

## Relative Rates of Nucleotide Substitution in Frogs

Andrew J. Crawford\*

Committee on Evolutionary Biology, University of Chicago, Chicago, IL 60637, USA

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**Abstract.** Accurate estimation of relative mutation rates of mitochondrial DNA (mtDNA) and single-copy nuclear DNA (scnDNA) within lineages contributes to a general understanding of molecular evolutionary processes and facilitates making demographic inferences from population genetic data. The rate of divergence at synonymous sites ( $K_s$ ) may be used as a surrogate for mutation rate. Such data are available for few organisms and no amphibians. Relative to mammals and birds, amphibian mtDNA is thought to evolve slowly, and the  $K_s$  ratio of mtDNA to scnDNA would be expected to be low as well. Relative  $K_s$  was estimated from a mitochondrial gene, *ND2*, and a nuclear gene, *c-myc*, using both “approximate” and likelihood methods. Three lineages of congeneric frogs were studied and this ratio was found to be approximately 16, the highest of previously reported ratios. No evidence of a low  $K_s$  in the nuclear gene was found: *c-myc* codon usage was not biased, the  $K_s$  was double the intron divergence rate, and the absolute  $K_s$  was similar to estimates obtained here for other genes from other frog species. A high  $K_s$  in mitochondrial vs. nuclear genes was unexpected in light of previous reports of a slow rate of mtDNA evolution in amphibians. These results highlight the need for further investigation of the effects of life history on mutation rates.

**Key words:** Substitution rate — Synonymous sites — Mitochondrial — Nuclear DNA — Amphibian — *c-myc* — *ND2*

### Introduction

Relative substitution rates of mitochondrial DNA (mtDNA) and single-copy nuclear DNA (scnDNA) vary among taxonomic groups, ranging from 1:1 to 1:14, depending on the taxonomic group and methods employed for estimating rates (Miyata et al. 1982; Powell et al. 1986; Vawter and Brown 1986; Sharp and Li 1989; Martin 1999; Pritchko and Moore 2000). Different genomes within the cell evolve at different rates (Brown et al. 1979). In general, substitutions in mitochondrial DNA are thought to occur 5- to 10-fold faster than in nuclear DNA (Slade et al. 1994; Moriyama and Powell 1997; Johnson and Clayton 2000; Sheldon et al. 2000), implying that different mechanisms underlie the stability of DNA sequence information in the two genomes. The finding that absolute rates differ among groups suggests that significant variation in the process of nucleotide substitution exists among taxa (Wu and Li 1985; Martin et al. 1992). One approach for deciphering the nature of control of substitution rates is comparative analysis of relative rates among taxonomic groups differing for traits known to influence, or suspected of influencing, the probability of nucleotide substitution (Martin and Palumbi 1993). We might expect that the ratio of rates for nuclear and mitochondrial genes varies with metabolic rate or generation time if, for example, such life-history variables influence

\*Current address: Smithsonian Tropical Research Institute, Apartado 2072, Balboa, Ancon, Republic of Panama  
Correspondence to: Andrew J. Crawford, STRI—Naos Lab, Unit 0948, APO, AA 34002-0948, USA; email: crawfordaj@naos.si.edu

mtDNA substitution rates to a greater extent than scnDNA substitution rates (Rand 1994). Alternatively, the ratio of substitution rates may be more or less constant across divergent taxa, implying that evolution has arrived at some fundamental constant determined by the balance between error minimization and energy investment. Although recent studies have found somewhat similar ratios across taxa (see above citations), comparative data are still largely lacking from many divergent groups. In vertebrates, most available data come from studies of mammals and birds. Notably absent are the amphibians.

