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# Phylogenetic analyses of mtDNA sequences reveal three cryptic lineages in the widespread neotropical frog *Leptodactylus fuscus* (Schneider, 1799) (Anura, Leptodactylidae)

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*Leptodactylus fuscus* is a neotropical frog ranging from Panamá to Argentina, to the east of the Andes mountains, and also inhabiting Margarita, Trinidad, and the Tobago islands. We performed phylogenetic analyses of 12S rRNA, 16S rRNA, tRNA-Leu, and ND1 mitochondrial (mt) DNA sequences from specimens collected across the geographic distribution of *L. fuscus* to examine two alternative hypotheses: (i) *L. fuscus* is a single, widely distributed species, or (ii) *L. fuscus* is a species complex. We tested statistically for geographic association and partitioning of genetic variation among mtDNA clades. The mtDNA data supported the hypothesis of several cryptic species within *L. fuscus.* Unlinked mtDNA and nuclear markers supported independently the distinctness of a 'northern' phylogenetic unit. In addition, the mtDNA data divided the southern populations into two clades that showed no sister relationship to each other, consistent with high differentiation and lack of gene flow among southern populations as suggested by allozyme data. Concordance between mtDNA and allozyme patterns suggests that cryptic speciation has occurred in *L. fuscus* without morphological or call differentiation. This study illustrates a case in which lineage splitting during the speciation process took place without divergence in reproductive isolation mechanisms (e.g. advertisement call in frogs), contrary to expectations predicted using a biological species framework. 

ADDITIONAL KEYWORDS: Amphibia – cryptic speciation – phylogeography – South America – species concept – species delimitation.

## INTRODUCTION

The leptodactylid frog *Leptodactylus fuscus* (Schneider, 1799) is distributed in the neotropical region ranging from Panamá to Argentina, to the east of the Andes mountains, as well as inhabiting the islands of Margarita, Trinidad and Tobago (Heyer & Reid, 2003). It occupies open habitats and is a good colonizer of river edges and recently modified habitats in forested regions (Wynn & Heyer, 2001). *L. fuscus* characterizes the specialized reproductive mode typical of the *L. fuscus* 'species group' that consists of the placement of a foam nest in an underground chamber (Heyer, 1978). Behavioural and physiological adaptations to water-stress conditions in marked seasonally dry climates have been found in larval and adult stages of *L. fuscus* (Downie, 1984, 1994a, b; Abe & Garcia, 1990; Downie & Smith, 2003).

The widespread distribution of *L. fuscus* across diverse climatic and physiographic zones has raised questions about the identity of *L. fuscus* as a single taxonomic unit. Heyer (1978) found morphometric differences between northern (Colombia and Panamá) and southern (Argentina) populations; however, he considered the differences to represent intraspecific

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geographic variation because of similar larval morphology and the lack of marked differences in the few advertisement call recordings available at the time. Karyotypic data also supported the single-species interpretation (Bogart, 1974), although geographic variation has been found subsequently in the morphology and banding patterns of chromosomes (Heyer & Diment, 1974; Silva, Haddad & Kasahara, 2000). In contrast, an immunological study suggested that more than one taxon might be hidden within *L. fuscus* (Maxson & Heyer, 1988).

Recently, the variabilities of 24 allozymic loci were screened for 16 populations throughout the geographic distribution of *L. fuscus* (Wynn & Heyer, 2001). The allozyme data suggested that populations of *L. fuscus* cluster into three genetic units: (i) Panamá, (ii) populations north of the Amazon River, and (iii) populations south of the Amazon River. Moreover, the allozyme data showed evidence of restricted gene flow among the southern populations. Although these authors could not determine the geographic boundaries between the genetic units, they concluded that *L. fuscus* is a complex of several species (Wynn & Heyer, 2001). Subsequently, the genetic units of *L. fuscus* were reconsidered using advertisement call characteristics that facilitate species recognition (Heyer & Reid, 2003). Call characteristics neither confirmed the three previously suggested genetic units nor suggested an alternative geographic partitioning of populations. Although advertisement call data supported the single-species hypothesis, Heyer & Reid (2003) argued that speciation in *L. fuscus* could have occurred without advertisement call differentiation.

In this study, we evaluated the relative support of mitochondrial (mt) DNA sequence data for two alternative hypotheses: (i) *L. fuscus* is a single widely distributed species, or (ii) *L. fuscus* is a species complex. We applied two complementary phylogenetic approaches to test these hypotheses: (i) a mtDNA tree-based method (Wiens & Penkrot, 2002; hereafter WP), and (ii) a phylogeographic nested clade analysis (Templeton, 1998, 2001; hereafter NCA). The WP method consists of a dichotomous protocol that makes species-level decisions based on the geographic exclusivity of mtDNA haplotypes. This tree-based method is effective in resolving species boundaries using a set of testable hypotheses about species limits. The WP protocol consists of: (1) assessing geographic differentiation through available representative localities for which most populations are represented by few samples, and (2) not assigning geographic variability a priori to discrete geographic units (as usually occurs in studies of well-sampled species). The geographic associations of mtDNA clades were tested using NCA, which has the potential to detect historical vicariant events and thus corroborate any proposed species units based on the WP method.

There is a growing number of species definitions and criteria to delimit species (reviewed by de Queiroz, 1998). We adopted the general lineage concept of species because of its focus on the concept of species as segments of population-level lineages (de Queiroz, 1998). A corollary criterion, compatible with the general lineage species concept, was utilized under the assumption that if lineage differentiation is occurring or has already occurred in *L. fuscus*, we should be able to detect these divergences and to reconstruct their genealogical relationships using mtDNA sequences.

MtDNA markers have been used widely to detect evolutionary lineages and to reconstruct their phylogenetic relationships (Avise, 2000). These markers are especially appropriate to address genealogies among closely related organisms, because they: (1) have high rates of evolution, (2) are maternally inherited, (3) have short allele coalescence times, and (4) are easily amplified using PCR techniques (Moritz, Dowling & Brown, 1987; Moore, 1995; but see caveats in Hoelzer, 1997). The phylogeographic patterns revealed in mtDNA lineages have been useful in resolving species limits in diverse taxa (Mulcahy & Mendelson, 2000; Riddle, Hafner & Alexander, 2000a, b; Dawood, Channing & Bogart, 2002; Wiens & Penkrot, 2002).

## MATERIAL AND METHODS

Frozen tissue samples of muscle and liver from the 16 populations of *L. fuscus* utilized by Wynn & Heyer (2001), plus an additional sample from Guyana, were included in this study. *L. mystaceus* and *L. mystacinus*, members of the *L. fuscus* species group, were used as outgroup taxa. Localities sampled (Fig. 1) and collection information of specimens utilized in this study are listed in the Appendix.

### DNA EXTRACTION, AMPLIFICATION, AND SEQUENCING

Total genomic DNA was extracted following the standardized protocols of Hillis *et al*. (1996). Fragments of the 12S ribosomal RNA (rRNA), 16S rRNA, the transfer RNA for leucine (tRNA-Leu), and ND1 mt genes were PCR amplified (Palumbi, 1996) using an MJ Research PTC-200 thermocycler. Double-stranded PCR amplifications were performed using the Promega Master Mix (Promega). A segment of about 400 base pairs (bp) from the 12S rDNA gene was amplified with primers 12Sa 5′-AAACTGGGATTA GATACCCCACTAT-3′ and 12Sb 5′-GAGGGTGA CGGGCGCTGTGT-3′ using the following thermal cycling conditions: initial denaturation at 94 °C for 2 min 30 s, followed by 30 cycles of (i) 94 °C for 1 min,


**Figure 1.** Map showing the localities sampled in this study. Dots correspond to localities grouped in basal clade A, triangles correspond to localities of basal clade B, and squares correspond to localities of basal clade C.

(ii) 53 °C for 1 min, and (iii) 72 °C for 1 min 30 s. A fragment approximately 2050 bp in length, including part of the 16S gene, tRNA-Leu, and part of the ND1 gene was assembled from two shorter fragments which had been amplified separately. The 5′-fragment was amplified using primers 16Sc 5′-GT(A/C)GGC CTAAAAGCAGCCAC-3′ and 16Sd 5′-CTCCGGTCT GAACTCAGATCACGTAG-3′ under the following conditions: initial denaturation at 94 °C for 2 min followed by 34 cycles of 94 °C for 1 min, 58 °C for 1 min, and 72 °C for 1 min 30 s. The primers ND1tmet 5′-TTGGGGTATGGGCCCAAAAGCT-3′ and ND116S 5′-TTACCCT(A/G)GGGATAACAGCGCAA-3′ were used to amplify the 3′-end under the following conditions: initial denaturation at 94 °C for 3 min, followed by 36 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min 30 s. Amplified segments were cleaned and purified using Wizard Preps DNA Purification System (Promega).

Purified products were cycle-sequenced with the dideoxy chain termination method using the SequiTherm Excel II DNA sequencing kit (Epicentre Technologies). Infrared-labelled primers were used in sequencing reactions (Licor Biotechnology) under the following conditions: initial denaturation at 95 °C for 2 min 30 s, followed by 30 cycles of 95 °C for 30 s, 58 °C for 30 s, and 70 °C for 30 s. In addition to the primers listed above, two internal primers were used in sequencing reactions only, 16SaR 5′-CGCCTGTT TACCAAAAACAT-3′ and ND1tmet2 5′-CCCTTTC(T/C)ATAGAAGTTCAAATCTTCTCG-3′, that anneal to approximately the middle of the 16Sc–16Sd and ND1tmet–ND116S fragments, respectively. Sequencing products were run in 6% and 4% acrylamide (44 cm and 66 cm in length, respectively) gels using a Licor DNA 4000 L automatic sequencer. Sequencing reactions were single stranded; double-stranded PCR fragments were sequenced in both directions.

 

GenBank accession numbers for the sequence data are AY905695–AY905717 for the 12S fragment and AY911264–AY911286 for the 16S + tRNA-Leu + ND1 fragment.

