Molecular Ecology (2003) doi: 10.1046/j.1365-294X.2003.01910.x

Huge populations and old species of Costa Rican and Panamanian dirt frogs inferred from mitochondrial and nuclear gene sequences

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Abstract

Molecular genetic data were used to investigate population sizes and ages of *Eleutherodactylus* (Anura: Leptodactylidae), a species-rich group of small leaf-litter frogs endemic to Central America. Population genetic structure and divergence was investigated for four closely related species surveyed across nine localities in Costa Rica and Panama. DNA sequence data were collected from a mitochondrial gene (*ND2*) and a nuclear gene (c-*myc*). Phylogenetic analyses yielded concordant results between loci, with reciprocal monophyly of mitochondrial DNA haplotypes for all species and of c-*myc* haplotypes for three of the four species. Estimates of genetic differentiation among populations (F_{ST}) based upon mitochondrial data were always higher than nuclear-based F_{ST} estimates, even after correcting for the expected fourfold lower effective population size (N_e) of the mitochondrial genome. Comparing within-population variation and the relative mutation rates of the two genes revealed that the N_e of the mitochondrial genome was 15-fold lower than the estimate of the nuclear genome based on c-*myc*. Nuclear F_{ST} estimates were ≈ 0 for the most proximal pairs of populations, but ranged from 0.5 to 1.0 for all other pairs, even within the same nominal species. The nuclear locus yielded estimates of N_e within localities on the order of 10^5. This value is two to three orders of magnitude larger than any previous N_e estimate from frogs, but is nonetheless consistent with published demographic data. Applying a molecular clock model suggested that morphologically indistinguishable populations within one species may be 10^7 years old. These results demonstrate that even a geologically young and dynamic region of the tropics can support very old lineages that harbour great levels of genetic diversity within populations. The association of high nucleotide diversity within populations, large divergence between populations, and high species diversity is also discussed in light of neutral community models.

Keywords: c-*myc* intron, effective population size, *Eleutherodactylus*, F_{ST}, molecular clock, mtDNA, Neotropics, phylogenetics

Received 13 January 2003; revision received 29 April 2003; accepted 2 June 2003

Introduction

The origin of species diversity in the tropics is an issue of long-standing interest among ecologists and evolutionary biologists (Wallace 1878; Dobzhansky 1950). Two alternative hypotheses are often invoked to explain this diversity: either the tropics have functioned as a cradle of speciation or a museum for older lineages (Stebbins 1974). Tropical communities are known for their manifold ecological interactions which may have facilitated diversification and increased speciation rates (Rohde 1992; Jablonski 1993). Tropical regions are also thought to have been historically stable relative to the temperate zone, which may have facilitated the preservation of species and the lowering of extinction rates (Fischer 1960; Connell & Orians 1964). No consensus has yet emerged regarding the extent to which the tropics are a cradle of diversity or a museum of antiquity (Chown & Gaston 2000; Bermingham & Dick 2001).

Understanding the origins of tropical diversity will require analysis of not only the tempo of clade diversification (e.g.

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Richardson *et al*. 2001; Ricklefs & Bermingham 2001; Buzas *et al*. 2002), but also the mode of population differentiation (Moritz *et al*. 2000). To this end, we need to collect data on the population genetics of tropical organisms. These data serve multiple purposes. Studies of genetic variation among populations can be used to reveal the geographical extent and identity of species that may harbour cryptic diversity (e.g. Bermingham & Martin 1998; García-París *et al*. 2000). Quantification of genetic variation within populations can provide estimates of the effective size (N_e) of populations (Wright 1931) and diverging lineages (Kliman *et al*. 2000). Such analyses are relevant to investigations of the role of population bottlenecks in population divergence and speciation (Carson & Templeton 1984), and whether tropical species might have smaller population sizes due to increased pressure from other trophic levels (Paine 1966) or due to finer niche partitioning within guilds (Klopfer 1959). Comparing within- and among-population variation provides indirect estimates of migration rates between pairs of populations (Wright 1951). Finally, we can estimate the relative ages of populations and species by quantifying differences in molecular sequences (Zuckerkandl & Pauling 1965). These analyses will reveal the tempo and mode of speciation in tropical organisms, and we can compare these findings with results from temperate zone lineages.

All of the above genetic parameters may be most readily investigated in tropical organisms that are speciose, widespread and abundant. One such group is *Eleutherodactylus* (Anura: Leptodactylidae), a genus of frogs endemic to the Neotropics comprised of over 600 species (e.g. Lynch & Duellman 1997; Duellman & Pramuk 1999), ≈ 13% of the world's frog diversity. This study focused on a group of *Eleutherodactylus* known as 'dirt frogs' endemic to Central America. Dirt frogs are found only on the ground in the leaf litter, and are the most abundant member of the leaf litter herpetofauna at many sites where they occur (Scott 1976), possibly numbering in the millions within one forest reserve (Lieberman 1986).

The focal group of dirt frogs I studied has, at various times in the past, been recognized as seven species (Taylor 1952), one species (Savage & Emerson 1970), two species (Miyamoto 1983), and most recently as a complex of four species (Savage 2002). The geographical distribution of the four species extends from the southern border of Nicaragua to the western border of Panama on the Pacific slope, and from central Nicaragua to just east of the Panama Canal on the Atlantic slope. *Eleutherodactylus stejnegerianus* is the sole Pacific slope species, and the three Atlantic slope species have the following distribution: *E. bransfordii* in the north, *E. persimilis* in central and southern Costa Rica, and *E. polyptychus* in the south and east. The distributions of *E. bransfordii* and *E. polyptychus* are parapatric, nearly meeting in central Costa Rica, while *E. persimilis* overlaps with both (Savage 2002). When this study began all Atlantic slope populations were still referred to as *E. bransfordii*. All four species are restricted to elevations below 1400 m (Savage 2002) and are members of the *rhodopis* species group, subgenus *Craugastor* (Hedges 1989; Lynch 2001).

I assayed genetic variation from samples of five Atlantic and four Pacific slope populations. Sample sizes of up to 12 frogs per population were assayed for both mitochondrial DNA (mtDNA) and nuclear DNA (nDNA) sequence variation in order to provide comparative measures of the population genetic structure of the sampled populations. Joint analysis of mtDNA and nDNA sequence data permits a detailed description and reliable interpretation of the evolutionary and demographic history. Mitochondrial genes provide a high degree of resolution and accuracy in the reconstruction of recent cladogenesis due to their rapid evolution (Brown *et al*. 1979) and small effective population size (Avise *et al*. 1984; Moore 1995). Comparing mtDNA and nDNA marker variation among dirt frog populations may permit the detection of differences in migration rates between the sexes (Avise *et al*. 1987; FitzSimmons *et al*. 1997; Slade *et al*. 1998). Comparing mtDNA and nDNA variation within individual populations allows us to test the frequent assumption (e.g. Crochet 2000; Palumbi *et al*. 2001) that the effective population size of mitochondrial genes (N_{mt}) is 1/4 that of nuclear genes (N_e). If the mitochondrial genome has little or no recombination, then selection at any nucleotide site should reduce variation in the whole molecule (Kreitman & Wayne 1994). Therefore, we should expect that $N_{mt} < 0.25\ N_e$. Higher variance in female reproductive success relative to males would produce a similar effect (Avise *et al*. 1987).

Measuring DNA sequence variation at multiple loci can also reveal the origin of that variation by comparing within-population nucleotide diversity among loci across an array of populations. Genetic drift and certain forms of natural selection can both reduce variation within populations. The former tends to operate at the genome level through the stochastic success or failure of gametes, whereas the latter tends to affect particular genes deterministically. Therefore, if variation at independent loci covaries significantly across populations, then genetic drift due to differences in N_e is the probable cause of variation at the marker loci. Otherwise a marker-specific force, such as a selective sweep, may have reduced variation at particular locus within one or a subset of local populations. Sex-linked markers are susceptible to marker-specific demographic effects, as well, e.g. a population bottleneck among females would disproportionately affect mtDNA variation relative to nDNA.