In this study I investigated rates of silent site divergence (abbreviated  $K_s$  or  $d_s$ ) in mitochondrial and nuclear gene sequences to understand better the process of nucleotide substitution in these amphibians. If silent sites are evolving neutrally,  $K_s$  provides an estimate of the mutation rate (Kimura 1983) and removes the influence of selective constraints as a confounding variable in comparing divergence ratios (Palumbi 1989). To this end, I collected DNA sequence data from *ND2* and *c-myc* for three lineages of tropical frogs (Anura: Leptodactylidae: *Eleutherodactylus*) of the subgenus *Craugastor* of Central America. The genus is comprised of over 600 species of direct developing frog (Lynch and Duellman 1997; Duellman and Pramuk 1999). Population genetic data from one of the three lineages showed remarkable levels of endemism and genetic divergence (Crawford 2003), conditions favorable to genetic drift over the long term. Studying three distinct but related lineages allowed multiple independent estimates of  $K_s$ . The mitochondrial-to-nuclear  $K_s$  ratio was estimated across a range of divergence levels to accommodate the nonlinear nature of DNA sequence divergence over time (Palumbi 1989). Absolute dating was possible in these frogs via molecular clock calibration. Finally, I estimated rates of substitution in other frog species based on publicly available DNA sequence data and compared the results with those from *Eleutherodactylus*.

## Materials and Methods

### Sampling

The three distinct phylogenetic groups used in this study are referred to here as the *rhodopis*, the “*mexicanus*,” and the *gollmeri* groups. Current taxonomy does not distinguish the former two groups (Lynch 2000), but phylogenetic analyses show that the reciprocal monophyly of each clade is very well supported by both mtDNA and scnDNA datasets (A.J. Crawford and E.N. Smith, unpublished). Furthermore, the assumption made here of a trichotomous relationship among the three groups is not rejected by either dataset. Two outgroup samples from South American lineages are included for molecular clock calibration. Species names, taxonomic grouping, and GenBank accession numbers are listed in the Appendix.

### Laboratory Techniques

MtDNA fragments were PCR amplified using the primers L4437 and H5934 from Macey et al. (1997). Cycle sequencing utilized d-rhodamine dye-terminator chemistry, and products were analyzed on an ABI Prism 377 automated DNA sequencer (Applied Biosystems Inc.). Sequences were aligned using Sequencher 3.0 (Gene Codes Corp.) and by eye, using both nucleotide and inferred amino acid sequences. These protocols yielded the complete NADH dehydrogenase subunit 2 (*ND2*) gene.

A fragment of the cellular myelocytomatosis (*c-myc*) gene was amplified using the primers of Crawford (2003). The *c-myc* protooncogene is one of five paralogous genes in the *myc* family. These paralogs originated around the time of the divergence of the ancestors of extant vertebrate classes and are therefore typically older than the orthologous genes found among taxa within orders of vertebrates (Atchley and Fitch 1995). PCR products were sequenced directly and in both directions. Sequences were aligned using Sequencher 3.0 and checked by eye. These protocols yielded a single sequence that included approximately 540 bp of exon 2, 500 bp of intron 2, and 300 bp of exon 3.

Two *c-myc* sequences were inferred to be heterozygous at one site in exon 2. For these two frogs, one of the two inferred haplotypes was arbitrarily chosen for inclusion in the analyses. In intron 2, four frogs were inferred to have single heterozygous sites, and again one haplotype was chosen arbitrarily. One frog (*E. mimus*) was inferred to have two heterozygous bases within intron 2, precluding inference of either haplotype. Therefore both of these sites were excluded from the analyses of the *gollmeri* species group. Based on population genetic sampling of intron sequences from *rhodopis* group taxa, the *c-myc* gene appears not to be sex linked (Crawford 2003).

For comparative analyses, DNA sequences of *ND2* and the homologous portion of *c-myc* exon 2 were obtained from GenBank for human (accession numbers NC\_001807 and V00568, respectively), chimpanzee (NC\_001643 and M38057), Norway rat (NC\_001665 and Y00396), and domestic mouse (AB042432 and L00038).