### Alignment and phylogenetic analyses

Sequences were first aligned using matching sequences from complementary strands. Then, the chromatographs generated in BaseImagr software (Licor Biotechnology) were inspected visually for mismatches of aligned positions to confirm or manually correct the automatic reading. Verified sequences were aligned with ClustalX (Thompson *et al.*, 1997) using the multiple alignment option. Alignments were improved considering secondary structure constraints (Kjer, 1995; Hickson *et al.*, 1996) using the models for the 12S and 16S genes of *Xenopus laevis* from R. R. Gutell's laboratory (Cannone *et al.*, 2002). Character incongruence among partitions of the dataset was evaluated with an incongruence length difference (ILD) test (Farris *et al.*, 1995) implemented as the partition-homogeneity test in PAUP* 4.0 (Swofford, 2002).

Maximum parsimony (MP) and maximum likelihood (ML) analyses were run as implemented in PAUP* 4.0 (Swofford, 2002). Weighted MP and the optimization of model parameters in ML followed a sensitivity analysis approach to detect the most stable clades under different analytical assumptions (Giribet, 2003). Character states were treated as unordered and gaps were considered as a fifth character; heuristic searches were performed with one hundred random additions of sequences and tree bisection-reconnection (TBR) branch swapping. Strict consensus trees were calculated when several equally parsimonious trees resulted from the MP searches. ML analyses were run using the general time-reversible model with a gamma distribution of across-site rate variation and an estimated proportion of invariable sites (GTR + $\Gamma$ + I, Swofford *et al.*, 1996). This model provided the best fit to our dataset as determined by ModelTest 3.0 (Posada & Crandall, 1998). ML was run applying a successive approximations approach, starting with a neighbour-joining tree, estimating model parameters with ML, and performing new searches using recalculated parameters from the previous run until log likelihoods converged on stable values. A final ML search with user-defined stabilized model parameter values was performed with a single random addition of sequences and TBR branch swapping.

Clade support of inferred trees was assessed by nonparametric bootstrapping (Felsenstein, 1985), based on 1000 pseudoreplicates (10 random additions on each pseudoreplicate) in MP and 100 pseudoreplicates (a single random addition on each pseudoreplicate) in ML to minimize computation time. We considered Bootstrap support of 70% or higher as strong support (Hillis & Bull, 1993).

Weighted MP analyses were run under two different schemes. First, the substitution bias toward transitions was considered separately for each of five data partitions (12S, 16S, and first, second, and third codon positions of ND1) using stepmatrices. To determine the codon positions, ND1 sequences were translated into proteins using MacClade 4.0 (Maddison & Maddison, 2000) and the reading frame was aligned with the complete ND1 sequence of *Rana nigromaculata* (Sumida *et al.*, 2001). Transition/transversion (ti : tv) ratios were estimated for each data partition using ML. The ti : tv weights applied to each data partition were: ti : tv = 1 : 4 (12S), ti : tv = 1 : 3 (16S), ti : tv = 1 : 6 (first codon position), ti : tv = 1 : 3 (second codon position), and ti : tv = 1 : 8 (third codon position). Second, loop positions in ribosomal sequences received twice the weight of stem positions whereas third codon positions of ND1 were downweighted once with respect to the other codon positions. The secondary structure models of 12S and 16S of *X. laevis* (Cannone *et al.*, 2002) were used to determine stem and loop positions in the dataset. Upon alignment with the ribosomal sequences of *X. laevis*, a total of 702 stem and 719 loop positions were determined in the dataset (available at http://learning.richmond.edu/Leptodactylus/matrices.html).

Phylogenetic analyses were also performed using a Bayesian inference approach as implemented in MrBayes 2.0 (Huelsenbeck & Ronquist, 2001). Bayesian analyses were run under the GTR + $\Gamma$ + I model with parameters estimated separately for each of six data partitions: 12S, 16S, tRNA-Leu, and first, second, and third codon positions of ND1. Four simultaneous Monte Carlo Markov Chains (MCMC) were run for 5 000 000 generations and trees were sampled every 100 generations. *L. mystacinus* was used as the outgroup in Bayesian analyses because this species was found to be more distantly related to *L. fuscus* compared with *L. mystaceus* based on our MP and ML analyses. Two independent runs were performed to check for convergence of log likelihoods in stationarity (Huelsenbeck & Ronquist, 2001; Leaché & Reeder, 2002). Convergence among different runs was evaluated by comparing the Bayesian support (posterior probability) of clades (Huelsenbeck & Imennov, 2002). Trees sampled in MCMC after the burn-in were summarized using 50% majority-rule consensus trees to obtain mean estimations of branch lengths and to calculate posterior probabilities of clades (Huelsenbeck & Ronquist, 2001).

### Phylogeographic analyses

ML and MP trees of the combined dataset were identical; we used the branch lengths of the unweighted

MP tree as a guide to make a nested clade design applying the nesting rules described in Templeton & Sing (1993) and Crandall (1996). The interior-tip status of clades was determined with outgroup rooting. The nested design was input in GeoDis 2.0 (Posada, Crandall & Templeton, 2000), supplying the geographic distances between localities to run a NCA based on 10 000 random permutations. NCA was performed on nesting clades that consisted of more than one locality; distinct localities were defined as samples collected from more than 40 km apart. Only the São Paulo sample had multiple individuals from a single locality. Significant associations between clades and geography were interpreted with the inference key published in Templeton, Routman & Phillips (1995) and updated on November 25th, 2003 in the GeoDis website (http://darwin.uvigo.es/software/geodis.html) to distinguish between demographic and historical factors.

In addition, significance of the association between geographic and genetic distances was examined with Mantel tests implemented in NTSYSpc software (Rohlf, 1998) using the raw statistic option with 1000 random permutations. We also performed an analysis of molecular variance (AMOVA) in Arlequin v2.0 (Schneider, Roessli & Excoffier, 2000) in order to assess the hierarchical structure of sequence variation distributed at three levels: within populations, among populations, and among major mtDNA clades as recovered in phylogenetic analyses. We ran 10 000 permutations of a pairwise matrix of uncorrected p-distances among haplotypes in order to obtain the null distributions and to test for the significance of variance components and $\phi$-statistics (Excoffier, Smouse & Quattro, 1992).

## RESULTS

### Sequence variation

Unique haplotypes were found at all localities, except for Arima and Nariva. Three out of five São Paulo individuals had the same haplotype (Table 1). The mean (± standard deviation) uncorrected distance among all localities was 4.1% ± 1.8%, with the greatest distances being those of the São Paulo vs. Palos Blancos and São Paulo vs. Guyana samples (7.0%) and the minimum distance being between the Arima and Icacos samples (0.0%) (Table 1). The maximum divergence within a population (São Paulo) was 0.9%. The mean distance between the ingroup and *L. mystaceus* was 11.3% ± 0.4% and between the ingroup and *L. mystacinus* it was 12.3% ± 0.4%; the uncorrected distance between the outgroup taxa was 13.2% (Table 1).

Genetic distances within *L. fuscus* revealed a pattern in which northern populations had less variation than did southern populations, although there was not a clear relationship between geographic and genetic distances on a smaller geographic scale. For example, the greatest geographic distances among Trinidad and Tobago, among Roraima, and between Argentinean samples were around 200 km. However, the maximum genetic distance among Trinidad and Tobago samples was 0.3% (which lies within the range of variation within a population), whereas the genetic distance among Roraima samples was 3.1%, and between Argentinean localities was 6.6%. This irregular geographic pattern across the species distribution caused a moderate but significant correlation between geographic and genetic distances ($r = 0.69$, $P = 0.001$). This raw measure of association suggests that some populations or groups of populations differed genetically more or less as expected by geographic distance alone. A phylogenetic analysis to assess the historical patterns of relationships among populations can be helpful when geographic distances do not account completely for the patterns of genetic differentiation.

### Phylogenetic relationships

The ILD test showed significant partition heterogeneity (10 000 replicates, $P = 0.001$) among the following two partitions: (1) the noncoding 12S and 16S gene sequences; (2) the protein-coding ND1 gene sequence. The tRNA segment was not included in the test. Consequently, we conducted separate parsimony analyses for the ribosomal, ND1, and combined sequences dataset to evaluate data partition incongruence (Wiens, 1998). The ribosomal dataset consisted of 1425 bp that included 306 (21.5%) variable positions – of which 116 (8.1%) were parsimony-informative – and 16 (1.1%) indels. The consensus of 19 most parsimonious trees of 369 steps (L = 369, and a consistency index, CI, of 0.718) only resolved a few clades composed of up to four samples. The ND1 dataset consisted of 955 bp, of which 323 (33.8%) were variable positions and 214 (22.4%) were parsimony informative. The ND1 data analysis resulted in a single most parsimonious tree (L = 633, CI = 0.649) that provided highly supported resolution at both deep and shallow divergences. The combined dataset consisted of 2453 bp, of which 555 (22.6%) were variable positions, including 333 (13.6%) parsimony-informative positions and 16 (0.7%) indels. The single MP tree recovered (L = 1062, CI = 0.642) was very similar to the trees found in weighted MP, ML, and Bayesian analyses (Fig. 2). MP weighting considering substitutional biases found four trees (L = 2095, CI = 0.747, not shown); two equally parsimonious trees (L = 1487, CI = 0.646, not shown) were recovered in the analysis considering secondary structure of ribosomal sequences and down-weighting third codon positions

 