In the past decade some ecologists have endeavoured to bring an historical perspective to studies of community assembly (Ricklefs & Schluter 1983), including one recent effort to unite community ecology with biogeography (Hubbell 2001). By combining population genetic and phylogenetic analyses with demographic data, I hope this study

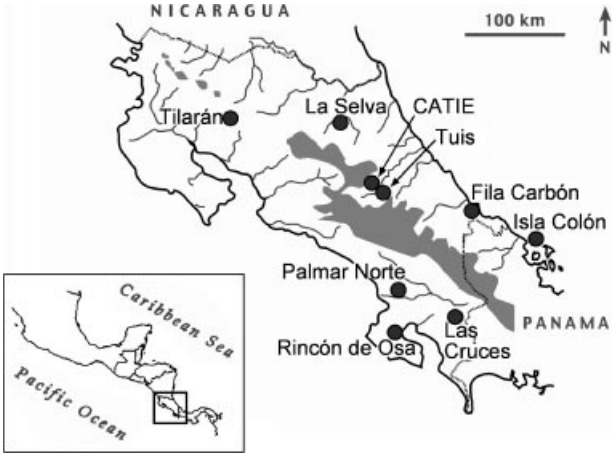

Fig. 1 Map of eight Costa Rican and one Panamanian (Isla Colón) *Eleutherodactylus* sampling localities. The grey area dividing much of Costa Rica represents terrain above 1500 m in elevation. Political map of Central American is inset at bottom left, with a box around Costa Rica. Species designations presented in Table 1.

may contribute to the continued integration of ecological and historical approaches. Here I present evidence that in *Eleutherodactylus* the numerical abundance within local populations, the age of populations and species, and the diversity of species within genera may be positively correlated. The task remains for future investigations to explore whether these factors are causally related as well (Darwin 1859; Hubbell 2001).

Materials and methods

Sampling

Frogs were collected from eight Costa Rican localities and one Panamanian locality during the dry seasons (January to April) of 1998 and 1999 (Fig. 1). To minimize the degree of consanguinity among frogs, samples were collected from across the length of each locality whenever possible, rather than from a single point (Table 1). Tissues were collected in the field and preserved in a 20% solution of dimethylsulfoxide (DMSO) saturated with NaCl (Amos & Hoelzel 1991, cited in Amos *et al*. 1992) with the addition of 0.125 M EDTA. Specimens were deposited in the collections of the Division of Amphibians and Reptiles at the Field Museum of Natural History, Chicago.

For phylogenetic reconstruction, one specimen from each of two related species was included in the analysis of both the mtDNA and nDNA sequence data sets as outgroups: the more closely related species, *Eleutherodactylus podiciferus* (FMNH257653), from the highlands of Costa Rica, and *E. rhodopis* from Guatemala (ENS8615). Phylogenetic analysis of mtDNA data includes an additional outgroup, the sister taxon of *E. rhodopis*, *E. 'loki'* (ENS10376), from Mexico. Only these latter two taxa are designated a priori as outgroups. This choice of outgroups is based upon a larger molecular phylogenetic analysis of the *rhodopis* species group (AJ Crawford & EN Smith, unpublished results).

Laboratory techniques

Genomic DNA was extracted from liver and/or thigh muscle tissues using either standard phenol–chloroform methods or the Qiagen QIAamp tissue kit. Mitochondrial DNA fragments were amplified using published primers (Table 2). Negative controls were used to monitor potential contamination. To guard against allelic contamination of within-population samples, approximately half of the samples from each locality were extracted, amplified and sequenced in separate rounds conducted two months apart.

Polymerase chain reaction (PCR) products were cleaned by PEG precipitation, Qiagen QIAquick columns, gel slicing and agarose digest, or *Exo*I/SAP digest. For each individual, both H (heavy) and L (light) strands were sequenced directly

Table 1 Summary of sampled localities for four species of Mesoamerican *Eleutherodactylus*. Transect refers to the approximate straight-line distance across which samples were collected at a given site. Description of site is qualitative, with reserves being the largest habitats, creeks the smallest. Connection to further habitat describes whether the sampling locality is an isolated island of habitat, or to what extent it may be a part of a larger forest

Locality	Versant	Taxon	Elevation (m)	Transect (km)	Description of site	Connection tp further habitat
La Selva	Atlantic	*E. bransfordii*	40–90	4.3	forested reserve	one side
Fila Carbón	Atlantic	*E. polyptychus*	50–150	2.0	forest remnant	no
Isla Colón	Atlantic	*E. polyptychus*	40	0.04	forest remnant	no
Tuis	Atlantic	*E. persimilis*	970–1010	0.15	forested reserve	two sides
CATIE	Atlantic	*E. persimilis*	540–560	0.08	forest remnant	no
Tilarán	Pacific	*E. stejnegerianus*	860	0.04	tree-lined creek	no
Las Cruces	Pacific	*E. stejnegerianus*	880–1130	3.1	forested reserve	no
Palmar Norte	Pacific	*E. stejnegerianus*	60–70	0.2	tree-lined creek	one side
Rincón de Osa	Pacific	*E. stejnegerianus*	20	0.6	forested reserve	three sides

Table 2 Primers used for the amplification (amp) and sequencing (seq) of the NADH dehydrogenase subunit 2 (*ND2*) gene and intron 2 of the cellular myelocytomatosis (c-*myc*) proto-oncogene in *Eleutherodactylus*

Primer	Use	Source	Sequence (5′ to 3′)
ND2			
H5934	amp	Macey *et al*. 1997	AGRGTGCCAATGTCTTTGTGRTT
L4437	both	Macey *et al*. 1997	AAGCTTTCGGGCCCATACC
H4980	seq	Macey *et al*. 1997	ATTTTTCGTAGTTGGGTTTGRTT
H4996	seq	this study	AGTATGCTAAGAGTTTTC
c-*myc*			
cmyc1U	amp	this study	GAGGACATCTGGAARAARTT
cmyc3L	amp	this study	GTCTTCCTCTTGTCRTTCTCYTC
cmyc3U	seq	this study	TCTTTCCTTACCCGTTGAATGATRC
cmyc6L	seq	this study	CAAAAGCCAGMCATTGGAAGATAA

from PCR products using d-Rhodamine dye-terminator reaction chemistry analysed on an ABI Prism™ 377 automated DNA sequencer (Applied Biosystems Inc). Resulting sequences were aligned using SEQUENCHER Version 3.0 (Gene Codes Corp.) and by eye, using both nucleotide and inferred amino acid sequences. These protocols yielded the first 510 bp of the mitochondrial NADH dehydrogenase subunit 2 (*ND2*) gene. *ND2* sequences are available under GenBank Accession numbers AY205579, AY205576, AY273135, AY273137, AY273139, AY273141, AY273212–AY273261, AY279081 and AY279082.

A fragment of the cellular myelocytomatosis (c-*myc*) gene was amplified using the primers cmyc1U and cmyc3L (Table 2) developed for this study. PCR protocols were performed in a mixture containing 1.5 mM Mg^{2+} and a profile involving 35 cycles with a touchdown procedure in the annealing phases from 58 to 55 °C. Other protocols were as above. Data analyses were based on 351 bp of the c-*myc* gene, consisting of 58 bp of exon 2 and 293 bp of intron 2. Indels of 1, 2 and 5 bp were observed. C-*myc* haplotypes are available under GenBank Accession numbers AY211293, AY211299, AY211317, AY211319–22 and AY269281–390.

Analyses

Nucleotide sites in the directly sequenced c-*myc* fragments were inferred to be heterozygous when the automated sequencing chromatograms from both strands of DNA showed strong double peaks of similar height, or when the particular base corresponding to the dominant peak alternated on the two chromatograms (Hare & Palumbi 1999). Heterozygous sites were determined independently within each population. For individual frogs with more than one heterozygous nucleotide site, the two haplotypes were resolved using the inference method of Clark (1990). To test for deviations from Hardy–Weinberg equilibrium (HWE) expectations, each population sample of c-*myc* haplotypes was evaluated using the exact test (Weir 1996; p. 98) applied independently to each nucleotide site. Although segregating sites are probably not independent, high nucleotide diversity in some samples precluded meaningful testing of HWE expectation at the haplotype level.

