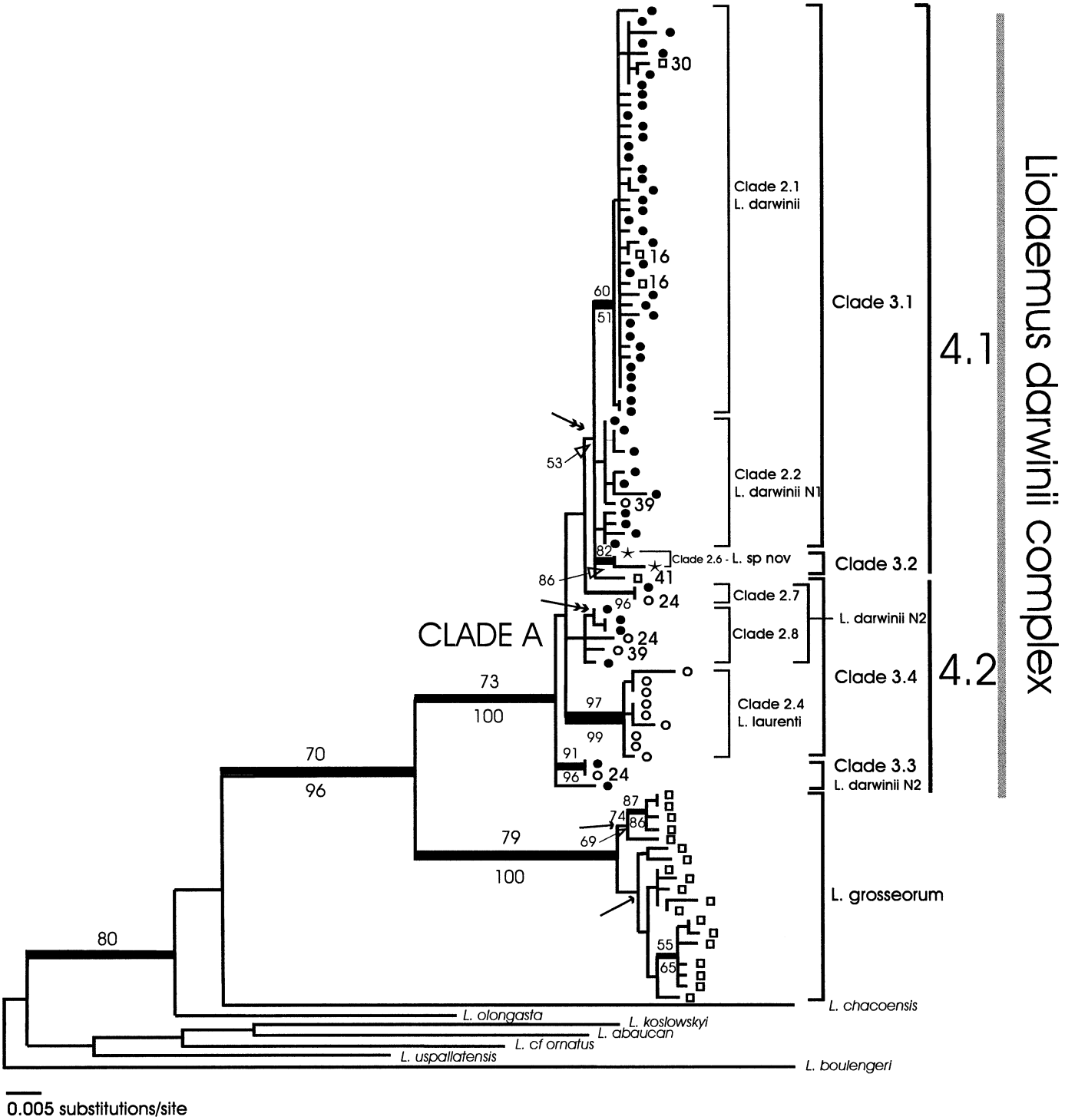

FIG. 2. Maximum likelihood tree (ln $L = -3350.4503$) for cytochrome *b* haplotypes of the *Liolaemus darwinii* group. Numbers above and below branches represent likelihood bootstrap and parsimony bootstrap values, respectively. Thicker branches have posterior probabilities between 0.9 and 1.0, branches indicated with a black double pointed arrow have posterior probabilities between 0.8 and 0.9, and those with black single pointed arrows have posterior probabilities between 0.7 and 0.8. Terminals identified by black circles represent haplotypes from *L. darwinii* (sensu lato), open circles represent *L. laurenti*, stars represent haplotypes from *L.* sp. nov., and open squares represent haplotypes from *L. grosseorum* (as in Fig. 1).

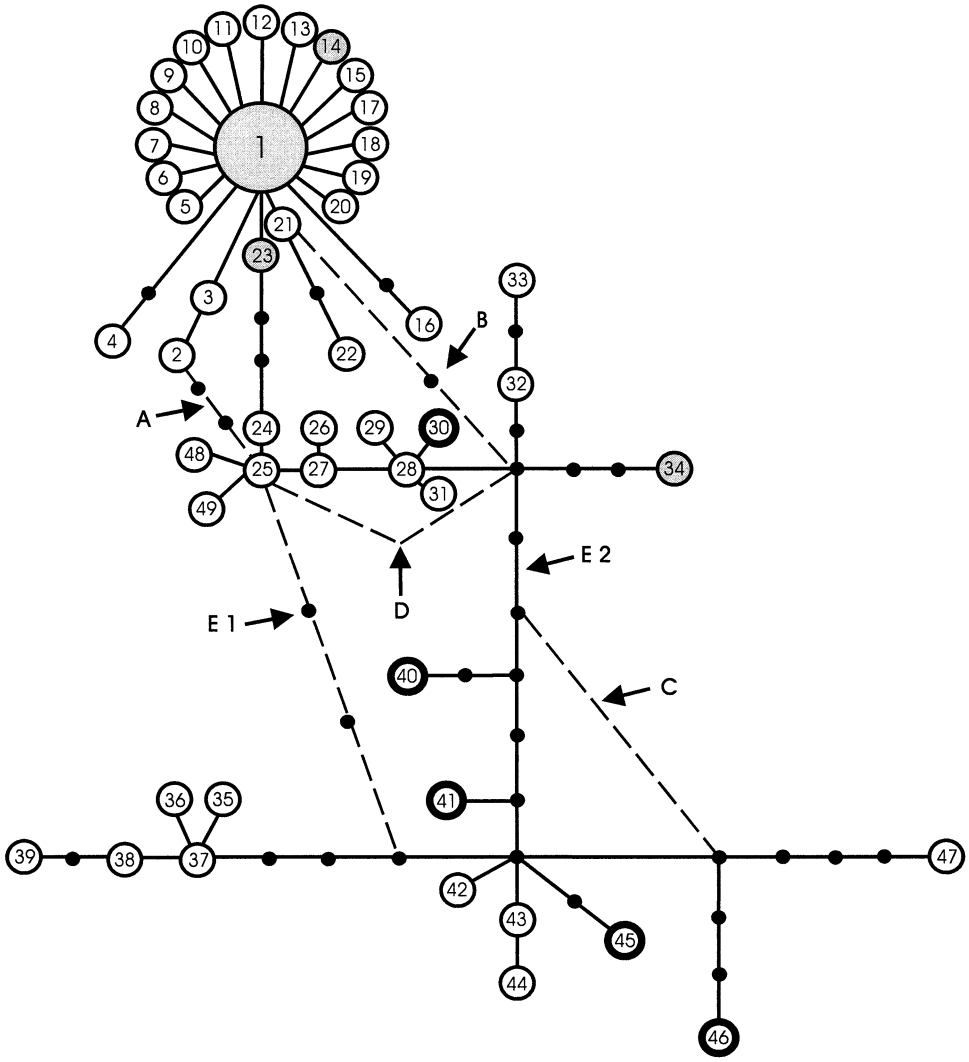

FIG. 3. Unrooted cytochrome *b* haplotype network for clade A of Figure 2; arrows and letters indicate alternative loop solutions (dotted lines). Haplotypes are designated by numbers (Appendix 1) and circle sizes are proportional to haplotype frequencies. Black dots are intermediate haplotypes not present in the sample, and each line represents a single mutational step connecting two haplotypes. Shaded circles, haplotypes unique to *L. grosseorum* or shared between *L. grosseorum* and *L. darwinii*; bold circles, *L. laurenti* haplotypes interdigitated within *L. darwinii* N1 and *L. darwinii* N2 haplotypes (*L. laurenti* A).

signs, and possibly different inferences about the population history. We therefore performed the NCAs for both options, and interpretations about the population history were very similar, so here we present the result only for the solution obtained by breaking loop E1 (Fig. 3). After resolution of the loops, the nested design presented in Figure 4 was used for the NCA, which is graphically summarized in Figure 5. Because the nested design yield clades that are strongly congruent with the observed structure in the tree (Fig. 2), the geographic criteria used to resolve some of the loops most probably will not bias the subsequent geographical analyses.