**Table 1.** Absolute number of sequence differences (upper matrix) and uncorrected p-distances (lower matrix) between *Leptodactylus fuscus* populations and outgroup taxa based on the entire dataset

| | Norm | Toba | São1 | PaBl | Pará | Pana | Nari | JVGo | Icac | FrGu | Emba | Beni | Arim | BoVi | IgCo | Pern | São2 | São3 | São4 | São5 | Guya | Taci | Myce |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Norm | | 71 | 163 | 98 | 36 | 67 | 65 | 147 | 64 | 12 | 91 | 99 | 64 | 68 | 75 | 145 | 161 | 153 | 163 | 163 | 21 | 296 | 260 |
| Toba | 0.029 | | 152 | 101 | 85 | 61 | 8 | 151 | 7 | 73 | 93 | 104 | 7 | 59 | 60 | 138 | 151 | 143 | 152 | 152 | 70 | 294 | 263 |
| São1 | 0.067 | 0.062 | | 171 | 132 | 162 | 158 | 129 | 157 | 167 | 161 | 145 | 157 | 157 | 156 | 120 | 3 | 21 | 0 | 0 | 169 | 315 | 286 |
| PaBl | 0.040 | 0.041 | 0.070 | | 111 | 103 | 95 | 165 | 94 | 98 | 50 | 52 | 94 | 107 | 99 | 143 | 170 | 165 | 171 | 171 | 94 | 297 | 288 |
| Pará | 0.015 | 0.035 | 0.054 | 0.046 | | 85 | 79 | 137 | 78 | 44 | 99 | 92 | 78 | 85 | 92 | 150 | 130 | 122 | 132 | 132 | 52 | 297 | 267 |
| Pana | 0.027 | 0.025 | 0.066 | 0.042 | 0.035 | | 55 | 163 | 54 | 67 | 96 | 109 | 54 | 21 | 69 | 143 | 161 | 156 | 162 | 162 | 68 | 295 | 271 |
| Nari | 0.027 | 0.003 | 0.065 | 0.039 | 0.032 | 0.023 | | 150 | 1 | 67 | 87 | 98 | 1 | 53 | 54 | 138 | 157 | 149 | 158 | 158 | 64 | 296 | 265 |
| JVGo | 0.060 | 0.062 | 0.053 | 0.068 | 0.056 | 0.067 | 0.062 | | 151 | 153 | 162 | 152 | 151 | 160 | 161 | 142 | 128 | 126 | 129 | 129 | 154 | 296 | 291 |
| Icac | 0.026 | 0.003 | 0.064 | 0.039 | 0.032 | 0.022 | 0.000 | 0.062 | | 66 | 86 | 97 | 0 | 52 | 53 | 139 | 156 | 148 | 157 | 157 | 63 | 295 | 264 |
| FrGu | 0.005 | 0.030 | 0.068 | 0.040 | 0.018 | 0.027 | 0.027 | 0.063 | 0.027 | | 93 | 103 | 66 | 68 | 77 | 142 | 165 | 159 | 167 | 167 | 25 | 297 | 262 |
| Emba | 0.037 | 0.038 | 0.066 | 0.020 | 0.041 | 0.039 | 0.036 | 0.066 | 0.035 | 0.038 | | 58 | 86 | 95 | 89 | 133 | 160 | 153 | 161 | 161 | 85 | 286 | 274 |
| Beni | 0.041 | 0.043 | 0.059 | 0.021 | 0.038 | 0.045 | 0.040 | 0.062 | 0.040 | 0.042 | 0.024 | | 97 | 109 | 103 | 154 | 144 | 139 | 145 | 145 | 99 | 281 | 289 |
| Arim | 0.026 | 0.003 | 0.064 | 0.039 | 0.032 | 0.022 | 0.000 | 0.062 | 0.000 | 0.027 | 0.035 | 0.040 | | 52 | 53 | 139 | 156 | 148 | 157 | 157 | 63 | 295 | 264 |
| BoVi | 0.028 | 0.024 | 0.064 | 0.044 | 0.035 | 0.009 | 0.022 | 0.066 | 0.021 | 0.028 | 0.039 | 0.045 | 0.021 | | 49 | 144 | 156 | 151 | 157 | 157 | 69 | 294 | 270 |
| IgCo | 0.031 | 0.025 | 0.064 | 0.041 | 0.038 | 0.028 | 0.022 | 0.066 | 0.022 | 0.032 | 0.036 | 0.042 | 0.022 | 0.020 | | 131 | 155 | 149 | 156 | 156 | 76 | 299 | 268 |
| Pern | 0.059 | 0.057 | 0.049 | 0.059 | 0.062 | 0.059 | 0.057 | 0.058 | 0.057 | 0.058 | 0.055 | 0.063 | 0.057 | 0.059 | 0.054 | | 119 | 115 | 120 | 120 | 150 | 302 | 282 |
| São2 | 0.066 | 0.062 | 0.001 | 0.070 | 0.053 | 0.066 | 0.064 | 0.053 | 0.064 | 0.068 | 0.066 | 0.059 | 0.064 | 0.064 | 0.064 | 0.049 | | 20 | 3 | 3 | 167 | 314 | 285 |
| São3 | 0.063 | 0.059 | 0.009 | 0.068 | 0.050 | 0.064 | 0.061 | 0.052 | 0.061 | 0.065 | 0.063 | 0.057 | 0.061 | 0.062 | 0.061 | 0.047 | 0.008 | | 21 | 21 | 161 | 309 | 280 |
| São4 | 0.067 | 0.062 | 0.000 | 0.070 | 0.054 | 0.066 | 0.065 | 0.053 | 0.064 | 0.068 | 0.066 | 0.059 | 0.064 | 0.064 | 0.064 | 0.049 | 0.001 | 0.009 | | 0 | 169 | 315 | 286 |
| São5 | 0.067 | 0.062 | 0.000 | 0.070 | 0.054 | 0.066 | 0.065 | 0.053 | 0.064 | 0.068 | 0.066 | 0.059 | 0.064 | 0.064 | 0.064 | 0.049 | 0.001 | 0.009 | 0.000 | | 169 | 315 | 286 |
| Guya | 0.009 | 0.029 | 0.070 | 0.039 | 0.021 | 0.028 | 0.026 | 0.063 | 0.026 | 0.010 | 0.035 | 0.041 | 0.026 | 0.028 | 0.031 | 0.062 | 0.069 | 0.066 | 0.070 | 0.070 | | 293 | 265 |
| Taci | 0.121 | 0.121 | 0.129 | 0.122 | 0.122 | 0.121 | 0.121 | 0.121 | 0.121 | 0.122 | 0.117 | 0.115 | 0.121 | 0.121 | 0.123 | 0.124 | 0.129 | 0.127 | 0.129 | 0.129 | 0.121 | | 321 |
| Myce | 0.107 | 0.108 | 0.118 | 0.118 | 0.110 | 0.112 | 0.109 | 0.120 | 0.109 | 0.108 | 0.113 | 0.119 | 0.109 | 0.111 | 0.110 | 0.116 | 0.117 | 0.115 | 0.118 | 0.118 | 0.109 | 0.132 | |

Abbreviations for taxa are shown in Appendix.

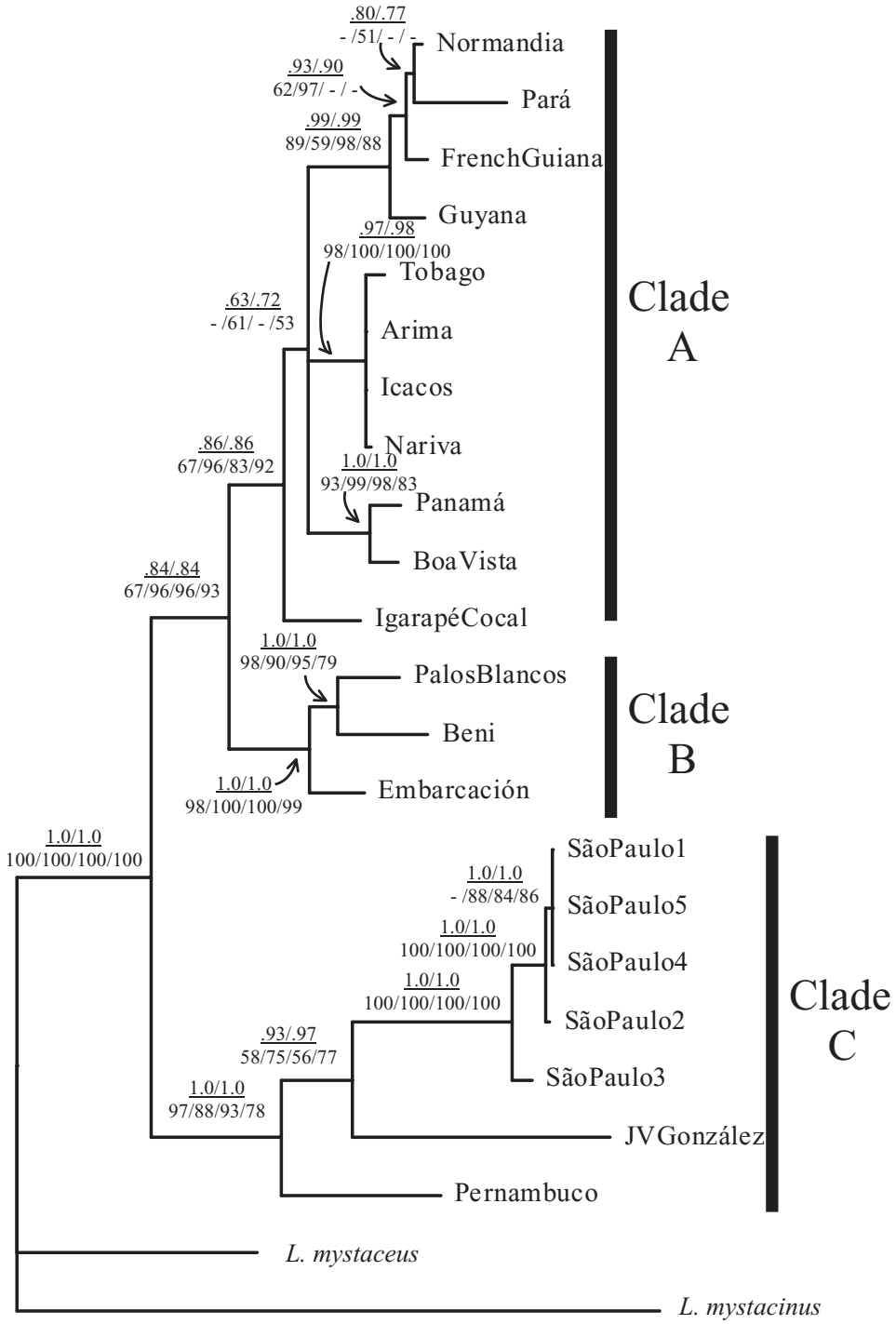


**Figure 2.** Consensus tree (50% majority-rule) from Bayesian analysis. Numbers above the branches are clade posterior probability values derived from two independent runs; values below branches are bootstrap values (> 50%) derived from maximum likelihood (ML)/unweighted maximum parsimony (MP)/weighted transition vs. transversion MP/weighted secondary structure and codon positions MP.