The phylogeny of these dirt frog samples was estimated in order to evaluate the newly proposed taxonomic changes to the group, and to understand the temporal and geographical context of the divergence of populations and species. Fitch (1971) parsimony trees were constructed from both DNA sequence data sets. Trees and attending bootstrap confidence limits (Felsenstein 1985) were estimated using PAUP*4.0b8 for the Power Macintosh (Swofford 2000). Indels of either 1 or 2 bp were treated as a single mutation to a fifth base. For both data sets, tree searches used the tree-bisection–reconnection branch-swapping algorithm, and character-state optimization employed the accelerated transformation option. Bootstrap support analyses involved 2000 replicates, each using 10 random sequences of addition of taxa to the tree. The index of consistency was calculated for both bootstrapped phylogenies (Kluge & Farris 1969).

Genetic differentiation between pairs of populations was measured using Wright's F_{ST} (Wright 1951) redefined for DNA sequence data in terms of genetic diversity (Lynch & Crease 1990; Charlesworth 1998). Calculation of mitochondrial F_{ST} (mtF_{ST}) involved a correction for multiple hits and is therefore equivalent to N_{ST} of Lynch & Crease (1990). Calculations were made using DNASP Version 3.5 (Rozas & Rozas 1999). Variation around the estimated F_{ST} value was gauged by bootstrapping, i.e. randomly sampling with replacement the values of within-population nucleotide diversity, π_S, and the values of the between-population divergence, π_B, 500 times and recalculating F_{ST} for each replicate. This method might result in misleadingly narrow bootstrap confidence limits for those calculations involving populations with low π_S. Therefore, the minimum and maximum F_{ST} of all 500 replicates, analogous to a 99.6% confidence limit, are reported here. These calculations were made using the software, SEQUENCER Version 6.1.0 (Kessing 2000).

If selection is reducing variation in the mitochondrial genome (Kreitman & Wayne 1994) then this effect should manifest itself in the estimation of mtF_{ST} as well as N_{mt}. For

comparison with nucF_{ST} values, mtF_{ST} values were also calculated as 'corrected' values (Crochet 2000). To obtain the corrected mtF_{ST}, each standard mtF_{ST} estimate was divided by the factor, $\{4 - 3(\text{mt}F_{ST})\}$. Corrected confidence limits for mtF_{ST} estimates were obtained by multiplying the minimum and maximum values by this same factor. Migration rates between populations were calculated using Wright's (1951) standard approximations: $N_e m \approx 0.25([\text{nuc}F_{ST}]^{-1} - 1)$ for the nuclear marker and $N_f m \approx 0.5([\text{mt}F_{ST}]^{-1} - 1)$ for the mitochondrial marker, where m is the per individual rate of migration and N_f is the effective size of the female portion of the population.

Genetic variation within each locality was quantified as follows and used to estimate N_e. Within population variation was estimated for each of the two loci as the per site nucleotide diversity, π (Nei & Li 1979; Li 1997), and the per site population mutation rate, θ_W (Watterson 1975). Calculations ignored sites with indels. Kendall's rank correlation coefficient, τ (Sokal & Rohlf 1995; p. 594), was used in one-tailed tests for a positive correlation between mtDNA and nDNA markers in either $\hat{\pi}$ or $\hat{\theta}_W$. One-tailed tests were employed because I wanted to know specifically whether levels of mtDNA and nDNA polymorphism were positively correlated, as expected in the absence of locus specific selective forces. I calculated 95% confidence intervals (CIs) for $\hat{\theta}_W$ using the recursion equation method of Kreitman & Hudson (1991). These parameters were then used to estimate effective population sizes as follows. Under the equilibrium neutral model (Kimura 1968) both of these parameters estimate the quantity, $4N_e\mu$, for nuclear genes (Tajima 1983), where μ is the per site, per generation mutation rate. For mitochondrial sequences, which are presumed to be haploid (no heteroplasmy was observed) and maternally inherited without paternal leakage, $\theta_W = 2N_f\mu$.

Estimating N_e from $\hat{\theta}_W$ requires that the sampled population fits the equilibrium neutral model. Departures from this model may alter the site frequency spectrum of a sample of DNA sequences. Such deviations can be evaluated by the parameter, D_T (Tajima 1989a). Under the infinite sites model (Kimura 1969), π and θ_W have the same expectation (Tajima 1983). Therefore, the standardized difference between these two parameters equals D_T and has an expectation of 0 and a variance of 1 (Tajima 1989a). Significantly negative D_T reveals an excess of rare nucleotides segregating in the sample which could be caused by population growth (Tajima 1989b), initial recovery of variation after a selective sweep (Braverman *et al*. 1995) or bottleneck (Fay & Wu 1999), or low frequency migrants from other populations. Significantly positive values are due to an excess of common variants relative to rare ones which may be caused by balancing selection or recent population bottleneck (Tajima 1989b).

Significant departure of Tajima's *D* from zero was evaluated in two ways. First, significance was tested using the analytical method of Tajima (1989a), assuming that D_T follows the beta distribution and assuming no recombination. For nDNA sequence data, however, the assumption of no recombination is unrealistic and comes at a great loss in power to reject neutrality (Wall 1999). If demographic and evolutionary inferences depend on the data conforming to the standard neutral model, then we should be rigorous in our attempt to reject that model. Therefore, significance of D_T estimates from the c-*myc* data were also analysed using coalescent simulations allowing for recombination. For each population, the probability of observing $\hat{D}_T$ was derived from 10^3 coalescent simulations conditioned on the sample size and the observed number of segregating sites (S) (Hudson 1990, 2002), assuming either no recombination or the estimated rate, $\hat{C}$ (Hudson 1987). Although the variance around $\hat{C}$ will be large for small data sets, the false assumption of no recombination could have a much stronger effect on $P(\hat{D}_T)$ than assuming a recombination rate that is incorrect (Przeworski *et al*. 2001). The extreme 2.5% of simulated values marked the 95% CI for $\hat{D}_T$.

Estimating μ, N_e, N *and time since divergence*

Estimating N_e from $\hat{\theta}_W$ requires an estimate of μ, which may be approximated by the substitution rate at silent sites, K_S (Kimura 1968). Point estimates and attending 95% CIs for K_S were made with Comeron's (1995) method using the software, K-ESTIMATOR (Comeron 1999). As no estimates of μ in amphibian nuclear genes are available, this parameter was estimated herein from the following data sets. I made the most relevant estimate of μ, that of c-*myc* in *Eleutherodactylus*, in two ways. First, I estimated K_S from 516 bp of c-*myc* exon 2 by comparing *Eleutherodactylus* species in each of two subgenera, *E. stejnegerianus* (GenBank Accession no. AY211317) and *E. bransfordii* (AY211321) of the Central American subgenus, *Craugastor*, vs. *E. ridens* (AY211306) from Costa Rica and *E.* sp. cf. *conspicillatus* group (AY211305) from Brazil (of the South American subgenus, *Eleutherodactylus*). Mean and 95% CI for K_S between subgenera were 0.199 (0.121–0.294). The ancestral *Craugastor* lineage dispersed into Central America from South America (Savage 1982), most likely ≈ 72 Myr BP (Iturralde-Vinent & MacPhee 1999). Assuming a generation equals 1 year as in *E. bransfordii* (Donnelly 1999), I estimated μ at $1.38\ (0.841–2.04) \times 10^{-9}$/lineage/site/generation. Also, molecular evolutionary analyses of additional *Eleutherodactylus* taxa (AJ Crawford, in press) revealed that K_S in c-*myc* is 16-fold lower than K_S in *ND2*. The mitochondrial clock calibration for the 5′ half of *ND2* (see below) provided a K_S estimate of 32.2×10^{-9} per silent site/lineage/generation. Thus, μ of c-*myc* was estimated indirectly at 2.01×10^{-9}.

As a check on the generality of the above values, I also estimated μ from two published sources. First, DNA sequences of the zinc finger transcription factor, *slug*, were

obtained from GenBank for the tetraploid frog, *Xenopus laevis* (Accession nos AF368041 and AF368043), and its diploid relative, *X.* (aka, *Silurana*) *tropicalis* (AF368039); 798 bp of aligned sequences were analysed. Mean K_S and 95% CI were 0.186 (0.122–0.256). Assuming that the common ancestor of these two taxa existed 90 Myr BP, based on fossil evidence (Báez 1996), and assuming a generation time of 1 year [although under optimal conditions this may as fast as 8 months (Tinsley & McCoid 1996)], I estimated μ at $1.03\ (0.679–1.42) \times 10^{-9}$. Finally, various estimates were made from the unrooted phylogeny of Old World ranid frogs (Bossuyt & Milinkovitch 2000) for ≈ 500 bp of the tyrosinase precursor gene exon 1 by assuming the common ancestor of all the sampled Asian and Madagascan frogs was divided when these two land masses separated 88 Myr BP. All codons containing ambiguities were excluded from these analyses. Resulting μ estimates ranged from $1.69\ (1.14–2.45) \times 10^{-9}$ for *Boophis xerophilus* (AF249167) vs. *Micrixalus fuscus* (AF249183), to $3.35\ (2.43–4.52) \times 10^{-9}$ for *Aglyptodactylus madagascariensis* (AF249166) vs. *Fejervarya syhadrensis* (AF249170).