In all clade levels there were significant distance values, and although at least two described species (and possibly three undescribed) are included in this network, no historical fragmentation was inferred at the highest clade level, but there was support for a range expansion (RE) and long distance colonization (LDC; Fig. 5).

The higher-level clade 4-2 (Fig. 4) in the northern part of the distribution (Fig. 6), for which restricted gene flow with isolation-by-distance (RGF/IbD) was inferred, includes all *L. laurenti* haplotypes (clades 1-7 and 2-4: RGF/IbD), and grouped in clades 2-8, 2-7, and 3-3, all *L. laurenti* A and *L. darwinii* N2 haplotypes. The genetic pattern of *L. darwinii* N2 haplotypes in clades 2-8 and 3-3 is very similar; they are from localities 24, 25, and 26, and past fragmentation (PF) or IbD was inferred for both clades (Table 2). In this region, the Famatina and Sañogasta Mountains separate *L. darwinii* N2 populations 24 and 25 from 26, and could serve as gene flow barrier. Of the four *L. laurenti* A haplotypes included in these two clades, three (Fig. 4, haplotypes 40, 41, 46) have a tip position (suggesting they are rarer or/and relatively younger), are from adult males, and all are from locality 24, where *L. laurenti* is in syntopy with *L. darwinii* N2. This suggests that introgression of *L. darwinii* N2 mtDNA into *L. laurenti* may be the underlying process at this locality, producing these *L. laurenti* A haplotypes (Table 2, clade 2-8).

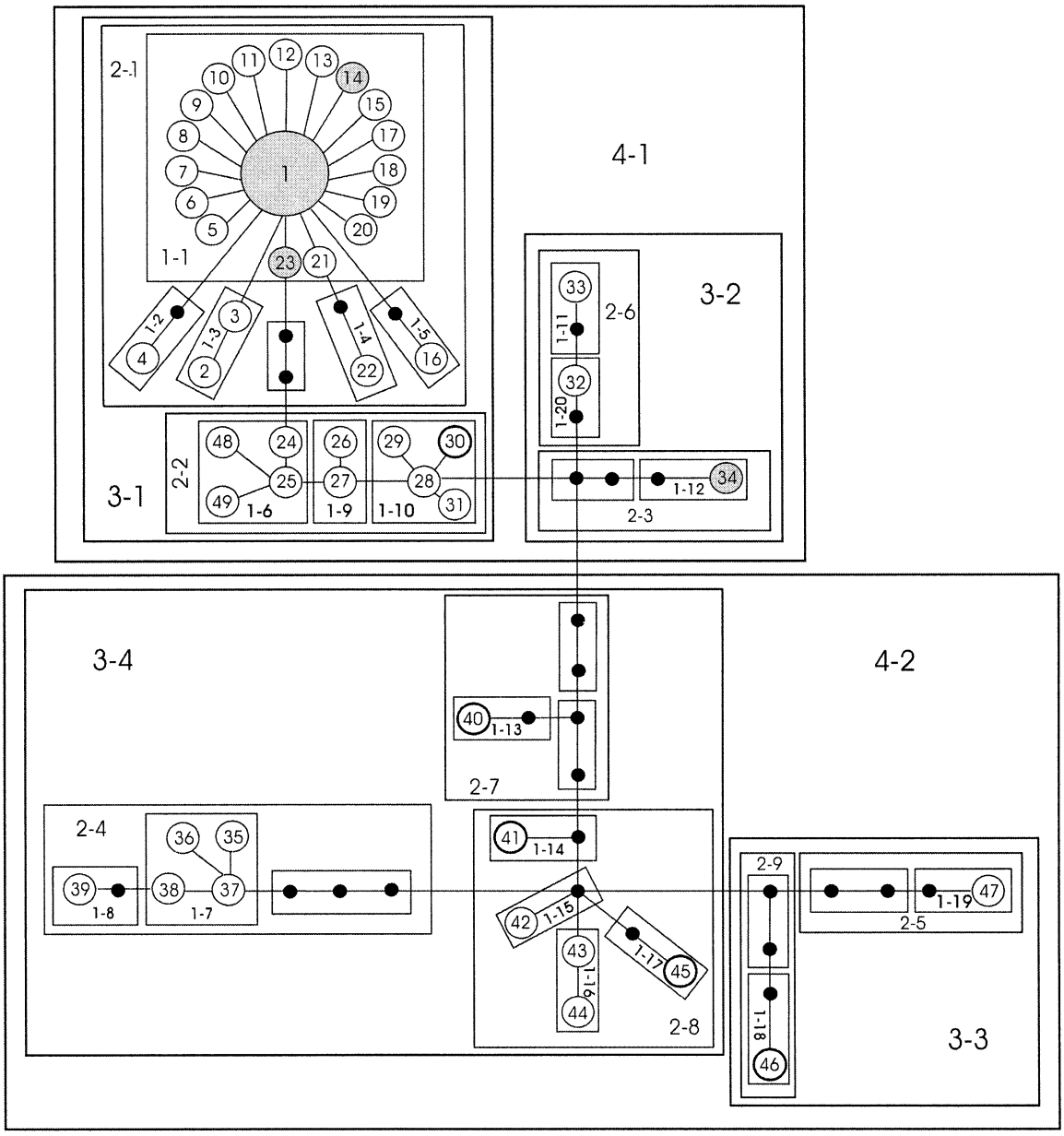

FIG. 4. Resolved haplotype network (from Fig. 3) with associated nested design. Haplotypes belonging to the same clade level are boxed, and all other symbols are as in Figure 3.

The fourth *L. laurenti* A haplotype (Fig. 4, haplotype 45) is from locality 39, and because there is no evidence for the presence of *L. darwinii* N2 at locality 39 (Fig. 6), this haplotype may represent an ancestral polymorphism; an old *L. darwinii* N2 haplotype retained in *L. laurenti* (Table 2, clade 2-8).

For clade 3-4 which includes clade 2-4 (*L. laurenti*) and haplotypes from *L. darwinii* N2 and *L. laurenti* A (clades 2-7 and 2-8), a past fragmentation (PF) between *L. laurenti* and *L. darwinii* N2 was inferred (Fig. 5).

Clade 3-1 (Figs. 4, 6) for which RE with LDC was inferred (Fig. 5), includes all haplotypes from *L. darwinii* (clade 2-1), haplotypes from *Liolaemus* sp. nov. (clade 2-6, F/ID), and from *L. darwinii* N1 (clade 2-2). Clade 2-2 includes clade 1-10 (Figs. 4, 5), which contains one haplotype (30) from *L. laurenti* A from locality 39. This pattern is comparable to that of clade 2-8, and likewise as with *L. darwinii* N2, suggesting that lineage sorting between *L. darwinii* N1 and *L. laurenti* may be incomplete (Table 2, clade 1-10).