 

of coding sequences. The Bayesian trees found in the two independent MCMC runs resulted from 50% majority-rule consensus trees of the last 30 000 trees. In both runs, the log likelihoods in stationarity converged after 100 000 generations; consequently, the first 20 000 trees were discarded. The topologies reconstructed in both runs were identical and branch support values were very similar, suggesting convergence in independent analyses (Fig. 2).

The tree using ribosomal sequences provided less resolution than did the tree from the combined dataset. Furthermore, it conflicted with the combined tree in having strong support for a [Boa Vista–Igarapé Cocal] clade, not found in the combined results (Fig. 2). The parsimony results for the ND1 data showed comparable resolution with the combined results and differed only in grouping São Paulo either with the Pernambuco or J. V. González samples (Fig. 2). Thus, applying Wiens's (1998) protocol, analyses of separate data partitions only identified two conflicts which were localized within two major clades supported in the combined-data analysis. However, the three deepest nodes, i.e. the three identified major clades, of the combined analyses were not in conflict with the separate data analyses, although, relationships within these clades were partially unresolved.

All analyses of the combined dataset recovered the same basic topology; consequently we describe phylogenetic relationships based on the Bayesian tree (Fig. 2) and mention deviations from this pattern. The ingroup was recovered as a strongly supported monophyletic clade in all analyses. Three major clades in the ingroup were recovered in all analyses: A, B, and C; among these, clades A and B showed closer relationships to each other. Clade A consisted of all samples from northern South America (north of the Amazon river) including Panama, Trinidad, and Tobago, with the exception of the Pará sample (Fig. 1). Clade A was supported strongly in all MP analyses and supported moderately in ML and Bayesian analyses. Populations from Beni and Palos Blancos (Bolivia) were sister taxa and formed a monophyletic group, clade B, with the Embarcación (Argentina) population. Clade B was restricted to western South America, south of the Amazon River (Fig. 1). Clade B was supported strongly in all analyses whereas clade [A + B] was recovered with high support in MP and moderate support in ML and Bayesian analyses. Clade C consisted of the Pernambuco, J. V. González, and São Paulo localities and represented the eastern, south-western, and southern borders of the species range (Fig. 1). Clade C was supported strongly in all analyses. Three haplotypes were found among five individuals in São Paulo, which formed a strongly supported cluster with slight differences among haplotypes (0.0%, 0.1% and 0.9%). Major clades A, B and C did not overlap geographically, although clades B and C may be parapatric.

Within the large clade A, a distinct insular subclade consisting of populations in Trinidad and Tobago islands was recovered in all phylogenetic analyses (subclade A1). Although relationships within this insular subclade were not resolved, samples from Trinidad were genetically more similar to each other than they were to Tobago (Table 1). The greatest variation within this insular group (0.3%) was lower than was the intrapopulation variation found in São Paulo; furthermore, the smallest genetic distance between samples of this insular subclade (Arima and Icacos) and a mainland population (Boa Vista) was 2.2%. Two other subclades within clade A were found in all analyses: one subclade grouped Normandia, Pará, French Guiana, and Guyana (subclade A2) while the other subclade included Panamá and Boa Vista (subclade A3). The other population from the State of Roraima (Brazil), Igarapé Cocal, was basal in clade A except in the ti : tv weighted MP analysis, in which Igarapé Cocal formed a polytomy with the three subclades (Fig. 2).

### Phylogeographic analyses

The complete nested design used in the NCA is available to view at http://learning.richmond.edu/Leptodactylus/matrices.html. The NCA rejected the null hypothesis of no geographic association of clades in: (i) clade 7-2, and (ii) the total cladogram (Fig. 3). We used the GeoDis inference key to determine what kind of demographic processes and/or historical events may have caused the significant associations. The permutation tests performed for clade 7-2 indicated that significant effects were as a result of the distance patterns of nested clade 6-3 only. Clade 6-3, including samples from Tobago, Trinidad, and Igarapé Cocal, showed both lower clade (Dc) and nested clade (Dn) distances than would be expected randomly. However, as interior-tip status of clades could not be determined from the nested design (all three nested clades containing samples were tip clades), we could not go any further with the inference key, resulting in an inconclusive outcome. The permutation analysis of the total cladogram revealed significant effects in tip clades as well as in the test of interior vs. tip clades. Nested clade 7-1, consisting of all samples north of the Amazon River except the samples in nested clade 7-2, showed lower Dc distances than would be expected randomly. The other tip clade consisting of J. V. González and São Paulo samples, nested clade 7-3, showed significantly lower Dc and higher Dn distances than would be expected by chance alone. The test of interior vs. tip clades (7-1 and 7-3 were tip clades and 7-2 was interior) found

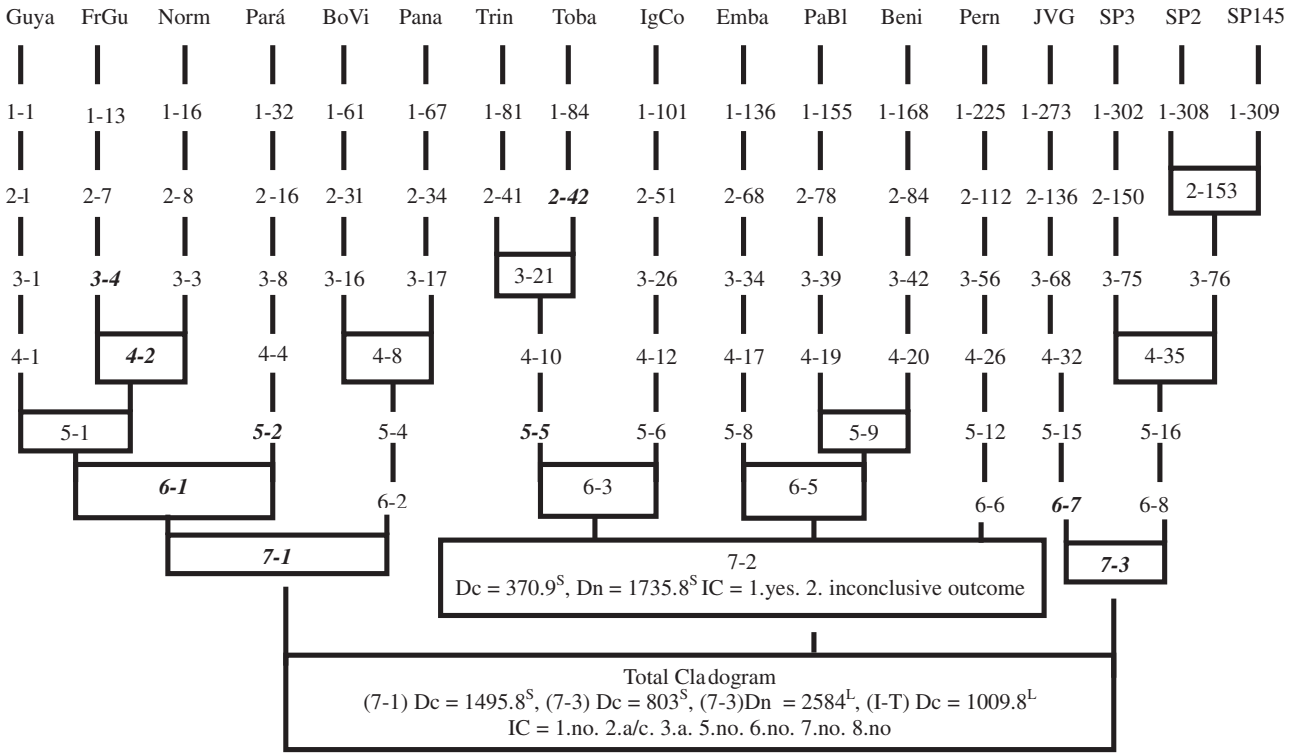


**Figure 3.** Nested clade analysis scheme. The numbering of clades refers to the nested design available to view at http://learning.richmond.edu/Leptodactylus/matrices.html. For clades showing significant association, the clade distance (Dc) and the nested clade distance (Dn) values as used in the inference chain (IC) are provided. $^{S}$ indicates distances significantly smaller than expected and $^{L}$ indicates distances significantly larger than expected; I-T indicates interior vs. tip distances, and the numbers of tip clades are italicized when their status could be determined from the nested design.

significantly higher Dc distances than would be expected randomly. Based on these significant associations and the patterns of geographic overlap among clades, the inference key did not allow discrimination between isolation by distance and long distance dispersal resulting from inadequate geographic sampling (lack of samples from intermediate areas between clades 7-1, 7-2, and 7-3).

An AMOVA was run to test for the significance of molecular variance statistics at three hierarchical levels: within populations, among populations within major clades, and among major clades. The AMOVA results showed significant differentiation among major clades and among populations within clades (Table 2). Variation among major clades accounted for about half of the total variation across the distributional range of *L. fuscus*, whereas the variation among populations within major clades accounted for 43%. Mantel tests found significant, low matrix correlations between patristic mtDNA distances and geography ($r = 0.63$, $P = 0.001$) and between patristic mtDNA distances and Nei's distances ($r = 0.52$, $P = 0.001$) based on the allozyme data.