In conclusion, either estimate of μ for the c-*myc* gene in *Eleutherodactylus* appeared to be reasonable. Therefore, to be conservative, I used the faster of the two rate estimates, $\mu = 2.01 \times 10^{-9}$ from the *Eleutherodactylus* data, to calculate N_e from $\hat{\theta}_W$, because assuming a higher μ would result in a lower N_e estimate.

Genetic estimates of N_e were compared with estimates of the demographic adult population size, N, based published surveys of dirt frog abundance at two sites. Lieberman (1986) counted leaf-litter amphibians and reptiles in 90 quadrats of 64 m² each, sampled across La Selva Biological Station throughout one year. In total, she captured 693 *E. bransfordii*, for a mean density of one frog per 8.31 m². La Selva Biological Station encompassed 1536 ha (Matlock & Hartshorn 1999). I estimated therefore that La Selva contained 1 848 000 individuals; 44.4% of these were breeding adults (Donnelly 1999), meaning N = 821 000 dirt frogs. Similarly, Scott (1976) surveyed just 10 plots of 58 m² each within Las Cruces Biological Station (235 ha) and found 266 *E. stejnegerianus* (reported as *E. bransfordii*) or one frog per 2.18 m². By assuming this reserve had the same proportion of breeding adults as La Selva, I estimated that for Las Cruces N = 479 000 adult dirt frogs.

Rates of divergence in the *ND2* gene region are remarkably similar across disparate lineages of amphibians and reptiles (reviewed in Macey *et al*. 2001). To estimate the age of dirt frog populations and species I used the mtDNA divergence data of Macey *et al*. (1998) obtained from Eurasian toads, but I recalibrated their clock by using only the homologous 510 nucleotide sites of *ND2* that were used in this study. Because uncorrected genetic distances may bias divergence time estimates towards the calibration point (Arbogast *et al*. 2002), I also applied a Tajima & Nei correction to the data of Macey *et al*. (1998) as well as the *Eleutherodactylus* data under investigation. I obtained an average divergence of 0.957% per lineage per million years across the 10 Myr BP calibration point of Macey *et al*. (1998). For all pairwise population comparisons I reported the minimum divergence, thereby reducing the contribution of within-population variation to divergence estimates. To obtain 95% confidence limits that portray accurately the evolutionary variance in DNA sequence divergence that arises from the stochastic nature of mutation (Bromham & Penny 2003), I conducted simulations conditional on the length of the sequence (510 bp), the observed GC content and the ratio of transitions to transversions. The above analyses again employed the software, K-ESTIMATOR (Comeron 1999).

Results

Taxonomy and phylogeny of dirt frogs

Molecular phylogenetic analyses strongly supported the current taxonomy, and provided genetic validation of the resurrection from synonymy by Savage (2002) of *Eleutherodactylus persimilis* and *E. polyptychus*. From each of the nine sampling localities, *ND2* sequences were obtained for five to eight frogs. The inferred mtDNA gene tree showed ≥ 90% bootstrap support for the reciprocal monophyly of each of the four recognized species (Fig. 2). Mitochondrial DNA sequence divergence was unexpectedly high among all taxa (Fig. 2). For example, the uncorrected genetic distance between the northern and southern Atlantic slope species pair, *E. bransfordii* and *E. polyptychus*, was 16%. Substantial divergence was also inferred at the level of the ND2 amino acid sequence (Fig. 2). In addition, the mtDNA phylogeny showed clearly that the Atlantic species do not form a monophyletic group with respect to *E. stejnegerianus* on the Pacific (Fig. 2).

C-*myc* data were collected from a total of 73 frogs yielding 146 observed or inferred sequences (alignment available from the author). HWE was not rejected for any nucleotide position within any population. No individuals were homozygous for an indel, but 10 were inferred to be heterozygous for one of three different indels. Only one DNA strand was effectively sequenced for these frogs, but they were included in all analyses. No c-*myc* haplotypes were shared among species. Conspecific c-*myc* haplotypes formed monophyletic clades with ≥ 80% bootstrap support for all species except *E. bransfordii* (Fig. 3).

Population genetic structure

Some conspecific populations were as diverged at *ND2* as Atlantic species were from each other. Within *E. stejnegerianus*, Las Cruces showed 16% uncorrected mtDNA sequence divergence from Tilarán in the north and 13% divergence