### Data Analysis

I calculated divergence at synonymous sites by two methods. The first is an “approximate” method of Comeron (1995) implemented in the software, K-Estimator 5.5 (Comeron 1999). I also used Comeron’s (1995, 1999) method to calculate the effective number of synonymous sites (abbreviated SES) and confidence intervals around  $K_s$  via 1000 replicate simulations. The ratio of mitochondrial-to-nuclear mutation rates was then calculated by plotting  $K_s$  and attending confidence intervals for *c-myc* exon 2 against the corresponding estimates for *ND2* for all pairwise comparison among taxa within each species group. A linear regression line was fitted to the data and forced to intercept the origin. The slope of this line provided an estimate of the ratio of mitochondrial to nuclear  $K_s$ .

The above analyses provided three independent estimates of the *ND2*-to-*c-myc* ratio of  $K_s$ , yet each estimate was derived from nondependent data points. Therefore, I repeated the analysis using a codon-based maximum likelihood (ML) method of estimating divergence at synonymous sites, abbreviated  $d_s$ , rather than  $K_s$ , that allowed for simultaneous comparison of multiple sequences (Goldman and Yang 1994). In all ML analyses, equilibrium codon frequencies were calculated from the average nucleotide frequencies at the three codon positions (the F3x4 option in PAML v. 3.0 [Yang 2000]). The key difference between this method and Comeron’s (1995) method is that the ML method allowed for the inference of an unrooted phylogeny of each species group based upon

**Table 1.** Summary of analyzed DNA sequence data from *Eleutherodactylus*

Taxa	bp	<i>c-myc</i>											
		ND2				Exon 2				Intron 2			
		Variable sites <sup>a</sup>	Mean SES <sup>b</sup>	Indel sites	bp	Variable sites <sup>a</sup>	Mean SES <sup>b</sup>	Indel sites	Mean ENC <sup>c</sup>	bp	Variable sites <sup>a</sup>	Indel sites	
<i>rhodopis</i> group	6	1047	562	318.6	0	516	27	130.5	3	60.8	406	44	9
" <i>mexicanus</i> " group	5	1026	563	308.2	6	516	16	137.6	0	53.8	396	32	20
<i>gollmeri</i> group	4	1026	321	328.7	0	465	7	130.3	0	48.6	401	13	1

<sup>a</sup> Not including sites with indels.

<sup>b</sup> Synonymous effective number of sites (Comeron 1995), excluding stop codon or splice site codon, TCT; mean of pairwise comparisons.

<sup>c</sup> Effective number of codons (Wright 1990).

$d_s$  for each gene. I then compared mitochondrial versus nuclear  $d_s$  distances. To explore the trade-off between too few mutations at the nuclear gene and saturation of sites in the mitochondrial gene, I partitioned the same data three different ways to obtain three estimates of the ND2-to-*c-myc* ratio of  $d_s$  values. First, I obtained this ratio just from the lengths of terminal branches leading to each taxon. Second, I calculated this ratio by comparing the ND2-versus-*c-myc*  $d_s$  separating two sister taxa (including one polytomous trio of taxa from the odd-numbered "*mexicanus*" group). Finally, I calculated the (ND2/*c-myc*)  $d_s$  ratio by comparing the total  $d_s$  tree lengths for each species group.

$K_s$  in exon 2 was also compared to the overall number of substitutions per site,  $K$ , in intron 2 to appraise the relative constraints on synonymous sites relative to non-protein coding sites of *c-myc*. Point estimates and confidence intervals were calculated using Comeron's (1995, 1999) methods, as above. Three divergent members of each species group were used to estimate the within-group ratio of  $K$  to  $K_s$ , again by linear regression. The  $K/K_s$  ratio was also calculated from an among-species group comparison using one arbitrarily selected representative of each species group, *Eleutherodactylus mexicanus*, *E. stejnegerianus*, and *E. mimus*.

To quantify the selective constraints on synonymous sites, I estimated codon bias using Wright's (1990) measure of the effective number of codons (ENC) as implemented in DnaSP 3.5 (Rozas and Rozas 1999). ENC values may range from 20 to 61, with values below 40 indicative of highly biased codon usage.