## DISCUSSION

### PHYLOGENETIC UNITS

All phylogenetic analyses performed under a variety of assumptions about DNA sequence evolution recovered three basal clades within *L. fuscus*. The basal clades did not overlap geographically suggesting that these three phylogenetic units are allopatric, although geographic sampling is necessary to verify this assertion. In particular, clades B and C seemed to approach each other in north-western Argentina and further sampling in this area promises to give insight into the degree of genetic isolation between the units. Fine-grained, multiple-character geographic sampling will be necessary to detect either abrupt or gradual changes in genetic patterns in contact zones between phylogenetic units. Genetic distances among basal clades of *L. fuscus* were higher than those values reported as indicative of interspecific differentiation in frogs. For example, intraspecific divergence in 16S mt sequences of morphologically, ecologically, and bioacoustically distinct species of Malagasy frogs never exceeded 2% (Vences *et al*., 2004). Furthermore, these

 

**Table 2.** Results of AMOVA applied on a hierarchical design in which populations were assigned to three groups representing basal clades A, B, and C as recovered in phylogenetic analyses

| Source of variation | d.f. | $\phi$-statistics | Variance components | Percentage of variation |
|---|---|---|---|---|
| Among major clades | 2 | 0.512 | 33.092* | 49.73 |
| Among populations within major clades | 14 | 0.860 | 28.855* | 43.36 |
| Within populations | 4 | 0.932 | 4.600* | 6.91 |

*$P < 0.005$.

authors suggest that the pairwise distances from 16S mt sequences among the basal clades in their study of *Ptychadena mascareniensis* represent cryptic species. In *L. fuscus*, average distances of 16S mt sequences among different basal clades were: 2.26% between clades A and B and 2.95% between clades B and C, and between clades C and A; consequently, in *L. fuscus* differentiation among basal clades has surpassed the 2% threshold of intraspecific variation for this mtDNA marker. Furthermore, genetic distances between clades using all available sequences in our study were: 3.99% between clades A and B, 6.31% between clades B and C, and 6.18% between clades C and A.

A phylogenetic approach has been used widely to uncover diagnosable phylogenetic units in mtDNA phylogenies of several cryptic taxa (Ashton & de Queiroz, 2001; Burbrink, 2002; Feldman & Spicer, 2002; Masta *et al*., 2002; Wiens & Penkrot, 2002; Zaldívar-Riverón, León-Regagnon & Nieto-Montes de Oca, 2004). As an example, the WP method applies a phylogenetic criterion to propose species boundaries considering geographically separated mtDNA clades as different phylogenetic species (Wiens & Penkrot, 2002). The application of the WP method on all phylogenetic trees of *L. fuscus* resulted in the following decisions: (i) the focal species (*L. fuscus*) comprised an exclusive set of haplotypes (i.e. it is monophyletic relative to the outgroup taxa), and (ii) there were three well-supported basal clades (A, B, and C) concordant with geography (clades did not overlap). We did not have multiple samples available to test for haplotype exclusivity of all localities (except for São Paulo) and therefore to evaluate the occurrence of gene flow among them. However, this sampling limitation did not contradict the fact that the major clades of *L. fuscus* were deep, well-supported clades of haplotypes that did not overlap geographically. Thus, based on this phylogenetic approach, the phylogenetic analyses of mtDNA supported the hypothesis of *L. fuscus* as a 'species complex'. The degree of support of the mtDNA data for several phylogenetic species within *L. fuscus* decreased in the following order: (i) two phylogenetic species consisting of the clades [A + B] and C; (ii) three phylogenetic species represented by each of the three clades A, B, and C; (iii) a fourth phylogenetic species restricted to the Trinidad and Tobago islands.

### Association between mtDNA variation and geography

The NCA analyses did not reveal any significant association of mtDNA clades with geography consistent with a pattern of historical fragmentation of populations. Indeed, the NCA analyses could not distinguish between different populations' processes to account for the distance patterns that occurred at the highest nesting levels that are supposed to provide high statistical power. Thus, the phylogeographic analysis did not corroborate the species delimitation proposed by the WP method based on a phylogenetic criterion, although this result by itself does not refute the hypothesis of multiple species within *L. fuscus*. It is important to note that the nested design did not allow us to contrast against each other the major clades, and it is precisely these phylogenetic units that could be proposed as different species. When a separate nested design was done for each major clade and then the three major clades were nested at the highest nesting level, the outcome of the NCA was inconclusive (results not shown). In this case, the inference key established that the sampling was inadequate because major clades did not overlap geographically and the species occurred in the nonsampled areas between the clades. Thus, the available samples limited the efficiency of this phylogeographic analysis to corroborate or reject the distinctiveness of the three phylogenetic units.

Beyond these results, the NCA analyses did find significant association patterns at the highest nesting level and within clade A. In the first case, the permutation tests indicated that samples from Trinidad, Tobago, and Igarapé Cocal (clade 6-3) were closer to each other than would be expected by random chance. However, the clustering of the inland sample, Igarapé Cocal, with the insular samples of nested clade 7-2 resulted in clade 6-3 appearing closer to the other nested clades (6-1 and 6-3) than would be expected by random chance. Supposing that we were to include all

insular samples in a single clade (likely producing a low Dc and a high Dn), the lack of samples from the mainland, just across from Trinidad, would prevent us from inferring fragmentation between the insular and mainland populations. In the second case, the permutation tests indicated that (i) most samples from northern South America were significantly close to each other, and (ii) that J. V. González and São Paulo (clade 7-3) were significantly close to each other but relatively far from the other clades (7-1 and 7-2) (an effect probably resulting from the multisampled São Paulo locality). In addition, the tip vs. interior tests found larger Dc distances than would be expected by random chance.

The NCA found significant associations between cladistic patterns and geography at several nesting levels because some clades were closer or further away than would be expected by chance. The cladistic patterns within basal clades A, B, and C did not correlate well with geographic distances as indicated by a Mantel test performed on patristic mtDNA distances. Although there was a lack of geographic structuring within the basal clades, the similar partitioning of genetic variability within and among clades may have obscured the underlying, deep differentiation among basal clades A, B, and C. The AMOVA results indicated substantial interclade and interpopulational differentiation within clades with variability among basal clades being just greater than that found within those clades. This suggests that the differentiation within each basal clade has been so large that it masks their relationships. Consequently, the historical, genealogical diversification into three mtDNA units (clades A, B, and C) could have been blurred in the phylogeographic analyses while still being recovered in phylogenetic trees.

## Concordance between mtDNA and other datasets

The distinction of different species using mtDNA genealogies alone can be misleading if not corroborated by independent datasets (Moritz, 1994; Wake & Jockusch, 2000; Jockusch & Wake, 2002). The evidence in favour of several phylogenetic units within *L. fuscus* would be strengthened if our mtDNA-defined units were concordant with patterns of variation found in other datasets (Sites & Crandall, 1997). There are three alternative sources of information with which to compare our mtDNA data: allozymic (Wynn & Heyer, 2001), morphological (Heyer, 1978), and advertisement call data (Heyer & Reid, 2003).

### *Allozymes and mtDNA integration*

Comparing allozyme and mtDNA markers has been very useful in understanding historical vs. current patterns of genetic cohesion among and within species units in other amphibian studies (Shaffer & McKnight, 1996; Wake & Schneider, 1998; Donnellan *et al*., 1999; Garcia-Paris *et al*., 2000; Wake & Jockusch, 2000; Mead, Tilley & Katz, 2001; Jockusch & Wake, 2002).

All localities sampled for allozyme variation in *L. fuscus* were also included in the mtDNA analysis. A detailed comparison of the mtDNA and allozyme-based trees revealed a general agreement between both datasets on a continental scale (Fig. 4). This north-to-south pattern of differentiation throughout the range distribution was consistent with an overall low but significant association between both datasets in the Mantel test. Samples from northern South America, Panamá, Trinidad, and Tobago formed a cluster in the phylogenetic analysis using allele frequency data (Fig. 4A, Wynn & Heyer, 2001); this cluster was almost identical to basal clade A (Guyana was not included in the allozyme analyses). In addition, the low Nei's distances among these northern samples were consistent with the shallow topology of clade A in the mtDNA analysis. However, relationships within the 'northern' allozymic unit differed from those within clade A, reflecting a lack of concordance between datasets on a regional scale. For example, in the allozyme results, Tobago clustered with mainland samples and not with Trinidad, and the Roraima samples appeared more closely related to each other than in mtDNA-based clade A. However, the relationship between Tobago and mainland samples derived from allele frequencies rather than from discrete character changes. The Trinidad samples formed a cluster in the allozyme analysis that was consistent with the mtDNA data (Table 1).

In both datasets, the southern samples showed greater divergence among themselves than did the northern samples. This was reflected in their separation into two nonsister basal clades (clades B and C) in the mtDNA analysis and the lack of consistent clustering of southern populations (because of substantial genetic isolation) in the allozyme analyses.

The mtDNA-defined basal clades B and C were not recovered in the allozyme analysis since the J. V. González sample formed a highly supported clade with the Embarcación sample. Furthermore, the ML bootstrap of the allozyme data only provided convincing support for the two Argentinean samples, in strong contrast to our mtDNA results. Beyond sampling problems that could affect the comparisons, it is interesting to notice that these two localities, only 160 km apart, are close to a potential contact zone between mtDNA clades B and C. Previous studies have found asymmetrical geographic patterns in nuclear- vs. mt-linked markers in areas believed to represent contact zones between divergent phylogenetic units (Wake,

 