Clade 1-1 (Figs. 4–6) includes all haplotypes from *L. darwinii* and three haplotypes from *L. grosseorum* (haplotypes 1, 14, 23). Two of these haplotypes are from locality 16 (Fig. 6), where *L. grosseorum* is in syntopy with *L. darwinii*, and the third is from locality 30, which is very close to 16. These results in combination with the tip position of two of these haplotypes (Fig. 3, haplotypes 14, 23), suggest that in this

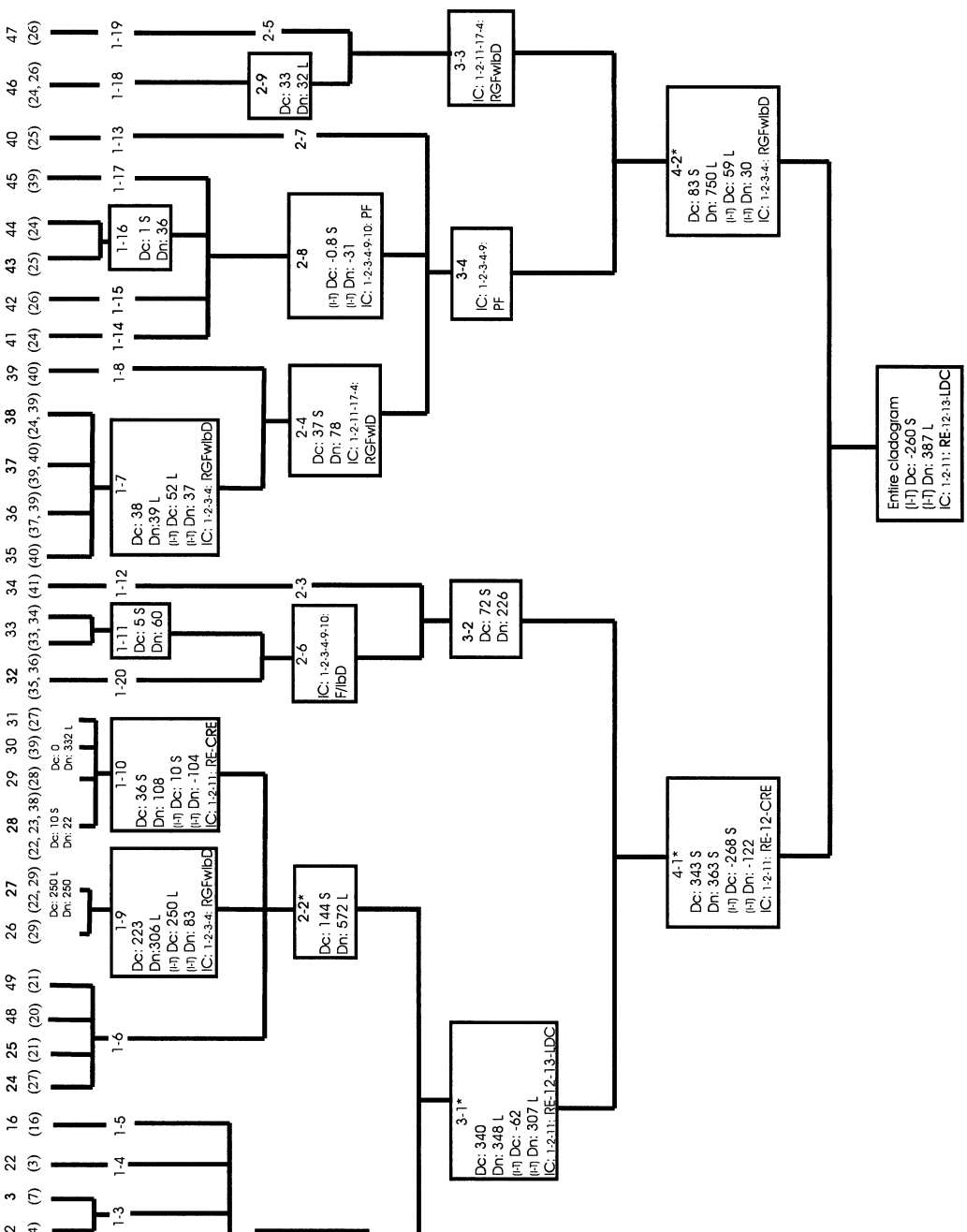

FIG. 5. Graphical summary of nested clade analysis results for haplotypes of the *Liolaemus darwinii* complex, based on the nested design shown in Figure 4. Nesting levels extend from top clades with individual haplotypes (0-step clades) to bottom more inclusive clades, and counting only clades with observed haplotypes. The numbers in parentheses just below the 0-step clades (haplotypes) are the localities (Table 1) from which each haplotype is known. Clades identified by an asterisk (2-2, 3-1, 4-1, and entire cladogram) had significant results in the exact contingency test (GeoDis Part I). For clades that show a significant ($P < 0.05$) probability of association between geography and haplotype distribution, the clade distance (Dc) and the nested clade distance (Dn) are given and distances significantly smaller or larger than expected (under a null model of no association) are indicated with an S or L; results contrasting interior versus tip clades are indicated by I-T (numbers of interior clades are italized and in bold). The inference chain (IC) from the key provided by Templeton (2001) is used to interpret the statistical results; RE, range expansion; CRE, continuous range expansion; LDC, long-distance colonization; RGF/lbD, restricted gene flow with isolation by distance; PF, past fragmentation; D, dispersal; LDD, long-distance dispersal; RD/GF, restricted dispersal/gene flow.

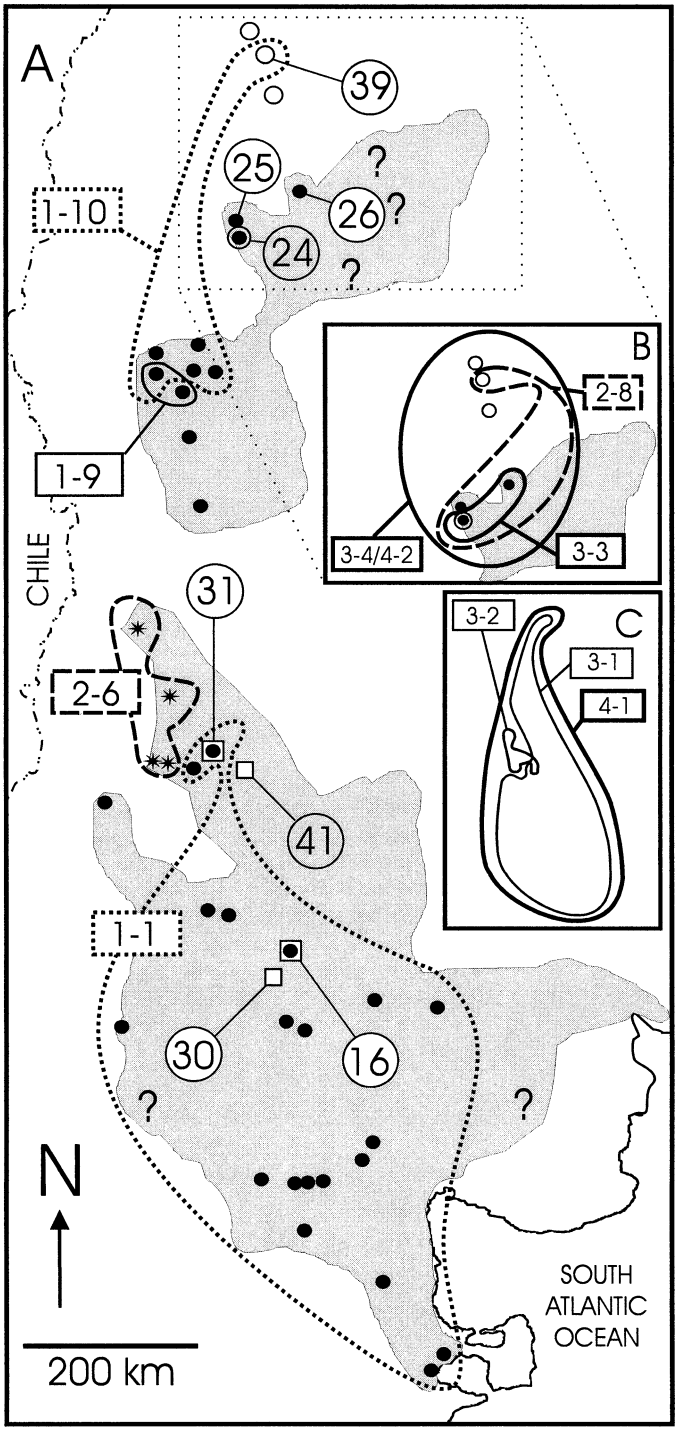

FIG. 6. (A)-Distribution of haplotypes of the *Liolaemus darwinii* complex, with the associated nesting design for some clades relating the haplotypes from these localities (Fig. 4, Table 2). Numbers correspond to localities in Table 1. (B) Extended area corresponding to the upper dotted square. (C) Schematic representation of clades 3-1 and 3-2 and the most inclusive clade, 4-1.