Estimates of absolute rates of substitution were made as follows. The genus *Eleutherodactylus* originated in South America, while the subgenus *Craugastor* represents an endemic Central American radiation (Savage 1966). *Eleutherodactylus* is thought to have arrived in Central America during the late Cretaceous or Paleocene (96–58 million years before present [mybp]) via a proto-Antillean land bridge (Savage 1966, 1982; Rosen 1976). Geological evidence suggests that such a connection may have existed 80–60 mybp (Burke 1988; Iturralde-Vinent and MacPhee 1999). Absolute  $K_s$  was calibrated from the average pairwise  $K_s$  for *c-myc* exon 2 between *Craugastor* (represented by *E. mexicanus*, *E. bransfordii*, and *E. mimus*) and the South American group (i.e., *E. sp.* from eastern Brazil and *E. ridens*), using the maximum and minimum divergence time estimates above. Generation time is assumed to be 1 year, as has been shown for *E. bransfordii* (Donnelly 1999).

No published estimates of  $\mu$  in amphibian nuclear genes were available in the literature. Therefore, to compare the above results from *c-myc* in *Eleutherodactylus* with other genes from other frogs, I made the following two additional estimates of  $\mu$ . First, I analyzed 798 bp of the zinc finger transcription factor, *slug*, from the tetraploid frog, *Xenopus laevis* (GenBank accession numbers AF368041 and AF368043), and its diploid relative, *X.* (aka, *Silurana*) *tropicalis* (AF368039). Mean  $K_s$  and 95% CI were 0.186 (0.122–0.256). Based on fossil evidence (Báez 1996) the common ancestor of these two taxa existed 90 mybp. Assuming a generation time of 1 year (Tinsley and McCoid 1996), I estimated  $\mu$  as 1.03

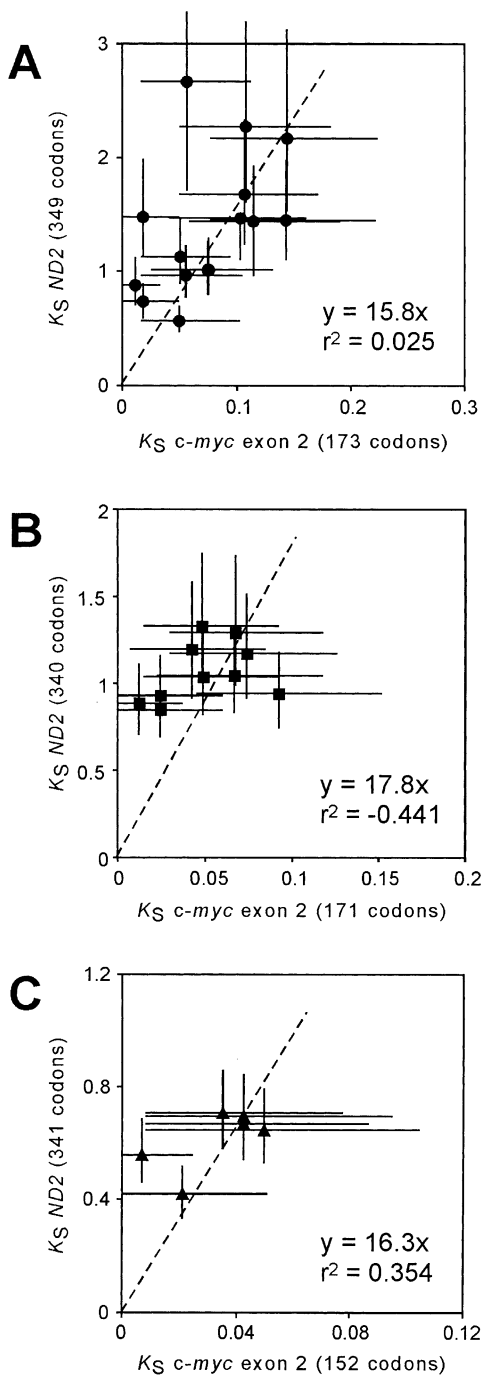
(0.679–1.42)  $\times 10^{-9}$  per synonymous site per year. Finally, various estimates were made from the unrooted phylogeny of Old World ranid frogs (Bossuyt and Milinkovitch 2000) for roughly 500 bp of the tyrosinase precursor gene exon 1 by assuming that the common ancestor of all the sampled Indian and Malagasy frogs was divided when these two land masses separated 88 mybp. All codons containing ambiguities were excluded from the analysis. Resulting  $\mu$  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). Because the ranid phylogeny could not be rooted, the higher rate estimates could be due to underestimating the age of evolutionary divergences that might have predated the vicariant event used in the calibration.