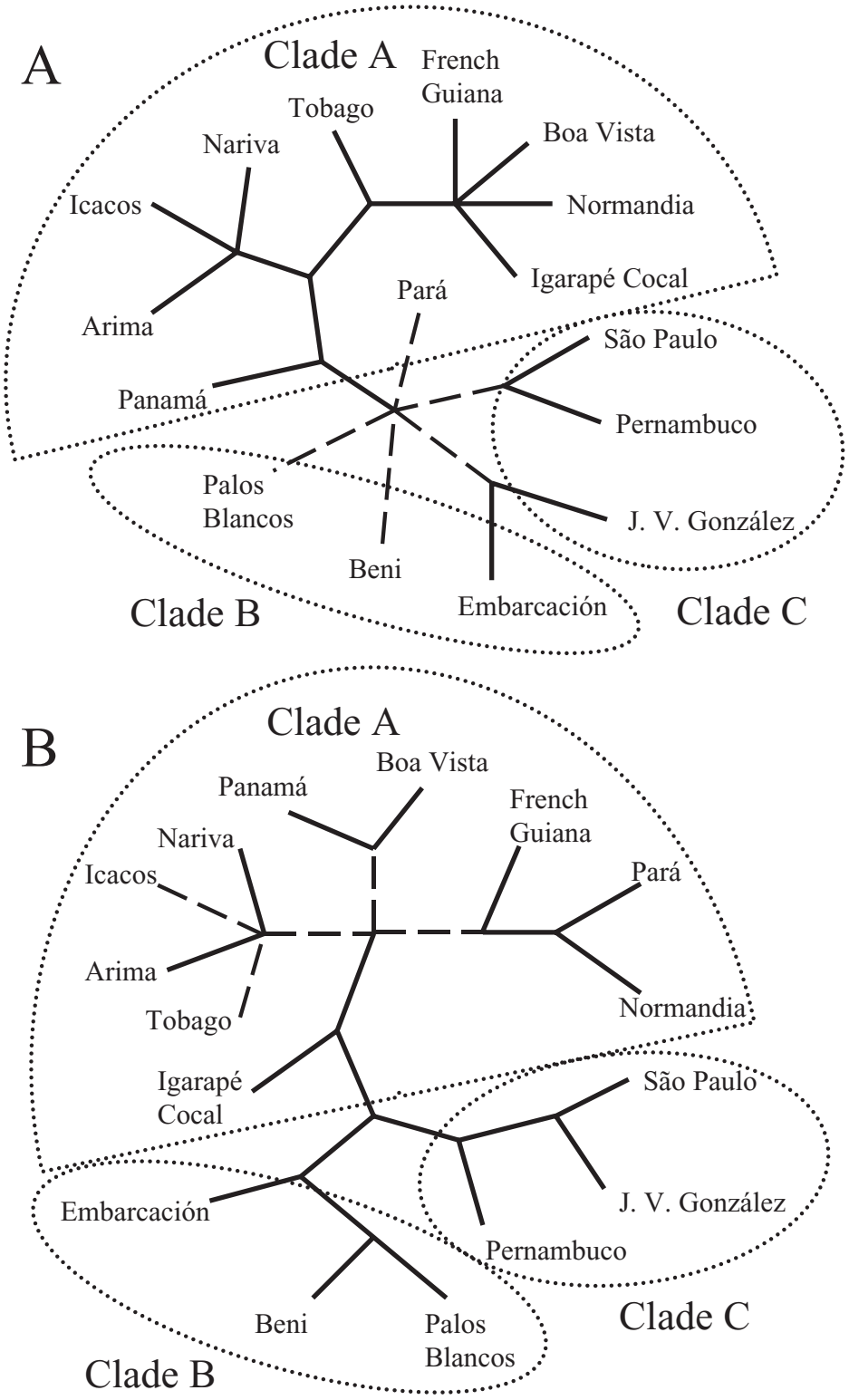


**Figure 4.** Phylogenetic relationships among the populations sampled for allozyme and mtDNA data. A, Strict consensus of the three networks reported by Wynn & Heyer (2001) based on allozymes. Dashed branches represent disagreement among the networks. B, Strict consensus of Bayesian, maximum likelihood, and maximum parsimony trees of combined data reported in this study. For simplification, São Paulo is represented by a single sample and Guyana is excluded because it was not sampled in the allozyme study.

1997; Mead *et al*., 2001). Thus, instead of considering these conflicting patterns as a methodological problem, these asymmetries are usually interpreted as isolation and differentiation of genetic units with subsequent contact between them (Wake & Schneider, 1998; Wake & Jockusch, 2000; Jockusch & Wake, 2002).

Both molecular markers supported the distinctiveness of a rather homogeneous 'northern' phylogenetic unit with low genetic distances among the samples across northern South America that remain connected by gene flow. Allozymes placed southern populations in a deeply differentiated cluster with reduced or no gene flow among many of the samples suggesting the presence of more than one lineage in southern South America. The mtDNA analysis also showed marked differentiation among southern samples and supported their separation into two basal and nonsister clades, clades B and C. Clade B was more closely related to the 'northern' A clade than it was to clade C.

In summary, both datasets supported the existence of a phylogenetic unit within *L. fuscus* restricted to northern South America. In addition, the allozyme data was consistent with the mtDNA data in that there were deep divergences among the samples south of the Amazon River. The mtDNA analyses suggested a split of southern samples into two additional phylogenetic units, one restricted to the western portion of southern South America and the other occupying the southern and south-eastern parts of the geographic distribution of *L. fuscus* (with the exception of the J. V. González sample). The lack of agreement between these datasets regarding the insular samples precluded recognition of a distinct phylogenetic unit restricted to Trinidad and Tobago. The mtDNA differentiation among Trinidad and Tobago populations and their distinctness from mainland samples requires further sampling to elucidate their status.

### *Morphology, advertisement call, and mtDNA integration*

Morphological variation within *L. fuscus* is relatively extensive and similar to that of other widely distributed taxa of the *L. fuscus* species group (Heyer, 1978). Previous morphological analysis (Heyer, 1978) revealed poor geographic structuring of populations except for the discrimination of some peripheral localities such as Colombia, Panamá, and Argentina. Although morphological differentiation occurs in the periphery of its geographic distribution, the variation is continuous without discrete breaks preventing recognition of diagnosable units. Although this peripheral distinctiveness in morphology is, at most, compatible with the genetic units, the overall morphological variation does not show the level of geographic structuring seen in the allozyme and mtDNA data. Likewise, variation in advertisement calls throughout the distribution of *L. fuscus* does not show geographic structure, since variability within a locality is equal to or greater than the variability between localities (Heyer & Reid, 2003). Moreover, there is much less variation in the call features of *L. fuscus* than that found among closely related species of the *L. fuscus* species group.

The morphological and advertisement call variation in *L. fuscus* is characterized by continuous change throughout its geographic distribution. Consequently, both types of data support the hypothesis that *L. fuscus* is a single widely distributed taxon. Concordance between morphology and advertisement call differentiation patterns across taxonomic boundaries represents the common pattern for the genus *Leptodactylus* (Heyer, 1978). However, there are exceptions to this pattern, with two morphologically identical species that differ strikingly in their calls (Heyer, García-Lopez & Cardoso, 1996; Kwet, Di-Bernardo & Garcia, 2001) and are not sister taxa (de Sá, Heyer & Camargo, 2005). The mate recognition information carried by advertisement call features serves an important role in assessing species boundaries among frogs (Gerhardt, 1988). Nevertheless, there are a few exceptions among frogs, with genetic data revealing a lack of correlation between speciation and advertisement call characteristics (Sullivan, Malmos & Given, 1996; Gergus, Sullivan & Malmos, 1997; Gergus, 1998). For example, there are cases in which speciation processes in frogs apparently occur without divergence in advertisement call features. Recently, Heyer & Reid (2003) cited examples where differentiated genetic units occurring in allopatry have not come into secondary contact and their advertisement calls have not diverged. In other cases, isolation mechanisms other than advertisement call may be involved in maintaining species identity in spite of the occasional production of viable hybrids in contact zones (e.g. *Bufo microscaphus* complex, Gergus *et al*., 1997). A lack of divergence in advertisement calls among closely related species occurring in allopatry or parapatry could be the result of selective pressures imposed by a similar acoustic environment (Gergus *et al*., 1997). Similar behavioural aspects of the reproductive biology of *L. fuscus* at several localities suggest that the populations share a common acoustic environment across the geographic distribution (Sazima, 1975; Solano, 1987; Martins, 1988; Rossa-Feres, Menin & Izzo, 1999). Thus, the advertisement call is not always a reliable indicator of species-level differentiation, as is generally assumed in frog systematics. However, these cases are perfectly congruent with a lineage concept of species because divergence in the two daughter lineages can occur in a set of characters that does not affect directly their reproductive

 

compatibility. De Queiroz (1998) advises that the most effective criterion to detect newly originated lineages will differ according to how the speciation process occurred. A biological species criterion based on a behavioural premating isolation mechanism (e.g. advertisement call) that is under strong selective pressure will not always be as efficient in detecting splitting of lineages compared with other characters that started to diverge as soon as the daughter lineages became genetically isolated, such as neutral molecular markers. We argue that *L. fuscus* exemplifies a case in evolutionary biology in which speciation is not necessarily linked to changes in a premating isolation mechanism as required by a biological species criterion. However, Wynn & Heyer (2001) noted that allozymic Nei's distances within *L. fuscus* suggest that genetic differentiation is high enough to allow the evolution of post zygotic isolation, i.e. it surpasses the threshold of Nei's distance = 0.3 for hybrid inviability (Sasa, Chippindale & Johnson, 1998). The case of *L. fuscus* also shows that the practice of species delimitation demands the use of (i) multiple types of data (even if incongruent), and (ii) a phylogenetic approach to address appropriately cases in which the speciation process has resulted in the origin of cryptic taxa in terms of both morphology and advertisement calls.

## CONCLUSIONS

Non-molecular data (e.g. morphology and advertisement calls) support a single-species hypothesis for *L. fuscus*, whereas molecular data (e.g. mtDNA and allozymes) support a multiple-species hypothesis. Based on the molecular data, we could assign the three mtDNA basal clades A, B and C of *L. fuscus* occurring in northern, southern and south-eastern, and western South America, respectively, to different phylogenetic units that are following independent evolutionary trajectories, consistent with different species under the general lineage concept of species. However, the absence of morphological or call diagnostic characters for these clades, our limited geographic sampling, and the uncertainty about current levels of gene flow among them, does not warrant at this time the assignment of formal taxonomic status to these phylogenetic units. This study offers a hypothesis of species delimitation of *L. fuscus* into three geographic lineages to be tested with further field, natural history, and molecular data. The single new dataset with the highest probability of providing lineage resolution is mt and nuclear DNA markers of an intensive geographic sampling transect between J. V. González and Embarcación, Argentina.

*L. fuscus* probably exemplifies a case in species-level systematics where species delimitation cannot be approached from the usual, biological-species perspective because lineage diversification occurs in characters that are not associated with the reproductive isolation/recognition system.