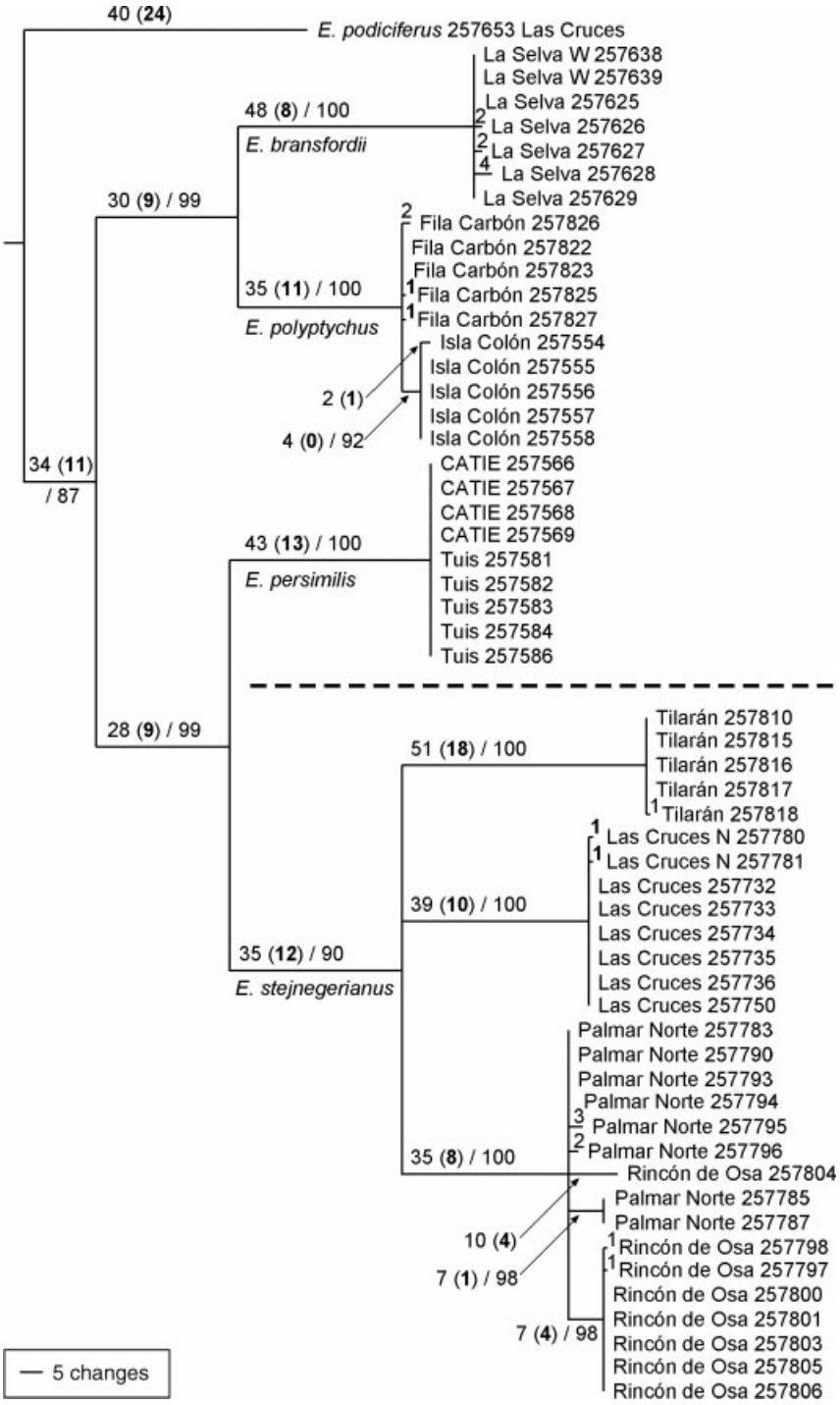

Fig. 2 Bootstrapped maximum parsimony phylogram of mtDNA sequences sampled from eight populations of *Eleutherodactylus* (note, localities Tuis and CATIE clearly represent the same genetic population). Dashed line represents the continental divide. Taxonomic designation for each population is indicated at corresponding ancestral node. For each node, the first value indicates the inferred number of character state changes (branch length), the bold value in parentheses (parentheses absent on some terminal nodes) indicates the subset of the previously indicated changes that represent inferred nonsynonymous changes, and the value following the slash indicates percent bootstrap support. All internodes receiving < 80% bootstrap support are collapsed. The six-digit codes refer to FMNH Accession nos. Data consist of the first 510 bp of the *ND2* gene, yielding 259 parsimony informative and 22 variable but uninformative characters. This tree was rooted on *Eleutherodactylus rhodopis* and *E. 'loki.'* Total tree length is 620, scoring a consistency index (excluding uninformative sites) of 0.634 and a retention index of 0.940.

from nearby Palmar Norte (Fig. 2). Although this latter pair is only 57 km apart (Fig. 1), they are separated by at least 800 m in elevation (Table 1). One pair of localities, however, showed no differentiation. Tuis and CATIE are 10.5 km apart and separated by ≈ 400 m in elevation, yet the combined nine *ND2* sequences from these two localities possessed not a variable nucleotide site among them (Fig. 2). From this observation it was inferred that these two localities represent a single population and were so treated for all subsequent analyses.

The high mtDNA sequence divergence among species and most populations was reflected in the very large pairwise F_{ST} estimates (Tables 3 and 4). The smallest of the mtF_{ST} values, 0.54, was obtained for the comparison

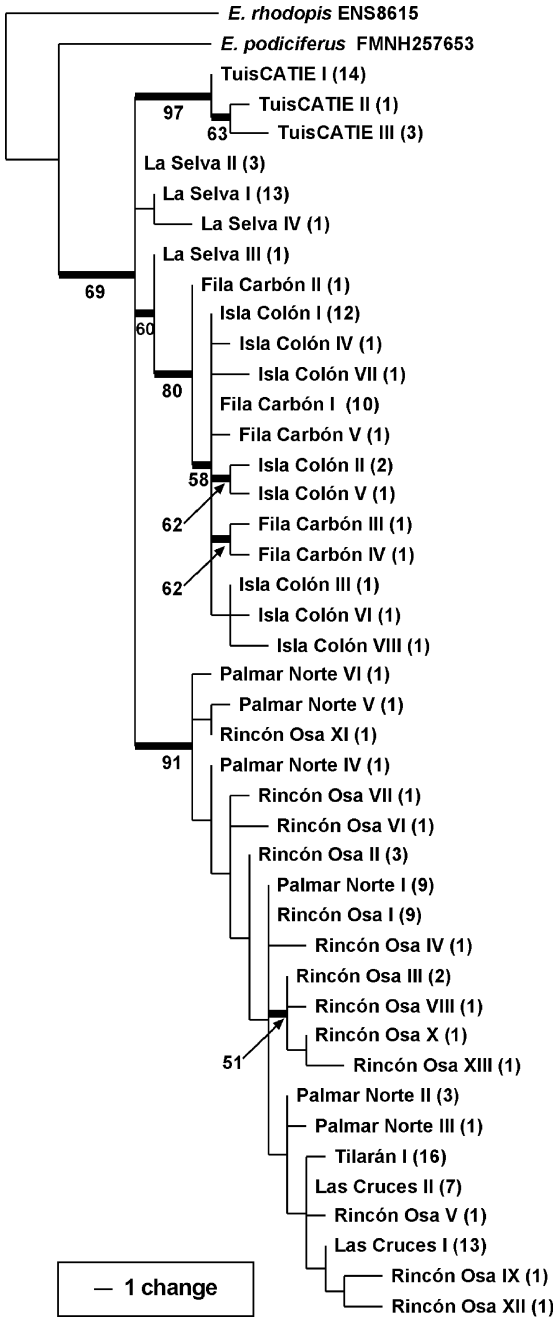

Fig. 3 One of > 10^5 equally parsimonious trees of length 86 inferred from *Eleutherodactylus* c-*myc* sequences. Nodes represented in all of the most parsimonious trees are indicated by thick lines. Bootstrap support values for each of these nodes is indicated below it. Within each population, each unique haplotype is assigned a Roman numeral and represented once on the genealogy, with the number of copies of the allele shown in parentheses (n). Note, the only haplotypes shared among populations are haplotype I in Isla Colón & Fila Carbón, and haplotype I in Palmar Norte & Rincón de Osa, the most common haplotype in each respective population. This tree is based upon 27 parsimony-informative characters and 37 variable but parsimony-uninformative characters. The resulting tree has a consistency index (CI) of 0.814 excluding uninformative sites, a retention index (RI) of 0.908.

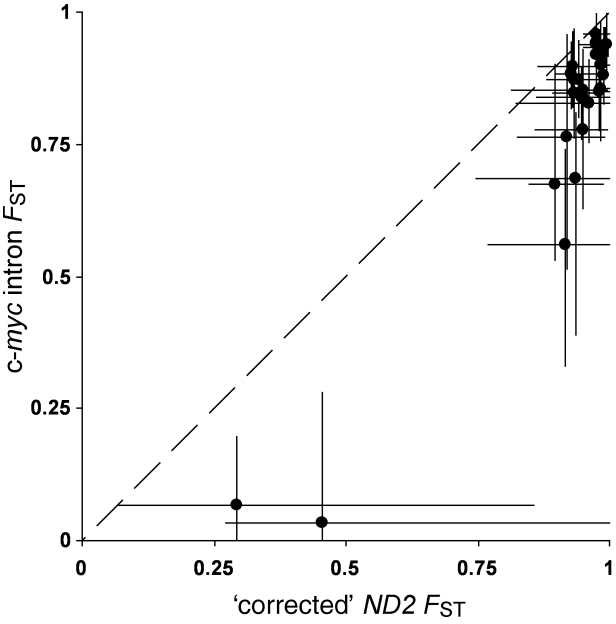

Fig. 4 Nuclear c-*myc* marker vs. mitochondrial *ND2* marker F_{ST} estimates and confidence limits for each pairwise comparison of *Eleutherodactylus* population samples. MtF_{ST} estimates and CIs are 'corrected' values under the assumptions of no heteroplasmy, no paternal inheritance, an equal sex ratio, and no sex-bias in migration rates among populations. After correction and all else being equal, nuclear and mitochondrial estimates of F_{ST} should be equivalent (dashed line).

of Palmar Norte vs. Rincón de Osa populations of *E. stejnegerianus*. These two are geographically very close, located just 32 km apart (Fig. 1). The next most genetically similar pair of populations was Fila Carbón and Isla Colón, two lowland Atlantic sites located 68 km apart representing *E. polyptychus*, with an estimated mtF_{ST} of 0.77.

Substantial population genetic structuring was also evident in the nDNA data, although to a lesser degree than in the mtDNA. The nucF_{ST} estimates were large and bounded well away from zero for all pairwise comparisons except two. Although the population pair Palmar Norte vs. Rincón de Osa and the pair Fila Carbón vs. Isla Colón both showed significant population structure at their mtDNA, they shared most of their nuclear haplotypes. The Tilarán and Las Cruces samples consisted of just one and two haplotypes, respectively, all of which were endemic. These haplotypes differed from some of the minor (i.e. low-frequency) conspecific haplotypes by a single point mutation or deletion. For example, Rincón de Osa haplotype 257803b differed from the Las Cruces minor allele by a 1 bp deletion.