area, *L. darwinii* mtDNA is introgressing into *L. grosseorum* (Table 2, clade 1-10). In clade 3-2 (Figs. 4, 6), haplotypes from *Liolaemus* sp. nov. are associated with one haplotype from *L. grosseorum* from locality 41 (Fig. 3, haplotype 34), near the known distribution of *Liolaemus* sp. nov. (clade 3-2, no NCA inference possible); in this area this two species are in sympatry (Ehteridge 2001; L. J. Avila, unpubl. data).

Neutrality Tests and Molecular Diversity Analysis

Results of the M-K test are consistent with neutral evolution (for *L. darwinii* complex: $P = 0.08$ with Fisher's exact test; $P = 0.12$ with *G*-test with Yate's correction; for *L. grosseorum*: $P = 0.36$ with Fisher's exact test; $P = 0.73$ with *G*-test with Yate's correction). Tajima's *D*-test was significant for the *L. darwinii* complex (Table 3), but this is due to a significant result only for clade 2-1 (*L. darwinii*). Similar results were obtained for Fu's test for selective neutrality; the *L. darwinii* complex and clade 2-1 have significant *P*-values, but clade 2-2 (*L. darwinii* N1) also gave a significant negative value. A negative value was also obtained for *L. laurenti* (clade 2-4), but because the *P*-value (0.043) is at the limit of significance (5% level, $P = 0.02$ is the acceptance level recommended by Excoffier 2001), we cannot make a strong inference about its cause for this clade. For the other clades within the *L. darwinii* complex, the results were not significant, and the null hypotheses of migration-drift equilibrium could not be rejected. Because Fu's statistic is particularly sensitive to range expansion, we performed mismatch distribution analyses for clades for which this test gave significant results (2-1, 2-2), and also for *L. grosseorum* (because, although nonsignificant, the *P*-value of the Fu test = 0.06); the small sample size of *L. laurenti* precluded this analysis. A model of demographic expansion was statistically supported for all three of these groups (Fig. 7): $P(SSD_{obs})$ values are 0.38, 0.86, and 0.33; raggedness indexes are 0.04, 0.05, and 0.02; and $P(Rag_{obs})$ values are 0.77, 0.55, 0.63, respectively. Both methods are consistent and fail to reject the expansion model.

These analyses were performed including and excluding haplotypes 30 (localities 39, clade 2-2), and 45 (localities 39, clade 2-8), which could be considered possible ancestral polymorphisms. Because the results were not significantly different, we present the ones obtained including them. Gene diversity and nucleotide diversity are highest in *L. grosseorum* (Table 3), and although the sample size of the *L. darwinii* complex is almost four times larger, its gene diversity is similar to but the nucleotide diversity is considerably lower than that of *L. grosseorum*. Between clades of the *L. darwinii* complex, nucleotide diversity is highest in clade 2-7 + 2-8 (where introgression of mtDNA from *L. darwinii* N2 into *L. laurenti* was inferred), from a relatively small geographic area in the northern part of the distribution, and lowest in clade 2–1, the southern part of this group's range.

The average corrected pairwise genetic distances between *L. grosseorum* and *L. darwinii* complex is 5.2%. Within the complex, the larger distances are between *L. laurenti* (clade 2-4) and southern clades (clade 2-1, 1.7%; 2-6, 1.8%), and the smallest (0.44%) is between clade 2-1 (*L. darwinii*) and clade 2-2 (*L. darwinii* N1).

DISCUSSION

Hybridization, Incomplete Lineage Sorting, and Phylogeography

In closely related species, introgression and random sorting of ancestral polymorphism can lead to discordance between

TABLE 2. Summary of possible causes for phylogenetic and nested-clade patterns for selected clades identified by resolved haplotype network/nesting design (Fig. 4) with nested clade analysis (NCA) inference (Fig. 5; CRE: contiguous range expansion; RE: range expansion; RGF: restricted gene flow; IbD: isolation by distance; LDC: long-distance colonization; LDD: long-distance dispersal; D: dispersal; and PF: past fragmentation); for each clade the haplotypes and associated localities, recognized morphological species, and the evidence for possible mechanisms are summarized.

Clade	NCA inference	Haplotype/Localities	*Liolaemus* species	Possible mechanisms
1-1	RGF/D/LDD	1/16	*grosseorum*	Localities 16 and 30 are possible secondary contact zones where *L. darwinii* mtDNA is introgressing asymetrically into *L. grosseorum*. Evidence: in locality 16 they are in sympatry, and in possible sympatry at locality 30; haplotypes are contained in the 0- and 1-step clades; species are morphologically distinct.
		1/1–11, 13, 14, 16–18, 31, 32	*darwinii*	
		5–15/1, 3, 4, 6, 8, 10, 14–16, 18	*darwinii*	
		14/16	*darwinii*	
		17–21/2, 12, 14, 17, 32	*darwinii*	
		23/1	*darwinii*	
		23/30	*grosseorum*	
1-10	RE-CRE	28/22, 23, 38	*darwinii* N1	There is no report of *L. darwinii* at locality 39, so haplotype 30 from this locality could be an ancestral polymorphism. This haplotype inflates the Dn value in the NCA, leading to this inference.
		29/28	*darwinii* N1	
		30/39	*laurenti* A	
		31/27	*darwinii* N1	
4-1	RE-CRE	clade 3-1	*grosseorum*	Possibly the ancestral polymorphism in clade 1-10 from locality 39 increases the estimate of Dn (this is the signature expected with incomplete lineage sorting, instead of fragmentation). This clade includes 2-1 and 2-2, for which independent evidence from neutrality tests and mismatch distribution suggest range expansion in this area.
		clade 3-2	*darwinii* N1	
			laurenti A	
			sp. nov.	
2-8	PF/IbD	41/24	*laurenti* A	Locality 24: *L. laurenti* and *L. dawinii* N2 are in sympatry, most parsimonious inference: introgression after secondary contact.
		42/26	*darwinii* N2	
		43/25	*darwinii* N2	
		44/24	*darwinii* N2	
		45/39	*laurenti* A	Locality 39: no report of *L. dawinii* N2 present at this locality, possibly incomplete lineage sorting and retention of a *L. darwinii* haplotype in *L. laurenti*.
3-4	PF	clade 2-4	*laurenti*	Locality 24 may be a zone of introgression, but the genetic signature is strong enough for the NCA to infer a fragmentation between *L. laurenti* and *L. darwinii* N2 + *L. laurenti* A.
		clade 2-8	*laurenti* A	
		40/24	*darwinii* N2	
		40/25		
3-3	RGF/IbD	clade 2-5: 47/26	*darwinii* N2	Because this *L. laurenti* A is from locality 24, this supports the inference that this could be a secondary contact area.
		clade 2-9: 46/24	*darwinii* N2	
		46/24	*laurenti* A	
		46/26	*darwinii* N2	
4-2	RGF&IbD	clade 3-3	*laurenti* A	The geographic area included in this clade presents a complex genetic structure, for which more sampling is needed.
		clade 3-4	*darwinii* N2	

TABLE 3. Estimates of gene and nucleotide diversity (π) and two different estimates of the parameter θ (θ_π and θ_S) for different clades identified in the phylogenetic and nested clade analyses. Standard errors for estimates are in parentheses. Tajima's *D*-statistic and Fu's test with associated level of significance: $^{**}P < 0.001$, $^{*}P < 0.02$, ns, nonsignificant.