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## REFERENCES

**Abe AS, Garcia LS. 1990.** Changes in the body fluids of the frog *Leptodactylus fuscus* during aestivation (Anura, Leptodactylidae). *Revista Brasileira de Biologia* **50:** 243–247.

**Ashton KG, de Queiroz A. 2001.** Molecular systematics of the western rattlesnake, *Crotalus viridis* (Viperidae), with comments on the utility of the D-loop in phylogenetic studies of snakes. *Molecular Phylogenetics and Evolution* **21:** 176–189.

**Avise JC. 2000.** *Phylogeography: the history and formation of species*. Cambridge, MA: Harvard University Press.

**Bogart JP. 1974.** A karyosystematic study of frogs in the genus *Leptodactylus* (Anura: Leptodactylidae). *Copeia* **1974:** 728–737.

**Burbrink FT. 2002.** Phylogeographic analysis of the cornsnake (*Elaphe guttata*) complex as inferred from maximum likelihood and Bayesian analyses. *Molecular Phylogenetics and Evolution* **25:** 465–476.

**Cannone JJ, Subramanian S, Schnare MN, Collett JR, D'Souza LM, Du Y, Feng B, Lin N, Madabusi LV, Müller KM, Pande N, Shang Z, Yu N, Gutell RR. 2002.** The comparative RNA web (CRW) site: an online database

of comparative sequence and structure information for ribosomal, intron, and other RNAs. *BioMed Central Bioinformatics* **3:** 2.

**Crandall KA. 1996.** Multiple interspecies transmissions of human and simian T-cell leukemia/lymphoma virus type I sequences. *Molecular Biology and Evolution* **134:** 115–131.

**Dawood A, Channing A, Bogart JP. 2002.** A molecular phylogeny of the frog genus *Tomopterna* in Southern Africa: examining species boundaries with mitochondrial 12S rRNA sequence data. *Molecular Phylogenetics and Evolution* **22:** 407–413.

**de Queiroz K. 1998.** The general lineage concept of species, species criteria, and the process of speciation: a conceptual unification and terminological recommendations. In: Howard DJ, Berlocher SH, eds. *Endless forms: species and speciation*. New York, NY: Oxford University Press, 57–75.

**de Sá RO, Heyer WR, Camargo A. 2005.** Are *Leptodactylus didymus and L. mystaceus* phylogenetically sibling species (Amphibia, Anura, Leptodactylidae)? *Russian Journal of Herpetology* **12** (Suppl.)**:** 90–92.

**Donnellan SC, McGuigan K, Knowles R, Mahony M, Moritz C. 1999.** Genetic evidence for species boundaries in frogs of the *Litoria citropa* species-group. *Australian Journal of Zoology* **47:** 275–293.

**Downie JR. 1984.** How *Leptodactylus fuscus* tadpoles make foam, and why. *Copeia* **1984:** 778–780.

**Downie JR. 1994a.** Developmental arrest in *Leptodactylus fuscus* tadpoles I: descriptive analysis. *Herpetological Journal* **4:** 29–38.

**Downie JR. 1994b.** Developmental arrest in *Leptodactylus fuscus* tadpoles II: does a foam borne factor block development? *Herpetological Journal* **4:** 39–45.

**Downie JR, Smith J. 2003.** Survival of larval *Leptodactylus fuscus* (Anura: Leptodactylidae) out of water: developmental differences and interspecific comparisons. *Journal of Herpetology* **37:** 107–115.

**Excoffier L, Smouse P, Quattro J. 1992.** Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. *Genetics* **131:** 479–491.

**Farris JS, Källersjö M, Kluge AG, Bult C. 1995.** Testing significance of incongruence. *Cladistics* **10:** 315–319.

**Feldman CR, Spicer GS. 2002.** Mitochondrial variation in sharp-tailed snakes (*Contia tenuis*): evidence of a cryptic species. *Journal of Herpetology* **36:** 648–655.

**Felsenstein J. 1985.** Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39:** 783–791.

**García-París M, Good DA, Parra-Olea G, Wake DB. 2000.** Biodiversity of Costa Rican salamanders: implications of high levels of genetic differentiation and phylogeographic structure for species formation. *Proceedings of the National Academy of Sciences, USA* **97:** 1640–1647.

**Gergus EWA. 1998.** Systematics and historical biogeography of the *Bufo microscaphus* complex: allozyme evidence. *Herpetologica* **54:** 317–325.

**Gergus EWA, Sullivan BK, Malmos KB. 1997.** Call variation in the *Bufo microscaphus* complex: implications for species boundaries and the evolution of mate recognition. *Ethology* **103:** 979–989.

**Gerhardt HC. 1988.** Acoustic properties used in call recognition by frogs and toads. In: Fritzsch B, Ryan MJ, Wilczynski W, Hetherington TE, Walkowiak W, eds. *The Evolution of the amphibian auditory system*. New York, NY: John Wiley & Sons, 455–483.

**Giribet G. 2003.** Stability in phylogenetic formulations and its relationship to nodal support. *Systematic Biology* **52:** 554–564.

**Heyer WR. 1978.** Systematics of the *fuscus* group of the frog genus *Leptodactylus* (Amphibia, Leptodactylidae). *Science Bulletin of the Natural History Museum of Los Angeles County* **29:** 1–85.

**Heyer WR, Diment MJ. 1974.** The karyotype of *Vanzolinius discodactylus* and comments on usefulness of karyotypes in determining relationships in the *Leptodactylus* complex (Amphibia, Leptodactylidae). *Proceedings of the Biological Society of Washington* **87:** 327–336.

**Heyer WR, García-Lopez JM, Cardoso AJ. 1996.** Advertisement call variation in the *Leptodactylus mystaceus* species complex (Amphibia: Leptodactylidae) with a description of a new sibling species. *Amphibia-Reptilia* **17:** 7–31.

**Heyer WR, Reid YR. 2003.** Do advertisement calls define species boundaries in the genetically diverse frog taxon currently known as *Leptodactylus fuscus* (Amphibia: Leptodactylidae)? *Annals of the Brazilian Academy of Sciences* **75:** 39–54.

**Hickson RE, Simon C, Cooper A, Spicer GS, Sullivan J, Penny D. 1996.** Conserved sequence motifs, alignment, and secondary structure for the third domain of animal 12S rRNA. *Molecular Biology and Evolution* **13:** 150–169.

**Hillis DM, Bull JJ. 1993.** An empirical test of bootstrapping as a method for assessing confidence in phylogenetic analysis. *Systematic Biology* **42:** 182–192.

**Hillis DM, Mable BK, Larson A, Davis SD, Zimmer EA. 1996.** Nucleic acids IV: sequencing and cloning. In: Hillis DM, Moritz C, Mable BK, eds. *Molecular systematics*. Sunderland, MA: Sinauer Associates, 321–381.

**Hoelzer GA. 1997.** Inferring phylogenies from mtDNA variation: mitochondrial-gene trees versus nuclear-gene trees revisited. *Evolution* **51:** 622–626.

**Huelsenbeck JP, Imennov NS. 2002.** Geographic origin of human mitochondrial DNA: accommodating phylogenetic uncertainty and model comparison. *Systematic Biology* **51:** 155–165.

**Huelsenbeck JP, Ronquist F. 2001.** MrBayes: bayesian inference of phylogeny. *Bioinformatics* **17:** 754–755.

**Jockusch EL, Wake DB. 2002.** Falling apart and merging: diversification of slender salamanders (Plethodontidae: *Batrachoseps*) in the American West. *Biological Journal of the Linnean Society* **76:** 361–391.

**Kjer KM. 1995.** Use of rRNA secondary structure in phylogenetic studies to identify homologous positions: an example of alignment and data presentation from the frogs. *Molecular Phylogenetics and Evolution* **4:** 314–330.

**Kwet A, Di-Bernardo M, Garcia PCA. 2001.** The taxonomic

status of *Leptodactylus geminus* Barrio, 1973. *Journal of Herpetology* **35:** 56–62.

**Leaché AD, Reeder TW. 2002.** Molecular systematics of the eastern fence lizard (*Sceloporus undulatus*): a comparison of parsimony, likelihood, and Bayesian approaches. *Systematic Biology* **51:** 44–68.

**Maddison D, Maddison W. 2000.** *MacClade 4.0: analysis of phylogeny and character evolution*. Sunderland, MA: Sinauer Associates.

**Martins M. 1988.** Biologia reprodutiva de *Leptodactylus fuscus* em Boa Vista, Roraima (Amphibia: Anura). *Revista Brasileira de Biologia* **48:** 969–977.

**Masta SE, Sullivan BK, Lamb T, Routman EJ. 2002.** Molecular systematics, hybridization, and phylogeography of the *Bufo americanus* complex in eastern North America. *Molecular Phylogenetics and Evolution* **24:** 302–314.

**Maxson LR, Heyer WR. 1988.** Molecular systematics of the frog genus *Leptodactylus* (Amphibia: Leptodactylidae). *Fieldiana, Zoology New Series* **41:** 1–13.

**Mead LS, Tilley SG, Katz LA. 2001.** Genetic structure of the blue ridge dusky salamander (*Desmognathus orestes*): inferences from allozymes, mitochondrial DNA, and behavior. *Evolution* **55:** 2287–2302.

**Moore WS. 1995.** Inferring phylogenies from mtDNA variation: mitochondrial gene trees versus nuclear gene trees. *Evolution* **49:** 718–726.

**Moritz C. 1994.** Applications of mitochondrial DNA analysis in conservation: a critical review. *Molecular Ecology* **3:** 401–411.

**Moritz C, Dowling TE, Brown WM. 1987.** Evolution of animal mitochondrial DNA: relevance for population biology and systematics. *Annual Review of Ecology and Systematics* **18:** 269–292.

**Mulcahy DG, Mendelson JR III. 2000.** Phylogeography and speciation of the morphologically variable, widespread species *Bufo valliceps*, based on molecular evidence from mtDNA. *Molecular Phylogenetics and Evolution* **17:** 173–189.