## Results

### *Silent Site Divergence in Mitochondrial versus Nuclear Genes*

I obtained DNA sequence data for four to six taxa from each of the three species groups. ND2 of the *rhodopis* group contained seven additional codons relative to the other two groups. The small *gollmeri* group sample showed the least overall divergence among samples relative to other species groups. ENC values were high for all *c-myc* sequences, suggesting that in *Eleutherodactylus* this gene uses synonymous codons with little to no bias (Table 1).

Regression of ND2 on *c-myc*  $K_s$  estimates (Comeron 1995) yielded a mean slope among the three species groups of 16.6 (Fig. 1), implying that the rate of nucleotide substitution at synonymous sites is over 16-fold higher in mitochondrial genes than nuclear genes. Using the codon-based ML method of estimating divergence at synonymous sites (Goldman and Yang 1994), I obtained three mean mtDNA-to-scDNA  $d_s$  ratios by averaging across the three hierarchical partitioning (tree tips, species pairs, and whole trees; see Methods) within each of the three independent frog clades. These  $d_s$  ratios were 21.8 for the least diverged *gollmeri* group (after removing the outlier of 66 obtained by comparing species pairs), 16.4 for the "*mexicanus*" group, and 11.4 for the highly divergent *rhodopis* group, for a similar overall mean of 16.5.



**Fig. 1.** Pairwise comparison of divergence at silent sites of *ND2* vs. *c-myc* among taxa within species group. Linear regression equation calculated by constraining line to pass through origin; 95% confidence limits estimated by simulation. Note the different scales on the two axes. Also note that the spatial distribution of data points in these graphs depends on the shape of the phylogeny of sampled taxa: a nested phylogeny would produce points along a line, while a star phylogeny would produce a cluster of points. **A** Six taxa of the *rhodopis* group. **B** Five taxa of the “*mexicanus*” group. **C** Four taxa of the *gollmeri* group.

#### Substitution Rates in the Exon versus the Intron

Within species groups, regressing total intron divergence,  $K$ , on  $K_S$  for exon 2 yielded an average slope of

0.48 (0.43 for the *rhodopis* and *gollmeri* groups, but 0.58 for the “*mexicanus*” group). For the *rhodopis* group, the 95% confidence limits around this ratio excluded unity, specifically, equal rates. The comparison made between species groups yielded a slope of 0.44, and again, a value of 1 was significantly rejected. Thus, the intron 2 nucleotide sites overall appear to have diverged about half as fast as exon 2 silent sites.