For all pairwise population comparisons, mtF_{ST} estimates exceeded those of nucF_{ST} (Tables 3 and 4), as expected (Crochet 2000). However, the 'corrected' mtF_{ST} values were also larger than nucF_{ST} in all pairwise comparisons. In most comparisons even the minimum value for the corrected

Table 3 For each pairwise comparison among *Eleutherodactylus stejnegerianus* populations the following parameter estimates are presented based on DNA sequence data from the mitochondrial *ND2* gene (above the diagonal) and the nuclear c-*myc* gene (below the diagonal): point estimate (upper value) and confidence limits (range of values, in parentheses) in uncorrected F_{ST} (**bold**) and either N_em for nDNA or N_fm for mtDNA below in plain font

	Tilarán	Las Cruces	Palmar Norte	Rincón de Osa
Tilarán		**0.9956** **(0.992–1.000)** 0.00 (0.000–0.004)	**0.9781** **(0.949–0.998)** 0.01 (0.001–0.027)	**0.9835** **(0.921–1.000)** 0.01 (0.000–0.043)
Las Cruces	**0.8549** **(0.756–0.936)** 0.04 (0.017–0.081)		**0.9724** **(0.957–0.997)** 0.01 (0.001–0.023)	**0.9733** **(0.930–1.000)** 0.01 (0.000–0.038)
Palmar Norte	**0.7642** **(0.512–0.957)** 0.08 (0.011–0.238)	**0.6740** **(0.530–0.903)** 0.12 (0.027–0.221)		**0.5443** **(0.222–0.960)** 0.42 (0.021–1.755)
Rincón de Osa	**0.6847** **(0.387–0.810)** 0.12 (0.059–0.396)	**0.5605** **(0.330–0.740)** 0.20 (0.088–0.508)	**0.0655** **(–0.26–0.199)** 3.57 (–1411–2383)	

Table 4 See table 3 for explanation

	Isla Colón	Fila Carbón
Isla Colón		**0.7703** **(0.599–1.000)** 0.15 (0.000–0.335)
Fila Carbón	**0.0335** **(–0.41–0.281)** 7.22 (–238–262)	

mtF_{ST} was larger than the corresponding nucF_{ST} point estimate (Fig. 4).

Genetic variation within populations

Among *ND2* sequences, one to four haplotypes were identified in each of the population samples (Table 5). $\hat{\pi}$ ranged from 0 for TuisCATIE to 0.00749 for Palmar Norte. Between 0 and 13 segregating sites were observed in each population, yielding $\hat{\theta}_W$ from 0 again for TuisCATIE to 0.00983 for Rincón de Osa. Note, the large number of segregating sites among *ND2* sequences in the Rincón de Osa sample (and consequently the low $\hat{D}_T$) were due to a single, very divergent (2.5%) haplotype, FMNH25780 (Table 5; Fig. 2). No population showed a significantly skewed $\hat{D}_T$ for the mitochondrial dataset, although power to reject the null hypothesis may have been hampered by small sample sizes.

Between 16 and 24 inferred c-*myc* sequences were sampled per population and 1–13 unique haplotypes were found in each sample (Table 6). The number of segregating sites in each sample also varied widely, ranging from 0 to 15. Aside from the invariant sample from Tilarán, $\hat{\pi}$ ranged from 0.00136 for Las Cruces, to 0.00653 for Rincón de Osa. This latter population also had the largest $\hat{\theta}_W$ at 0.01148. The second smallest value of $\hat{\theta}_W$, after Tilarán, was again from Las Cruces, with its single segregating site. All six populations with $S > 1$, showed a negative $\hat{D}_T$. Using Tajima's (1989a) method of calculating P($\hat{D}_T$), the Isla Colón c-*myc* sample was the only one to show a significantly negative $\hat{D}_T$. However, when P($\hat{D}_T$) was evaluated by coalescent simulation using the estimated recombination rates, the Rincón de Osa sample also rejected the null hypothesis at the $\alpha = 0.01$ level (Table 6).

The values of $\hat{\theta}_W$ and $\hat{\pi}$ were remarkably similar between the rather dissimilar nuclear and mitochondrial markers (Tables 5 and 6). Furthermore, populations that showed higher nucleotide diversity at one locus tended to show higher diversity at the other, and vice versa. The notable exception to this trend was TuisCATIE. This population showed the biggest difference in relative rank order of $\hat{\theta}_W$ values between marker datasets. TuisCATIE was devoid of mtDNA sequence variation, despite being the largest sample of mitochondrial haplotypes (n = 9) assembled from across the widest geographical distance (10.5 km), while moderately high variation was revealed at the nuclear marker. The rank correlation of $\hat{\pi}$ values between the two loci was significant only with the post hoc removal of the TuisCATIE sample (Kendall's $\tau = 0.619$, one-tailed $P = 0.05$ for $n = 7$).

Table 5 Summary of genetic polymorphism data for *Eleutherodactylus* population samples of *ND2* sequences, listed in ascending order of $\hat{\theta}_W$. Length = 510 bp. *N*, *n* and *S* are the number of sequences, haplotypes and segregating sites, respectively

Population	*N*	*n*	*S*	$\hat{\pi}$	$\hat{\theta}_W$	$\hat{\theta}_W$ 95% CI	$\hat{D}_T$	Pr ($\hat{D}_T$ | null)
TuisCATIE	9	1	0	0.00000	0.00000	0.00000–0.00383	NA	NA
Las Cruces	8	2	1	0.00084	0.00076	0.00002–0.00756	0.3335	> 0.1
Tilarán	5	2	1	0.00078	0.00094	0.00002–0.01059	–0.8165	> 0.1
Isla Colón	5	2	2	0.00157	0.00188	0.00019–0.01456	–0.9725	> 0.1
La Selva	7	3	4	0.00336	0.00320	0.00072–0.01541	0.2390	> 0.1
Fila Carbón	5	4	4	0.00314	0.00376	0.00081–0.02353	–1.0938	> 0.1
Palmar Norte	8	4	9	0.00749	0.00681	0.00230–0.02552	0.4944	> 0.1
Rincón de Osa	8	3	13	0.00693	0.00983	0.00366–0.03379	–1.4928	> 0.1

Table 6 Summary of genetic polymorphism data for *Eleutherodactylus* population samples of c-*myc* sequences, listed in ascending order of $\hat{\theta}_W$. Length, *L* = [(351 bp) – (# sites with indels)]. 2*N*, *n* and *S* are the number of sequences inferred, different haplotypes and segregating sites, respectively. An asterisk (*) in the column, $\hat{D}_T$, indicates a departure from standard neutral expectation ($\hat{D}_T = 0$) significant at the 0.05 level when significance is calculated using the method of Tajima (1989a). For each population, 95% CIs for $\hat{D}_T$ were calculated by coalescent simulations (with fixed *S*), assuming both a recombination rate of zero (upper interval in each row) and the estimated value, $\hat{C}$ (lower interval). Two-tailed probability tests of significance were derived from the simulations, and an asterisk in the final column indicates a significant departure from expectation. No a priori hypothesis is being tested so two-tailed tests are employed. Therefore, *P* = 0.025 is borderline significant at the 0.05 levels

Population	2*N*	*L*	*n*	*S*	$\hat{C}$	$\hat{\pi}$	$\hat{\theta}_W$	$\hat{\theta}_W$ 95% CI	$\hat{D}_T$	$\hat{D}_T$ 95% CI by simul. ($C = 0$ / $C = \hat{C}$)	2-tailed *P* from simul.
Tilarán	16	351	1	0	NA	0.00000	0.00000	0.00000–0.00401	NA	NA	NA
										NA	NA
Las Cruces	20	351	2	1	10^5	0.00136	0.00080	0.00002–0.00577	1.2618	–1.1644 to 1.5313	0.186
										–1.1644 to 1.565	0.207
La Selva	18	349	4	3	0.0989	0.00169	0.00250	0.00045–0.01000	–0.9027	–1.7130 to 2.0892	0.214
										–1.7130 to 1.8607	0.180
TuisCATIE	18	351	3	4	0.0000	0.00304	0.00331	0.00079–0.01180	–0.2517	–1.8531 to 1.9228	0.462
										–1.8531 to 1.906	0.461
Fila Carbón	14	350	4	4	0.0046	0.00198	0.00359	0.00085–0.01348	–1.4810	–1.7976 to 1.8862	0.090
										–1.7976 to 1.7999	0.096
Palmar Norte	16	350	6	5	0.0383	0.00345	0.00431	0.00119–0.01453	–0.6528	–1.9286 to 1.7712	0.263
										–1.6917 to 1.5707	0.255
Isla Colón	20	349	8	9	0.0515	0.00332	0.00727	0.00273–0.01999	–1.8693*	–1.8693 to 1.9193	0.025*
										–1.4198 to 1.4127	0.005*
Rincón de Osa	24	350	13	15	0.0654	0.00653	0.01148	0.00502–0.02744	–1.5224	–1.6276 to 1.7478	0.039
										–1.2610 to 1.2920	0.006*