**Palumbi SR. 1996.** Nucleic acids II: the polymerase chain reaction. In: Hillis DM, Moritz C, Mable BK, eds. *Molecular systematics*, 2nd edn. Sunderland, MA: Sinauer Associates, 205–247.

**Posada D, Crandall KA. 1998.** Modeltest: testing the model of DNA substitution. *Bioinformatics* **14:** 817–818.

**Posada D, Crandall KA, Templeton AR. 2000.** GeoDis: a program for the cladistic nested analysis of the geographical distribution of genetic haplotypes. *Molecular Ecology* **9:** 487–488.

**Riddle BR, Hafner DJ, Alexander LF. 2000a.** Phylogeography and systematics of the *Peromyscus eremicus* species group and the historical biogeography of North American warm regional deserts. *Molecular Phylogenetics and Evolution* **17:** 145–160.

**Riddle BR, Hafner DJ, Alexander LF. 2000b.** Comparative phylogeography of Baileys' pocket mouse (*Chaetodipus baileyi*) and the *Peromyscus eremicus* species group: historical vicariance of the Baja California peninsular desert. *Molecular Phylogenetics and Evolution* **17:** 161–172.

**Rohlf FJ. 1998.** *NTSYSpc: numerical taxonomy system*, Version 2.0. Setauket, NY: Exeter Publishing, Ltd.

**Rossa-Feres D, Menin M, Izzo TJ. 1999.** Seasonal occurrence and territorial behavior in *Leptodactylus fuscus* (Anura, Leptodactylidae). *Iheringia, Serie Zoologia* **87:** 93–100.

**Sasa MM, Chippindale PT, Johnson NA. 1998.** Patterns of postzygotic isolation in frogs. *Evolution* **52:** 1811–1820.

**Sazima I. 1975.** Hábitos reprodutivos e fase larvária de *Leptodactylus mystacinus* e *L. sibilatrix* (Anura, Leptodactylidae). Unpublished Master's Thesis, Universidade de São Paulo.

**Schneider S, Roessli D, Excoffier L. 2000.** *Arlequin*, Version 2.000: *a software for population genetics data analysis.* Distributed by the authors via http://anthro.unige.ch/arlequin. Genetics and Biometry Laboratory, University of Geneva, Switzerland.

**Shaffer HB, McKnight ML. 1996.** The polytypic species revisited: genetic differentiation and molecular phylogenetics of the tiger salamander *Ambystoma tigrinum* (Amphibia: Caudata) complex. *Evolution* **50:** 417–433.

**Silva APZ, Haddad CFB, Kasahara S. 2000.** Chromosomal studies on five species of the genus *Leptodactylus* Fitzinger, 1826 (Amphibia, Anura) using differential staining. *Cytobios* **103:** 25–38.

**Sites JW Jr, Crandall KA. 1997.** Testing species boundaries in biodiversity studies. *Conservation Biology* **11:** 1289–1297.

**Solano H. 1987.** Algunos aspectos de la biología reproductiva del sapito silbador *Leptodactylus fuscus* Schneider (Amphibia, Leptodactylidae). *Amphibia-Reptilia* **8:** 111–128.

**Sullivan BK, Malmos KB, Given MF. 1996.** Systematics of the *Bufo woodhousii* complex (Anura: Bufonidae): advertisement call variation. *Copeia* **1996:** 274–280.

**Sumida M, Kanamori Y, Kaneda H, Kato Y, Nishioka M, Hasegawa M, Yonekawa H. 2001.** Complete nucleotide sequence and gene rearrangement of the mitochondrial genome of the Japanese pond frog *Rana nigromaculata*. *Genes and Genetic Systems* **76:** 311–325.

**Swofford DL. 2002.** *PAUP*. Phylogenetic analysis using parsimony (*and other methods)*, Version 4.0. Sunderland, MA: Sinauer Associates.

**Swofford DL, Olsen GJ, Waddell PJ, Hillis DM. 1996.** Phylogenetic inference. In: Hillis DM, Moritz C, Mable BK, eds. *Molecular systematics*, 2nd edn. Sunderland, MA: Sinauer Associates, 407–514.

**Templeton AR. 1998.** Nested clade analyses of phylogeographic data: testing hypotheses about gene flow and population history. *Molecular Ecology* **7:** 381–397.

**Templeton AR. 2001.** Using phylogeographic analyses of gene trees to test species status and processes. *Molecular Ecology* **10:** 779–792.

**Templeton AR, Routman E, Phillips CA. 1995.** Separating population structure from population history: a cladistic analysis of the geographical distribution of mitochondrial DNA haplotypes in the tiger salamander, *Ambystoma tigrinum*. *Genetics* **140:** 767–782.

**Templeton AR, Sing CF. 1993.** A cladistic analysis of phenotypic associations with haplotypes inferred from restriction

endonuclease mapping. IV. Nested analyses with cladogram uncertainty and recombination. *Genetics* **134:** 659–669.

**Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG. 1997.** The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* **24:** 4876–4882.

**Vences M, Kosuch J, Rödel MO, Lötters S, Channing A, Glaw F, Böhme W. 2004.** Phylogeography of *Ptychadena mascareniensis* suggests transoceanic dispersal in a widespread African-Malagasy frog lineage. *Journal of Biogeography* **31:** 593–601.

**Wake DB. 1997.** Incipient species formation in salamanders of the *Ensatina* complex. *Proceedings of the National Academy of Sciences, USA* **94:** 7761–7767.

**Wake DB, Jockusch EL. 2000.** Detecting species borders using diverse data sets: examples from plethodontid salamanders in California. In: Bruce RC, Jaeger RG, Houck LD, eds. *The biology of plethodontid salamanders*. New York, NY: Kluwer Academic/Plenum Publishers, 95–119.

**Wake DB, Schneider CJ. 1998.** Taxonomy of the plethodontid salamander genus *Ensatina*. *Herpetologica* **54:** 279–298.

**Wiens JJ. 1998.** Combining data sets with different phylogenetic histories. *Systematic Biology* **47:** 568–581.

**Wiens JJ, Penkrot TA. 2002.** Delimiting species using DNA and morphological variation and discordant species limits in spiny lizards (*Sceloporus*). *Systematic Biology* **51:** 69–91.

**Wynn A, Heyer WR. 2001.** Do geographically widespread species of tropical amphibians exist? An estimate of genetic relatedness within the neotropical frog *Leptodactylus fuscus* (Anura, Leptodactylidae). *Tropical Zoology* **14:** 255–285.

**Zaldívar-Riverón A, León-Regagnon V, Nieto-Montes de Oca A. 2004.** Phylogeny of the Mexican coastal leopard frogs of the *Rana berlandieri* group based on mtDNA sequences. *Molecular Phylogenetics and Evolution* **30:** 38–49.

## APPENDIX

Specimen data of samples used in this study

| Sample (Abbreviation) | Locality | Museum Number | Field Number |
|---|---|---|---|
| Tobago (Toba) | Saint Paul; Roxborough, Tobago | USNM 306067 | USNMFS175556 |
| Arima (Arim) | Saint George; Arima, Trinidad | USNM 306149 | USNMFS175674 |
| Nariva (Nari) | Manzanilla Mayaro Road; Nariva, Trinidad | USNM 306123 | USNMFS 175651 |
| Icacos (Icac) | Saint Patrick; Icacos Point, Trinidad | USNM 287012 | USNMFS 175494a |
| Guyana (Guya) | East Berbice; Region 10 cty., Guyana | USNM 497739 | USNMFS 207166 |
| French Guiana (FrGu) | Cayenne; Sinnamary, French Guiana | USNM 291363 | USNMFS 175602 |
| Igarapé Cocal (IgCo) | Roraima; Igarapé Cocal, Brazil | MZUSP 76019 | USNMFS 8618 |
| Normandia (Norm) | Roraima; Caracaranã, near Normandia, Brazil | MZUSP 67073 | USNMFS 8739 |
| Boa Vista (BoVi) | Roraima; Bôa Vista, Brazil | MZUSP 67039 | USNMFS 8763 |
| São Paulo1 (São1) | São Paulo; Luiz Antonio, Brazil | USNM 303149 | USNMFS 52998 |
| São Paulo2 (São2) | São Paulo; Luiz Antonio, Brazil | USNM 303154 | USNMFS 53025 |
| São Paulo3 (São3) | São Paulo; Luiz Antonio, Brazil | USNM 303155 | USNMFS 53026 |
| São Paulo4 (São4) | São Paulo; Luiz Antonio, Brazil | USNM 303156 | USNMFS 53027 |
| São Paulo5 (São5) | São Paulo; Luiz Antonio, Brazil | USNM 303157 | USNMFS 53028 |
| Pará (Pará) | Pará; Serra de Kukoinhoken, Kenpore, Brazil | MZUSP 66954 | MZUSP 930112 |
| Pernambuco (Pern) | Pernambuco, Brazil | USNM 284551 | USNMFS 8541 |
| PalosBlancos (PaBl) | La Paz; Palos Blancos, Bolivia | CBF? | USNMFS 174020 |
| Beni (Beni) | Beni; Beni Biosphere Reserve, Bolivia | CBF 02908 | USNMFS 174133 |
| Embarcación (Emba) | Salta; Embarcación, Argentina | FML 04789 | USNMFS 175829 |
| J. V. González (JVGo) | Salta; Joaquín V. González, Argentina | FML 04788 (7) | USNMFS 175758 |
| Panamá (Pana) | Tocumen; Panamá City, Panamá | USNM 306189 | USNMFS 186541 |
| *L. mystacinus* (Taci) | San José; Sierra de Mahoma, Uruguay | | RdS 789 |
| *L. mystaceus* (Myce) | Pará; Serra de Kukoinhokren, Brazil | MZUSP 70371 | 940034 |

Additional locality data are given in Wynn & Heyer (2001). See also Figure 1.

CBF, Colección Boliviana de Fauna (Bolivia); FML, Fundación Miguel Lillo (Argentina); MZUSP, Museum of Zoology of the University of São Paulo (Brazil); RdS, Rafael O. de Sá personal collection; USNM, National Museum of Natural History (U.S.A.).

 