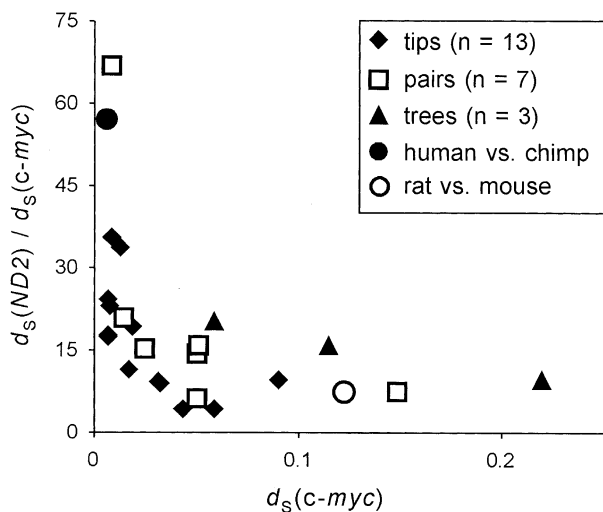
#### Absolute Substitution Rates

Average pairwise  $K_S$  for *c-myc* exon 2 between the subgenus *Craugastor* (represented by *E. mexicanus*, *E. bransfordii*, and *E. mimus*) and the subgenus *Eleutherodactylus* (i.e., *E. sp.* from eastern Brazil and *E. ridens*) equaled 0.178. Using the maximum and minimum divergence time estimates reported above, the absolute synonymous site divergence rate for *c-myc* was estimated as  $0.924 \times 10^{-9}$  to  $1.53 \times 10^{-9}$  substitutions per synonymous site per year. This range of values is very similar to the independent estimates of  $\mu$  obtained for *slug* and the tyrosinase precursor gene, above. Additionally, because  $K_S$  in *Eleutherodactylus* mtDNA is 16-fold faster than in *c-myc*, the above rate calibration would imply that the absolute  $K_S$  for *ND2* is between  $14.8 \times 10^{-9}$  and  $24.5 \times 10^{-9}$  substitutions per synonymous site per year.

#### Discussion

The rate of divergence at synonymous sites in *Eleutherodactylus* was 16 times faster in mtDNA than in the nuclear gene, and this ratio is among the highest yet reported for protein coding genes of any taxon. This result was robust across three datasets and between the two methods of estimation. I found the same overall ratio either using the “approximate” method of Comeron (1995) applied to three lineages independently (Fig. 1) or using the ML method of Goldman and Yang (1994), which provided independent, topology-based comparisons of synonymous site divergence. This high ratio of mtDNA-to-scndDNA divergence was not caused by codon bias (Table 1) slowing  $K_S$  (or  $d_S$ ) in the *c-myc* gene, nor was  $K_S$  slow relative to  $K$  in the neighboring intron.

The high ratio of mtDNA-to-scndDNA synonymous substitutions does not appear to be a consequence of an *ND2* substitution rate that is higher in *Eleutherodactylus* than in other species. The absolute rate estimated above for *ND2* in *Eleutherodactylus* is slightly lower than that estimated from mammals (Pesole et al. 1999) or from another frog: my analysis of silent site divergence at *ND2* in Eurasian toads, based on the data and calibration time of Macey et al. (1998), yielded a  $K_S$  of  $37.4 \times 10^{-9}$ . Thus,  $K_S$  in *ND2* of leaf litter frogs is not elevated relative to that of these other lineages.



**Fig. 2.** Ratio of *ND2*-to-*c-myc*  $d_s$  values as a function of  $d_s$  at *c-myc*. The same data are partitioned three ways: comparing lengths of external nodes (tips),  $d_s$  values between sister taxon pairs, and whole trees. Two taxa showed an external branch length of zero at the *c-myc* gene and are excluded from tips only. Comparative data are presented for the homologous gene regions of human vs. chimpanzee and rat vs. mouse.