	N	Gene diversity	Nucleotide diversity (π in %)	θ_π	θ_S	Tajima's *D*	Fu's *p*
Clade 2-1 (*L. darwinii*)	54	0.786 ± 0.06	0.228 ± 0.15	1.48 (1.00)	5.5 (1.82)	−2.5**	**
Clade 2-2 (*L. darwinii* N1)	14	0.945 ± 0.04	0.434 ± 0.27	2.86 (1.79)	3.77 (1.71)	−1.3 ns	**
Clade 2-6 (*Liolaemus* sp. nov.)	4	0.667 ± 0.2	0.348 ± 0.28	2.29 (1.87)	1.64 (1.18)	2 ns	ns
Clade 2-4 (*L. laurenti*)	9	0.917 ± 0.07	0.356 ± 0.24	2.40 (1.63)	2.57 (1.38)	−0.8 ns	0.043
Clade 2-7 + 2-8 (*L. darwinii* N2, *L. laurenti* A)	8	0.928 ± 0.08	0.844 ± 0.52	5.59 (3.41)	4.62 (2.32)	−0.17 ns	ns
Clade 3-3 (*L. darwinii* N2, *L. laurenti* A)	4	0.5 ± 0.26	0.623 ± 0.46	4.44 (3.30)	3.81 (2.4)	−0.8 ns	ns
L. darwinii complex	91	0.921 ± 0.02	1.156 ± 0.60	7.36 (3.85)	12 (3.28)	−1.82*	**
L. grosseorum	24	0.953 ± 0.03	7.735 ± 3.870	47.57 (23)	15.5 (5.3)	−1.4 ns	ns

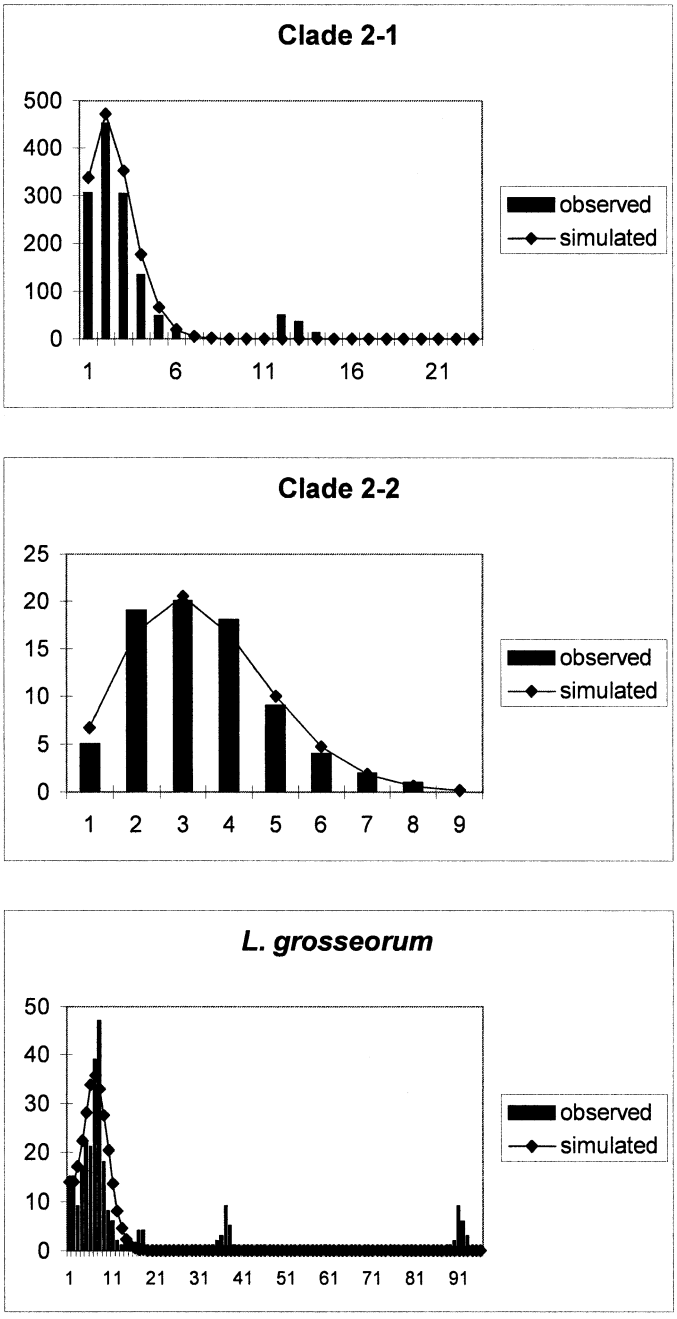

FIG. 7. Mismatch distributions of pairwise number of mutational differences between individuals from the *Liolaemus darwinii* complex for clades 2-1, 2-2, and *L. grosseorum*. Black bars represent the observed data, and the bold curve is the model fitted to the data.

gene trees and species trees (Harrison 1991; Maddison 1997; Knowles 2001; Sullivan et al. 2002). Hybridization between species or populations may result in the incorporation of alleles from one species or population into the gene pool of another (Ferris et al. 1983). The use of these markers for phylogenetic or phylogeographic analysis will give a different view of population histories than would markers that show no evidence of introgression (Harrison 1991). Also, when the level of divergence among closely related species or populations is low, introgression will be difficult to discriminate from ancestral polymorphism (Avise and Ball 1990). But because these two processes predict different spatial patterns, a phylogeographic analyses can be used to differentiate them (Goodman et al. 1999). If recent hybridization took place, we expect introgressed alleles to be more common in contact zones and areas that are relatively short dispersal distances from sympatric localities, while ancestral polymorphisms should be sampled at approximately equal frequencies throughout a distributional range (Barbujani et al. 1994).

Introgression results in haplotypes of one group being shared with a sympatric but distinct group, and differing from haplotypes of conspecific populations. Several examples from our data suggest that this may be the process underlying some of the observed patterns. In locality 24 (Fig. 6), where *L. laurenti* is in syntopy with *L. darwinii* N2, individuals from these two species share tip haplotypes 40 and 46 (Fig. 4, clades 1-13 and 1-18, respectively). Because rarer or younger haplotypes are expected to occur at higher frequencies at the tips of the cladogram (prediction empirically validated in Crandall and Templeton 1993), sharing tip haplotypes between two syntopic species is more likely due to introgression than to shared ancestral polymorphism (in which older, more interior haplotypes are expected to be shared; Maddison 1997).

Clade 1-1 (Fig. 4) presents another example where the pattern is consistent with introgression in localities 16 and 30 (Fig. 6), regions of sympatry or syntopy between *L. darwinii* and *L. grosseorum*. In locality 16 one individual of *L. grosseorum* was characterized by a *L. darwinii* haplotype (Fig. 4, haplotype 1), which has the highest frequency (n = 27) and is present throughout the distributional range of *L. darwinii*. Two more individuals of *L. grosseorum* from this same area (Fig. 6, localities 16, 30) had haplotypes separated by only one step from haplotype one (Fig. 4). Thus, in locality 16 there are two lizards that are unambiguously morphological *L. grosseorum*, but characterized by haplotypes that are more closely related to *L. darwinii* haplotypes than to other *L. grosseorum*, a pattern consistent with unidirectional introgression of mtDNA from *L. darwinii* into *L. grosseorum* (Table 2).