Estimating N_e

The above result supported the validity of inferring N_e from $\hat{\pi}$ for each population. However, the significantly nonzero D_T estimated from the c-*myc* sequences of Isla Colón and Rincón de Osa (Table 6) implied that these two samples did not conform to the assumptions of the standard neutral model. Violation of these assumptions invalidates the equality, $\theta = 4N_e\mu$ (because $\hat{D}_T$ values were negative, N_e would be overestimated). For this reason, and because demographic estimates of *N* exist for only two populations (see Materials and methods), N_e was estimated only for La Selva and Las Cruces. From Table 6, the mean and 95% CI values of $\hat{\theta}_W$ for the La Selva sample of c-*myc* haplotypes were 0.0025 (0.00045–0.010), implying $N_e = 3.1 \times 10^5$ (56 000–1 200 000). Therefore, the ratio of the variance effective population size to the breeding population size (N_e/N) = 0.38 (0.068–1.5). The Las Cruces sample had only one segregating site and $\hat{\theta}_W = 0.0008$ (0.00002–0.0058). Thus, at Las Cruces $N_e = 1.0 \times 10^5$ (2500–730 000), and $N_e/N = 0.21$ (0.005–1.5).

Ages of populations and species

Applying the recalibration of the *ND2* divergence rate estimate of Macey *et al*. (1998) to dirt frog lineages yielded

the following divergence dates and 95% CI accounting for stochastic variance. First, within populations, the one unusual mtDNA haplotype (FMNH257804; Fig. 2) in Rincón de Osa diverged from the other seven sympatric haplotypes 1.37 (0.622–2.13) Myr BP. This haplotype could represent a migrant from an unsampled locality. Comparing proximal populations, the minimum genetic distance between Palmar Norte and Rincón de Osa samples suggested that these populations diverged 0.727 (0.204–1.46) Myr BP, although they show no differentiation at the nuclear marker. This lowland pair of *E. stejnegerianus* populations was estimated to have diverged from the upland Las Cruces population 8.09 (6.25–10.3) Myr BP, while these three populations last shared a common ancestor with the northerly Tilarán population of conspecifics 10.3 (7.98–12.4) Myr BP. The most recent interspecific divergence is found between lowland Atlantic species, *E. bransfordii* and *E. polyptychus*, which diverged from their common ancestor 10.0 (7.63–12.3) Myr BP as well. All other interspecific divergence times would be older still.

Discussion

Origins of dirt frog diversity

The taxa studied here, whether they are more properly called populations or species, show remarkably ancient divergences over short geographical distances. These age estimates are not a result of unsampled taxa since even populations within the single taxonomic species, *Eleutherodactylus stejnegerianus*, diverged 8–12 Myr BP. From this we may infer that dirt frogs are older than the dirt they are standing on because much of lower Central America was under water as recently as 5 Myr BP, and Costa Rica was probably an archipelago 10 Myr BP (reviewed in: Coates & Obando 1996; Denyer *et al*. 2000; Montero 2000). Therefore, genetic and geological data suggest that these mainland dirt frog populations and species could have originated on islands. Certainly, the phylogeny and geographical distribution of the four dirt frog species does not support a simple model of vicariance by the uplifting of mountains because the mtDNA data support unequivocally the sister relationship between the Pacific slope species, *E. stejnegerianus*, and the mid-Altantic slope species, *E. persimilis* (Figs 1 and 2). These two species diverged 11.8 (9.32–13.7) Myr BP, apparently prior to the rise of the intervening mountain range 8 to 5 Myr BP (Coates & Obando 1996). Although the age of taxa may suggest that the tropics is a museum of antiquity, the cause of species diversity could have less to do with the stability of the tropical environment, per se (Connell & Orians 1964), and more to do with the millions of years of remarkable geological dynamism (García-París *et al*. 2000).

The old age of dirt frog lineages is not surprising, however, as this study indicates that effective population sizes in dirt frogs can be enormous. That large populations must be old, and abundant lineages should also be speciose, are fundamental predictions of the neutral theory of biodiversity and biogeography (Hubbell 2001). A positive correlation between abundance and diversity is also predicted by a more deterministic mechanism outlined by Darwin (1859), who took as practically self-evident the positive correlation between numbers of individuals and numbers of species. Darwin inferred that abundance leads to increased variation which facilitates adaptation and leads to increased competitive ability over rare species (p. 177). These adaptations are passed on to descendent species, and abundant species thereby lead to speciose genera (p. 428) that contain the numerically dominant species (p. 54), just as in *Eleutherodactylus*. Thus, high species diversity is not correlated with small N_e locally, and the many species of *Eleutherodactylus* did not result solely from recent isolation of small populations.

Population size

Dirt frogs are as common as dirt (Lieberman 1986) and have apparently been so for a long time. The level of DNA polymorphism within local populations of dirt frogs is enormous, especially in relation to previous findings in frogs. For various temperate zone species the numbers of breeding adults have been estimated at 2–200, based on genetic (Scribner *et al*. 1997) and demographic studies (Breden 1987; Berven & Grudzien 1990; Driscoll 1999; Seppä & Laurila 1999). Local N_e of tropical dirt frogs, however, would appear to be two to three orders of magnitude larger. Such comparisons are difficult to interpret as no previous studies have ever used nDNA sequence data to estimate N_e in frogs. We can compare mtDNA data sets between tropical and temperate zone frogs, however. The sample of 8 dirt frogs taken from within a 200-m transect in Palmar Norte yielded the identical θ estimate as a sample of 32 Columbia spotted frogs taken from across the state of Utah (Bos & Sites 2001). Dirt frogs pack a large amount of genetic variation into a small area.

The N_e/N ratio corroborates the finding of N_e of the order of 10^5. The mean N_e/N estimate among plants and animals has been calculated at 0.10, whereas the mean of four different amphibian species was 0.23 (Frankham 1995). Ratios of 0.38 and 0.21 for La Selva and Las Cruces populations accord well with these previous findings.

Isolation of populations

Appreciable genetic structuring at geographical scales of 1–50 km is not uncommon in temperate zone frogs (Scribner *et al*. 1994; Hitchings & Beebee 1997; Driscoll 1998; Rowe *et al*. 1998; Shaffer *et al*. 2000; Newman & Squire 2001). However, the magnitude of genetic structuring in dirt frogs is remarkable. Although populations of *E. stejnegerianus*

have not evolved any morphological differences noticeable by humans, these conspecific populations (Savage 2002) show as much *ND2* sequence divergence as does the average pair of myobatrachin frog genera at this same gene (Read *et al*. 2001). Whether such extreme subdivision at local scales occurs in other Neotropical frogs is not yet known. All previous population genetic studies have focused on widely ranging species and used allozyme data to uncover population structuring at scales approaching 1000 km (Gascon *et al*. 1996, 1998; Ryan *et al*. 1996; Wynn & Heyer 2001).

Highly structured genetic variation in dirt frogs is especially surprising in light of their natural history. Significant genetic structuring among temperate zone frog populations over short distances is usually explained by invoking metapopulation dynamics among ponds (reviewed in: Alford & Richards 1999; Marsh & Trenham 2001). Like all *Eleutherodactylus*, dirt frogs are direct developers, however, meaning there is no tadpole stage and no ponds or streams are required for ovipositing or development. These frogs may breed anywhere there is forest. Because Costa Rica was until very recently covered extensively with continuous forest (Sader & Joyce 1988), dirt frogs could have dispersed and bred historically across wide areas. Indeed, no evidence for limited dispersal was uncovered within the 4.3 km diameter of La Selva reserve or between Tuis and CATIE, separated by 10.5 km and a major river, the Reventazón. In contrast, Palmar Norte and Rincón de Osa showed a high and significant mtF_{ST} of 0.54, yet these populations are located just 32 km apart. This latter population pair diverged in the mid-Pleistocene. Forest refugia (Simpson & Haffer 1978) probably played no roll here, however, as the Osa Peninsula likely maintained continuous forests during glacial maxima (Piperno & Pearsall 1998). A potential riverine barrier (Lougheed *et al*. 1999), the Grande de Térraba, separates these two localities, though a different river has not impeded panmixia between CATIE and Tuis. Thus, it is not clear what factors have maintained isolation between lowland populations in the presumed absence of metapopulation dynamics.