The high ratio of mtDNA-to-scndDNA  $K_s$  in *Eleutherodactylus* does not appear to be due to low values in the denominator, specifically, too few *c-myc* substitutions. When the ratio of mtDNA-to-scndDNA  $K_s$  (or  $d_s$ ) is high, accurate estimation of this ratio involves a trade-off between a small number of substitutions in scndDNA observed at low divergences and a large number of multiply substituted sites observed in mtDNA at high divergences. The axes in Fig. 1 show the levels of divergence and saturation in the *Eleutherodactylus* data. The trade-off may be visualized on a graph of the  $d_s$  ratio (*ND2/c-myc*) versus the overall divergence, measured here by  $d_s$  of the nuclear gene: for recently diverged taxa, such as humans and chimps, the mitochondrial-to-nuclear ratio may appear inordinately high, while for distantly related taxa, such as mice and rats, this ratio may be depressed (Fig. 2 [cf. Palumbi 1989]). For *Eleutherodactylus*, similar mean ratios were obtained whether I focused on recent divergences, specifically, just the tips of the phylogeny, or focused on large-scale divergence, specifically, whole species groups. Across the three species groups, I obtained average  $d_s(ND2)$ -to- $d_s(c-myc)$  ratios of 18.1 for tips, 13.6 for pairs of taxa, and 15.2 for the comparison of  $d_s$  tree lengths for whole species groups (see Results and Fig. 2). The relative consistency among ratios across hierarchical partitions suggests that I have obtained a balance between insufficient nuclear substitutions and excessive mitochondrial substitutions. Therefore, 16 is a reasonable estimate of the ratio of mitochondrial-to-nuclear mutation rates based on these data.

The notion that we can estimate robustly the mtDNA-to-scndDNA  $K_s$  ratio is surprising given the

low  $r^2$  values and the divergent point estimates of the mtDNA-to-scndDNA divergence ratio (Fig. 1). However, the spatial distribution of data points in a plot such as Fig. 1 reflects the shape of the phylogeny of the sampled sequences. Pairwise comparisons among samples from a hierarchically nested topology will tend to produce points along a line with a modest  $r^2$  value (e.g., Fig. 1A). Pairwise comparisons among equidistantly related sequences, in particular, from a star phylogeny, would yield a clustering of points and a very low  $r^2$  value (e.g., Fig. 1B). Therefore, the data should be analyzed as a unitary set providing a single estimate, not as a series of points to be evaluated individually (D. Bernoulli 1778, cited in Stigler 1999).

The high ratio of mitochondrial-to-nuclear silent site divergence in *Eleutherodactylus* provides further evidence of the variation among taxonomic groups in the process of nucleotide substitution (Wu and Li 1985; Martin et al. 1992). The variation was in the opposite direction from our expectation for an amphibian. Poikilothermic vertebrates have been shown to have low rates of mtDNA evolution (Martin and Palumbi 1993), yet this study found no evidence of a rate slowdown in *Eleutherodactylus*. However, new data could reveal that many taxa have high mtDNA-to-scndDNA  $K_s$  ratios, similar to *Eleutherodactylus*. Understanding the influence of life-history variables on the ratio of mtDNA-to-scndDNA divergence in vertebrates will require further comparative data, notably from reptiles and fish. As highlighted in the preceding analysis, future work should focus on divergence at silent sites, employ a consistent analytical methodology, use multiple lineages within a taxonomic group, and compare results across a range of divergence levels.

## Appendix

Species of *Eleutherodactylus* used in this study, by species group, and their corresponding GenBank accession numbers are as follows. The first of each pair of accession numbers corresponds to the *ND2* sequence; the second corresponds to *c-myc*. The single GenBank number for the Brazilian outgroup, “*E. sp.*,” represents a *c-myc* sequence. Institutional specimen voucher numbers are provided in the GenBank records, **rhodopsis group**: *E. bransfordii* (AY205576, AY211321); *E. polyptychus* (AY273139, AY211322); *E. persimilis* (AY273141, AY211299); *E. stejnegerius* (AY205579, AY211317); *E. podiciferus* (AY273135, AY211319); *E. rhodopsis* (AY273131, AY211294). **“mexicanus” group**: *E. sp. nov.* (AY273120, AY211310); *E. pygmaeus* (AY273119, AY211313); *E. saltator* (AY273122, AY211311); *E. sartori* (AY273121, AY211308); *E. mexicanus* (AY273118, AY211312). **gollmeri group**: *E. mimus* (AY273125, AY211281); *E. noblei* (AY273127, AY211280); *E. gollmeri* Fortuna (AY273124, AY211279); *E. gollmeri* Santa Fe

(AY205590, AY211278). **Outgroups:** *E. ridens* (AY273101, AY211306); *E. sp. Brazil* (AY211305).

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