As an alternative to hybridization, retention of ancestral polymorphisms can account for mixing of haplotypes between species or distinct populations (Avise et al. 1983; Tajima 1983; Neigel and Avise 1986; Nei 1987; Pamilo and Nei 1988). Lineage sorting eliminates ancestral polymorphism over time such that sister species eventually become reciprocally monophyletic with respect to mtDNA, but this is expected to be incomplete when the rate of lineage splitting or speciation exceeds the rate of stochastic sorting of allelic polymorphisms within lineages (Sullivan et al. 2002). In these cases the phylogeny of alleles sampled will differ from larger species phylogeny (Pamilo and Nei 1988; Moran and Kornfield 1993), and a single gene genealogy may be misleading (Moritz et al. 1992; McMillan and Palumbi 1995; Redenbach and Taylor 2002). Under conditions of rapid speciation and maintenance of large effective population sizes, the effects of genetic drift are reduced and rates of lineage sorting slowed (Parker and Kornfield 1997). But when we observe evidence of incomplete lineage sorting (paraphyly

of the gene genealogy and recent introgression is ruled out), there is a key distinction to make: these shared haplotypes may represent ancestral polymorphisms maintained since the original divergence of the populations, or they may be shared since the last old introgression event between the populations. No single method currently available is capable of discriminating between incomplete lineage sorting and other population processes that could produce a similar pattern (Masta et al. 2003). Here we argue that the presence of ancestral polymorphisms appears to be the best explanation for some of the observed patterns, but we cannot make the distinction between sorting as a result of old introgression versus the original population divergence. It may be possible to distinguish between these with sufficiently variable unlinked nuclear markers.

In all phylogenetic hypotheses (Fig. 2) *L. darwinii* N1 and *L. darwinii* N2 are recovered as paraphyletic, and in the second case, NCA clade 2-8 includes *L. darwinii* N2 haplotypes and one haplotype from *L. laurenti* A (Fig. 4, haplotype 45, locality 39). Because at present there is no evidence for the distribution of *L. darwinii* N2 so far north, this haplotype may represent an ancestral polymorphism between *L. darwinii* N2 and *L. laurenti*.

Probably a clearer example of incomplete lineage sorting is in clade 1-10 (Fig. 4), which includes one haplotype from *L. laurenti* (haplotype 30) from locality 39 (Fig. 6), closely related to *L. darwinii* N1 haplotypes (clade 2-2 in Fig. 4). Because there is no evidence for the presence of *L. darwinii* N1 at this locality, this result suggests that incomplete lineage sorting is most likely responsible for this pattern (Table 2). In the NCA this haplotype produces a significantly larger Dn, leading to the range expansion inference for this clade (nested clade 1-10 in Fig. 5). Although we can hypothesize this was the process by which this lineage expanded at some time in the past, on the basis of pronounced morphological differentiation between *L. darwinii* N1 and *L. laurenti*, we would intuitively expect historical fragmentation to have isolated the ancestors of these two populations. If this interpretation is correct, then it is probable that more sampling will reveal a signature of historical fragmentation, unless speciation has been very recent, and driven largely by natural selection for morphological divergence in response to different ecological conditions (Schneider et al. 1999; Ogden and Thorpe 2002).

Divergence Times and Speciation

Although we have no *Liolaemus*-specific evidence to support the general rate of 0.5–1.4% divergence per million years, which is a range given for other ectothermic vertebrate mtDNAs (Avise et al. 1992; Caccone et al. 1997), studies of mtDNA divergence in other iguanian lizards (Macey et al. 1998, 1999) provide estimates of approximately 0.65% change per million years per lineage, thus about 1.3% sequence divergence is expected to accumulate over 1 million years for a pairwise comparison. The 5.2% average genetic difference between *L. grosseorum* and the *L. darwinii* complex suggest that they diverged from each other about 4 million years ago, placing this split in the late Pliocene. Evolution within the *L. darwinii* complex has been more recent (Pleistocene), with the oldest split between *L. laurenti* and the southern clades (2-1 and 2-6) occurring approximately 1.4 million years ago, and more recently between these two southern clades approximately 340,000 years ago. We are aware of potentially large error terms associated with these estimates (Edwards and Beerli 2000), but the general pattern suggests that the earliest speciation within the complex was in the northern part of its distribution, followed by later expansions toward the south. Different lines of evidence suggest range expansion for the southern clades: (1) NCA inferences for clade 3-1: CRE and LDC; and clade 4-1: RE-CRE (Fig. 5); (2) high levels of gene diversity but low nucleotide diversity, significant Fu's statistics for clades 2-1, 2-2 (Table 3); and nonsignificant M-K tests; and (3) significant signature of recent expansion inferred by mismatch distribution (Fig. 7). It is very probable that in the past *L. grosseorum* was also distributed further north, and a recent southern expansion (Table 3, marginally significant Fu's statistic; Fig. 7, mismatch distribution) brought populations from this species into recent contact with *L. darwinii* (*sensu stricto*), causing the introgression observed in our results.

Clades in the northern area show more extensive retention of mtDNA variation; clades 2-7 and 2-8, have high gene diversity values, but also the highest nucleotide diversity of the complex (Table 3), and because neutral variation may be retained in large populations in the absence of gene flow (Neigel and Avise 1986), this pattern may reflect the recent emergence and historically large population sizes of these groups. We consider this our best working hypothesis for the haplotypes that we interpret as ancestral polymorphisms in this area (Table 2, clades 1-10, 2-8). Pleistocene climatic changes may have had a role in isolating these populations, but present climatic conditions likely fostered their expansion and brought them into secondary contact, allowing for introgression between *L. laurenti* and *L. darwinii* N2 in locality 24 (Table 2, clade 3-3, clade 4-2).

However, some morphological traits can coalesce rapidly under strong directional (West-Eberhard 1983; Moran and Kornfield 1993), or sexual selection (Wikelski and Trillmich 1997; Abel 1998; LeBas 2001; Kwiatkowski and Sullivan 2002), and the degree of genetic differentiation may not reflect the degree of morphological differentiation (Bromham et al. 2002). A plausible alternative explanation then for populations of the *L. darwinii* complex, is that the degree of mtDNA differentiation does not reflect the degree of morphological differentiation, and that selective pressures (directional and/or sexual) may be acting on these morphological traits, leading to their faster differentiation (Schneider et al. 1999; Kwiatkowski and Sullivan 2002). Additional molecular and morphological data will be required to discriminate between these alternatives.

Cross-Validation of Nested Clade Analysis Inferences

As Masta et al. (2003) emphasized, NCA inferences are based on a combined analysis of patterns of geographical associations of within and between nested haplotype groups and assume that a significant association of geography and nested haplotypes is due to some form of restricted gene flow. The validity of the assumption underlying this pattern has been explored and appears validated by theoretical studies

(Neigel et al. 1991; Neigel and Avise 1993). In other cases, such as a contiguous range expansion or long-distance colonization, the NCA may not detect all such demographic events (Templeton 1998), but are thought to be conservative and not prone to false positives (Templeton 2002). Masta et al. (2003) cautioned that the NCA may especially incorrectly infer long-distance movements because it cannot detect localized extinction of haplotypes between two locations across which haplotypes are inferred to have moved. Because repeated cycles of range expansion and contraction have likely been common in species whose phylogeographic histories have been strongly influenced by Pleistocene cyclic climate changes, as we have suggested here for the *L. darwinii* complex, random extinction of some haplotypes in some lizard populations would be expected but not predictable. Masta et al. (2003) provide a modification of the NCA inference key to accommodate alternative inferences when the hypothesis of long-distance colonization is at odds with the biology of the organism under study. Our NCA inferences did not lead to the unambiguous conclusion of long-distance colonization for any nesting levels (Fig. 5), but we extend general comments about cross-validation to other NCA inferences not mentioned in the above sections.