Mitochondrial vs. nuclear population structure

The standard assumptions of dispersal unbiased by sex, equal sex ratios, no paternal transmission of mtDNA, and no heteroplasmy imply the following equalities: $N_e = 2N_f = 4N_{mt}$. For all pairwise population comparisons, subdivision was greater at the mtDNA marker than at the nDNA marker even after correcting for this expected fourfold smaller population size of mitochondrial genes relative to nuclear genes (Crochet 2000) (Fig. 4). When these assumptions hold, the quantity $\{mtF_{ST}(1 - nucF_{ST})/nucF_{ST}(1 - mtF_{ST})\}$ should be four. The average value of this factor over all pairs of dirt frog populations, however, is 23.3 (median = 17.9), not four. In other words, if $mtF_{ST} = (N_e m + 1)^{-1}$, then $nucF_{ST} \approx (23N_e m + 1)^{-1}$, suggesting that $N_e = 23N_{mt}$ in the average dirt frog population. The indirect estimate of $N_e m$ based on the nuclear data appears to be almost sixfold larger than our expectation based on the mitochondrial data. At least one of the initial assumptions is not valid, suggesting that the effective sex ratio and/or migration rates are male-biased, or that N_{mt} is reduced. Thus, mtDNA data were not used to estimate N_f.

Stronger population differentiation at mtDNA markers relative to nuclear markers is often used to infer reduced migration rates of females relative to males (e.g. Pardini *et al*. 2001), yet marked differences in mtF_{ST} vs. nucF_{ST} may also be due to differences in the evolutionary dynamics of the two genomes (Slade *et al*. 1998) that reduce N_{mt} rather than the female migration rate, m_f. Both purifying selection acting against the typically deleterious mutations and positive selection favouring the rare beneficial mutation would result in a reduction of genetic variation within populations (Maynard Smith & Haigh 1974; Kaplan *et al*. 1989; Charlesworth *et al*. 1993). These general effects on variation would be quite pronounced in the mitochondrial genome relative to the nuclear genome because of the higher mutational input and presumed lack of recombination in the former. Reduced $\hat{\theta}_W$ in mtDNA within populations means both reduced N_{mt} and higher mtF_{ST} (Charlesworth 1998).

Because fundamental population genetic forces such as background selection (Charlesworth *et al*. 1993) provide a reasonable and possibly more general explanation for inflated mtF_{ST} estimates, this hypothesis must be explored before assuming that females are philopatric. For example, one could explain the discrepancy in mtF_{ST} vs. nucF_{ST} estimates in dirt frogs by demonstrating that $N_e \approx 23N_{mt}$. The relative N_e and N_{mt} of dirt frogs may be calculated here from the estimates of within-population variation and relative mutation rates. Regressing nuclear on mitochondrial $\hat{\theta}_W$ values (Tables 5 and 6), and again ignoring the Tuis-CATIE sample, we find that the latter are 1.084 times larger ($r^2 = 0.60$). This result combined with the 16-fold difference in K_S between loci (AJ Crawford, in press) one obtains $N_e \approx 15N_{mt}$. Thus, although selective forces can account for a 15-fold reduction in N_{mt} relative to N_e (a much greater reduction than the fourfold factor expected from considering only the mode of transmission), reduced N_{mt} cannot explain entirely the discrepancy between mtF_{ST} and nucF_{ST} estimates. Therefore, demographic forces may be involved, too. These might include higher variance in reproductive success among females (Avise *et al*. 1984), large fluctuations in the female population size (Wright 1931), higher male migration (Palumbi & Baker 1994), or kin-structured colonization of new populations (Wade *et al*. 1994). Choosing among these explanations will have to await further details of the ecology of dirt frogs.

Conclusion

This study is unique in providing nDNA and mtDNA sequence based estimates of N_e, corroborated by demographic data, and placed in a geographical context within the tropics. The large divergence between populations and species suggest that the tropics have functioned as a museum of antiquity rather than as a cradle of speciation (Stebbins 1974) for these frogs. Not only are subgenera of *Eleutherodactylus* ancient (Hass & Hedges 1991), but so are individual species. Therefore, *Eleutherodactylus* owes its status as the most diverse vertebrate genus not to higher speciation rates (e.g. Richardson *et al*. 2001), but to old age (e.g. Bermingham & Dick 2001). This could be true for other speciose genera as well, e.g. some Amazonian populations and species of *Hyla* appear to be older than confamilial North American lineages (Chek *et al*. 2001).

Some unresolved issues remain. First, the correspondence of abundance, age and species diversity of dirt frogs matches, at least superficially, the predictions of a neutral biogeographical model (Hubbell 2001). However, this model also predicts that within this larger clade we should encounter rare, young taxa as well. Second, we do not yet know the full geographical extent of these older lineages or whether they also occur in sympatry with one another. Under a model of density-dependent dispersal, high local abundance should have led to expanded ranges and greater geographical mixing of lineages, unless species of dirt frog competitively exclude one another. Both of these issues are being addressed currently through more fine-scaled phylogeographical analyses. Finally, whether tropical populations in general tend to harbour more or less genetic diversity than their temperate zone counterparts is an open question, one that needs addressing if we are to understand the roles of speciation mode and lineage longevity in generating tropical diversity.

Acknowledgements

For permission to conduct research in Costa Rica I thank the MINAE, La Sistema Nacional de Areas de Conservacion, and J Guevara. For permission to collect samples at sites under their care, I thank LD Gomez, B Young, J Jimenez, R Matlock, L Erb at Rancho Naturalista (Tuis), the CATIE, and Señora Bianco and the Osa Pulcra Women's Collective. For logistical help in Costa Rica I thank the OTS, especially A Simpson, O Vargas, R Rojas and LD Gomez. For permission to conduct research in Panama, I thank ANAM. For logistical support, I thank the Office of Visitor Services at the STRI, especially M Leone and O Arosemena. Thanks to C Jaramillo and S Palma for help at Isla Colón. For training in molecular genetic techniques, my sincere thanks go to the Kreitman Laboratory: P Andolfatto, E Stahl, C Toomajian, J Gladstone, S-C Tsaur, C Bergman, M Ludwig, S Rollmann, and B Stranger. For assistance in the Bermingham Laboratory I thank G Reeves, B Quenoville, A Perdices, M González, J Eberhard, N Gomez, C Vergara, and V Aswani. Thanks to JR Macey for mtDNA primers and EN Smith for the *E. rhodopis* and E. *'loki'* samples. Thanks to B Kessing and J Comeron for help with software. Thanks to my thesis committee, SJ Arnold, MJ Wade, M Kreitman, W Wimsatt, A Graybeal, and M Leibold for their advice and support. For their patient consultation, I thank J Savage, F Bolaños, AS Rand, R Ibáñez, E Bermingham, A Carnaval and, most of all, thanks to E Stahl and P Andolfatto. Thanks to my thesis committee plus C Jiggins, H Lessios, J Merilä, M Chu, and especially E Bermingham for comments on various incarnations of this manuscript. This project was supported by an OTS graduate research fellowship from Peace Frogs, Inc., a STRI-OTS Mellon predoctoral fellowship, a Sigma Xi Grant-in-Research, a Gaige Award from the ASIH, a Grant-in-Aid from the Division of Ecology and Evolution of the SICB, and by the University of Chicago via the Park Fund, M Kreitman, and the Committee on Evolutionary Biology.

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This research formed most of a chapter from the Ph.D. thesis of Andrew J. Crawford on the evolution and maintenance of a colour pattern polymorphism in these frogs. The thesis project involved three interrelated approaches (ecological genetics, phylogenetics, and molecular evolution) and a succession of three different advisors. Meanwhile, Andrew is still fixated on dirt frog DNA, trying to understand various phenomenon ranging from the effect of 50 years of habitat modification on genetic variation to the effect of 90 million years of plate tectonics on species diversification.