For clade 2-1 (*L. darwinii*) the NCA inference was ambiguous (F/RE/IbD), and although the M-K test for the whole complex cannot reject the null hypotheses of neutral evolution, the Tajima and Fu tests were significant, and the mismatch distribution could not reject the null hypotheses of range expansion, thus narrowing the inference for this clade. For clade 2-2 (*L. darwinii* N1), a NCA inference was not possible, but a significant Fu test and the mismatch distribution (Fig. 7) allows an inference of range expansion. For the *L. darwinii* complex, where the NCA inference was range expansion with long-distance colonization, we can partially cross-validate this inference (range expansion with significant Tajima and Fu tests), but long-distance colonization will need independent evidence for validation (Masta et al. 2003). In clade 2-4 (*L. laurenti*), for which restricted gene flow with isolation-by-distance was inferred, we also obtained nonsignificant results with the Tajima and Fu tests, supporting the inference that this clade is in migration-drift equilibrium.

Taxonomic Implications

Figure 8 summarizes the known distribution of *L. grosseorum* and the different species and populations included in the *L. darwinii* complex. In this work we present evidence that *L. grosseorum* is the sister species of the *L. darwinii* complex (Fig. 2), a result consistent with the taxonomically broader study by Schulte et al. (2000). Although four *L. laurenti* haplotypes (*L. laurenti* A) were recovered interdigitated among the *L. darwinii* N2 samples, and one interdigitated within *L. darwinii* N1, this was interpreted as three cases of introgression and two examples of retention of ancestral polymorphisms. Morphologically the individuals that carried the introgressed haplotypes appear slightly more stout and larger than the morphologically homogeneous lizards that are recovered as a strongly supported monophyletic group (Fig. 2, clade 2-4), for which a fragmentation was inferred with NCA (Fig. 5, nested clade 3-4), thus supporting the validity of *L. laurenti*. Although haplotypes of *L. darwinii* N2 are paraphyletic, they do not interdigitate with haplotypes from *L. darwinii* N1, *Liolaemus* sp. nov., and *L. darwinii*, and future work should focus on independent evidence to test the proposition that populations from this area are different species. *Liolaemus* sp. nov. is very distinctive from all other members of the complex in a number of morphological characters

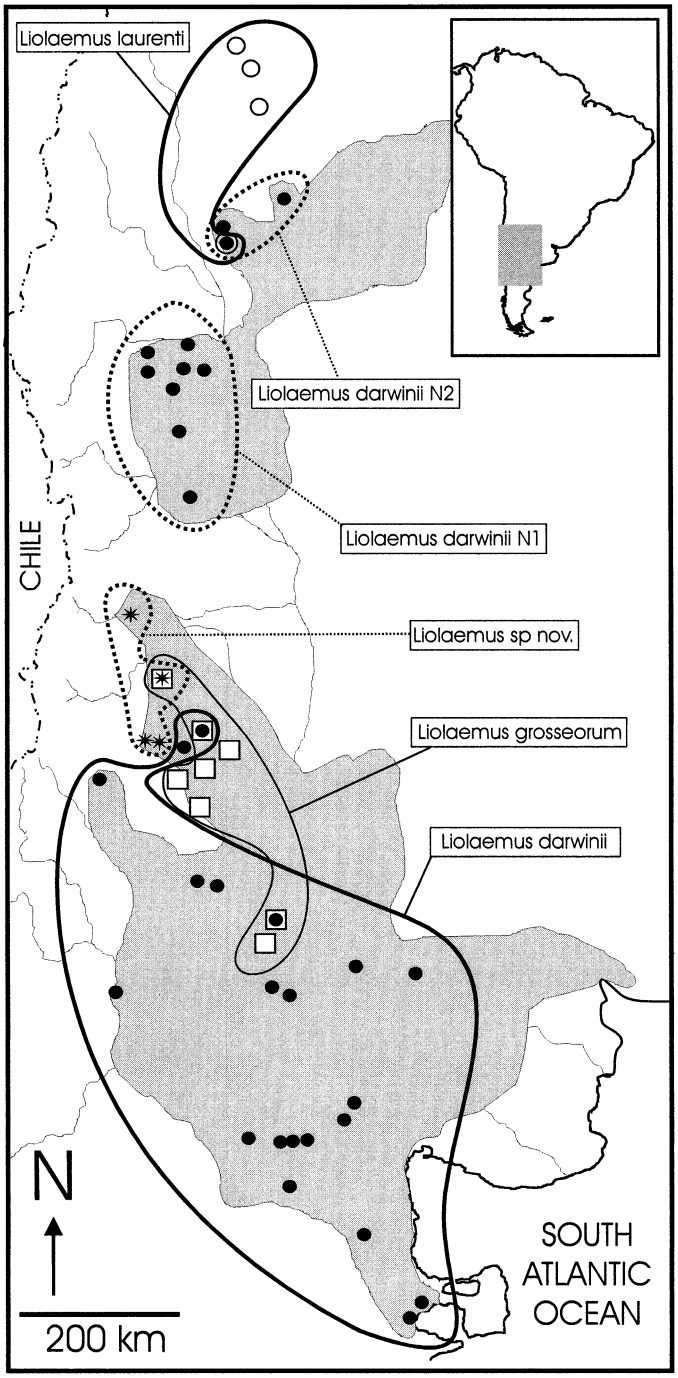

FIG. 8. Approximate geographic distribution of main groups in this study. Black circles, *Liolaemus darwinii* (*sensu lato*) localities; open circles, *L. laurenti* localities; stars, *Liolaemus* sp. nov. localities; squares, *L. grosseorum* localities. Syntopy localities are marked with two superimposed symbols.

(body size, color pattern, and squamation). This distinctiveness led Cei (1986) and Schulte et al. (2000) to consider it as a member of the *L. boulengeri* group, but here we present strong evidence for its inclusion within the *L. darwinii* complex. Although *L. darwinii* N1 is paraphyletic, none of its haplotypes interdigitate with *L. darwinii*, and the one from *L. laurenti* is interpreted as incomplete lineage sorting as mentioned above. Evidence from independent markers could corroborate this as a different species. There is strong support from the Bayesian analyses and weak bootstrap support for the monophyly of *L. darwinii* (*sensu stricto*; Fig. 2).

Conclusions

Most of the speciation events in this complex can be placed within the Pleistocene (with caveats), thus we can make the association that the formation of some of these species could have originated due to climatic changes that occurred during this period. These phenomena have been reported in phylogenetic analyses of other vertebrates in regions or the Northern Hemisphere characterized by Pleistocene climatic changes, tectonic activity, and increased aridity, and general causative factors can sometimes be implicated (Dowling and Secor 1997; Grandjean and Souty-Grosset 2000; Alexandrino et al. 2002; Redenbach and Taylor 2002; Sullivan et al 2002). Here we present the first evidence for introgression and incomplete lineage sorting in lizards (and possibly in any vertebrate) in temperate South America, which could also be related to Pleistocene climatic changes. Cycles of isolation and subsequent contact could provide repeated opportunities for gene exchange in recently diverged populations, and these processes could confound inferences about phylogeographic history. We have shown that a combination of methods can provide evidence to support or further resolve an ambiguous NCA inference, and thus provide more robust interpretations as well as to signal those requiring caution. The inability of any single method to unambiguously resolve all plausible hypotheses for a given phylogeographic patter is not a limitation as long as alternatives are clarified and presented in a testable manner. We applaud efforts to increase the rigor of phylogeographic analyses (Antunes et al. 2002; Knowles and Maddison 2002; Templeton 2002), and we agree that multiple approaches with currently available methods can narrow the range of plausible hypotheses about mechanisms and processes of divergence (Pfenninger and Posada 2002; Masta et al. 2003). Additional studies are needed in this group of lizards, and in other codistributed groups, to determine if Pleistocene climatic changes could be a general factor influencing the evolutionary history of a regional biota.

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APPENDIX 1.

Different haplotypes included in the nested clade analyses, with locality information (locality numbers match those in Appendix 2 and Fig. 1), and frequency for each one with voucher numbers that match with those in Appendix 2.

Haplotype number	Locality number	Number of individuals	*Liolaemus* species
1	1	1 (FML 13068)	*darwinii*
	2	1 (LJAMM 9fn)	*darwinii*
	3	1 (LJAMM 3030)	*darwinii*
	4	1 (MLP.S 2481)	*darwinii*
	5	1 (BYU 47325)	*darwinii*
	6	1 (BYU 47327)	*darwinii*
	7	1 (LJAMM 2619)	*darwinii*
	8	2 (BYU 47319-FML 13064)	*darwinii*
	9	3 (LJAMM 63–65 fn)	*darwinii*
	10	2 (LJAMM 3016-FML 13067)	*darwinii*
	11	3 (LJAMM 72–74 fn)	*darwinii*
	13	2 (MLP.S 2487/8)	*darwinii*
	14	3 (LJAMM 2406–2408)	*darwinii*
	16	2 (FML 13065/MLP.S 2490)	*darwinii*/*grosseorum*
	17	1 (LJAMM 2398)	*darwinii*
	18	2 (LJAMM 2402–2403)	*darwinii*
	31	1 (LJAMM 4037)	*darwinii*
	32	1 (LJAMM 4146)	*darwinii*
2	4	1 (MLP.S 2482)	*darwinii*
3	7	1 (LJAMM 2621)	*darwinii*
4	19	3 (BYU 47321-MLP.S 2506-FML 13082)	*darwinii*
5	16	1 (BYU 47317)	*darwinii*
6	4	1 (MLP.S 2483)	*darwinii*
7	14	1 (LJAMM 2410)	*darwinii*
8	1	1 (BYU 47318)	*darwinii*
9	10	1 (BYU 47326)	*darwinii*
10	3	1 (LJAMM 18 fn)	*darwinii*
11	8	1 (MLP.S 2485)	*darwinii*
12	6	1 (BYU 47328)	*darwinii*
13	15	1 (LJAMM 2641)	*darwinii*
14	16	1 (MLP.S 2491)	*grosseorum*
15	18	1 (LJAMM 2400)	*darwinii*
16	16	1 (MLP.S 2486)	*darwinii*
17	14	1 (LJAMM 2409)	*darwinii*
18	2	1 (MLP.S 2484)	*darwinii*
19	17	1 (LJAMM 2399)	*darwinii*
20	32	1 (LJAMM 4147)	*darwinii*
21	12	3 (BYU 47324-MLP.S 2489-FML 13066)	*darwinii*
22	3	1 (LJAMM 19 fn)	*darwinii*
23	1	1 (LJAMM 5 fn)	*darwinii*
	30	1 (LJAMM 4462)	*grosseorum*
24	27	1 (LJAMM 2295)	*darwinii* N1
25	21	2 (MLP.S 2507-FML 13084)	*darwinii* N1
26	29	1 (MLP.S 2493)	*darwinii* N1
27	29	1 (MLP.S 2492)	*darwinii* N1
	22	1 (LJAMM 2275)	*darwinii* N1
28	23	1 (IMCN 8LA)	*darwinii* N1
	22	1 (LJAMM 2276)	*darwinii* N1
	38	1 (IMCN 19LA)	*darwinii* N1
29	28	1 (LJAMM 2287)	*darwinii* N1
30	39	1 (MLP.S 2494)	*laurenti* A
31	27	1 (LJAMM 2297)	*darwinii* N1
32	36	1 (LJAMM 2860)	sp. nov.
	35	1 (LJAMM 4018)	sp. nov.
33	33	1 (LJAMM 4008)	sp. nov.
	34	1 (LJAMM 4004)	sp. nov.
34	41	1 (MLP.S 2500)	*grosseorum*
35	40	1 (MLP.S 2498)	*laurenti*
36	39	2 (LJAMM 4161–4162)	*laurenti*
	37	1 (LJAMM 2334)	*laurenti*
37	40	1 (MLP.S 2497)	*laurenti*
	39	1 (LJAMM 4160)	*laurenti*
38	24	1 (LJAMM 4118)	*laurenti*
	39	1 (LJAMM 4164)	*laurenti*
39	40	1 (LJAMM 4210)	*laurenti*
40	25	1 (LJAMM 1984)	*darwinii* N2

APPENDIX 1. Continued.

Haplotype number	Locality number	Number of individuals	*Liolaemus* species
	24	1 (LJAMM 4117)	*laurenti* A
41	24	1 (LJAMM 4115)	*laurenti* A
42	26	1 (LJAMM 4187)	*darwinii* N2
43	25	1 (LJAMM 1983)	*darwinii* N2
44	24	2 (BYU 47320-FML 13083)	*darwinii* N2
45	39	1 (MLP.S 2495)	*laurenti* A
46	24	2 (MLP.S 2505/LJAMM 4116)	*darwinii* N2/*laurenti* A
	26	1 (LJAMM 4186)	*darwinii* N2
47	26	1 (MLP.S 2496)	*darwinii* N2
48	20	1 (BYU 47323)	*darwinii* N1
49	21	1 (BYU 47322)	*darwinii* N1

APPENDIX 2.

Specimens of Reference

Liolaemus darwinii.—Locality 1: BYU 47318, FML 13068, LJAMM 5(fn); 2: MLP.S 2484, LJAMM 9(fn); 3: LJAMM 18–19(fn), 3030; 4: MLP.S 2481–2483; 5: BYU 47325; 6: BYU 47327–47328; 7: LJAMM 2619, 2621; 8: BYU 47319, FML 13064, MLP.S 2485; 9: LJAMM 63–65(fn); 10: BYU 47326, FML 13067, LJAMM 3016; 11: LJAMM 72–74(fn); 12: BYU 47324, FML 13066, MLP.S 2489; 13: MLP.S 2487–2488; 14: LJAMM 2406–2410; 15: LJAMM 2641; 16: BYU 47317, FML 13065, MLP.S 2486; 17: LJAMM 2398–2399; 18: LJAMM 2400, 2402–2403; 19: BYU 47321, FML 13082, MLP.S 2506; 20: BYU 47323; 21: BYU 47322; FML 13084, MLP.S 2507; 22: LJAMM 2275–2276; 23: IMCN 8LA; 24: BYU 47320, FML 13083, MLP.S 2505; 25: LJAMM 1983–1984; 26: LJAMM 4186–4187, MLP.S 2496; 27: LJAMM 2295, 2297; 28: LJAMM 2287; 29: MLP.S 2492–2493; 31: LJAMM 4037. 32: LJAMM 4146–4147; 38: IMCN 19LA.

Liolaemus sp. nov.—Locality 33: LJAMM 4008: 34: LJAMM 4004; 35: LJAMM 4018; 36: LJAMM 2860.

Liolaemus grosseorum.—Locality 16: MLP.S 2490–2491; 30: LJAMM 4462; 31: LJAMM 4035–4036; 35: LJAMM 4020–4022, MLP.S 2504; 41: LJAMM 4026, MLP.S 2500; 42: LJAMM 4045–4046, 4048, MLP.S 2501; 43: LJAMM 4105, 4107–4109, 4112–4113, MLP.S 2502–2503; 44: LJAMM 416–417 (fn); 45: 4029–4030.

Liolaemus laurenti.—Locality 24: LJAMM 4115–4118; 37: LJAMM 2334; 39: LJAMM 4160–4162, 4164, MLP.S 2494–2495; 40: LJAMM 4210, MLP.S 2497–2498.

Outgroups.—Liolaemus olongasta: LJAMM 2378; *Liolaemus chacoensis*: MLP.S 2508; *Liolaemus uspallatensis*: LJAMM 4459; *Liolaemus koslowskyi*: LJAMM 2330; *Liolaemus abaucan*: LJAMM 2372; *Liolaemus cf ornatus*: MLP.S 2531; *Liolaemus boulengeri*: LJAMM 2187.