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Cenozoic biogeography and evolution in direct-developing frogs of Central America (Leptodactylidae: *Eleutherodactylus*) as inferred from a phylogenetic analysis of nuclear and mitochondrial genes

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Abstract

We report the first phylogenetic analysis of DNA sequence data for the Central American component of the genus *Eleutherodactylus* (Anura: Leptodactylidae: Eleutherodactylinae), one of the most ubiquitous, diverse, and abundant components of the Neotropical amphibian fauna. We obtained DNA sequence data from 55 specimens representing 45 species. Sampling was focused on Central America, but also included Bolivia, Brazil, Jamaica, and the USA. We sequenced 1460 contiguous base pairs (bp) of the mitochondrial genome containing *ND2* and five neighboring tRNA genes, plus 1300 bp of the c-*myc* nuclear gene. The resulting phylogenetic inferences were broadly concordant between data sets and among analytical methods. The subgenus *Craugastor* is monophyletic and its initial radiation was potentially rapid and adaptive. Within *Craugastor*, the earliest splits separate three northern Central American species groups, *milesi*, *augusti*, and *alfredi*, from a clade comprising the rest of *Craugastor*. Within the latter clade, the *rhodopis* group as formerly recognized comprises three deeply divergent clades that do not form a monophyletic group; we therefore restrict the content of the *rhodopis* group to one of two northern clades, and use new names for the other northern (*mexicanus* group) and one southern clade (*bransfordii* group). The new *rhodopis* and *bransfordii* groups together form the sister taxon to a clade comprising the *biporcatus*, *fitzingeri*, *mexicanus*, and *rugulosus* groups. We used a Bayesian MCMC approach together with geological and biogeographic assumptions to estimate divergence times from the combined DNA sequence data. Our results corroborated three independent dispersal events for the origins of Central American *Eleutherodactylus*: (1) an ancestor of *Craugastor* entered northern Central America from South American in the early Paleocene, (2) an ancestor of the subgenus *Syrrhophus* entered northern Central America from the Caribbean at the end of the Eocene, and (3) a wave of independent dispersal events from South America coincided with formation of the Isthmus of Panama during the Pliocene. We elevate the subgenus *Craugastor* to the genus rank.

Keywords: Chortis block; *Craugastor*; Eleutherodactylinae; Paleogeography; Mesoamerica; Molecular phylogenetics; Proto-Antilles; Systematics

1. Introduction

With over 700 Neotropical species, *Eleutherodactylus* ranks as the most species-rich of all vertebrate genera, and the number of species continues to climb

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(AmphibiaWeb, 2005; Campbell, 1999; Duellman, 1999a; Frost, 2004; Hedges, 1999; Lynch and Duellman, 1997). For example, during a recent 4-year period, 1999–2002, *Eleutherodactylus* taxa were newly described or resurrected at a mean rate of one every month (e.g., Campbell and Savage, 2000; Duellman and Pramuk, 1999; Lynch, 2001a; Savage and Myers, 2002). Half of all the world's frog species are Neotropical, and a quarter of these are *Eleutherodactylus* (AmphibiaWeb, 2005; Duellman, 1999b). Many frog communities are dominated by *Eleutherodactylus*, both in terms of diversity and abundance (Lieberman, 1986; Scott, 1976), especially those in the highlands (Doan and Arriaga, 2002; Hofer and Bersier, 2001). This genus possesses an unusual, though not unique, mode of development among anurans in which the tadpole stage has been lost, eggs are laid terrestrially, and the young hatch out as tiny froglets (Callery et al., 2001; Elinson et al., 1990). Because of its direct development, *Eleutherodactylus* has provided a model system for studies of the evolution of development (Elinson and Ninomiya, 2003; Hanken et al., 2001). We find it to be no small tragedy, therefore, that this tremendous group of frogs is so poorly known regarding its biogeographic origins, phylogenetic relationships, taxonomic groupings, or in some cases even basic field identification.

The goals of this study are to estimate phylogenetic relationships among Central American *Eleutherodactylus*, estimate levels of genetic divergence among the major clades, and use these results to elucidate the biogeographic origins of these frogs. Although there are still points of contention among students of the genus, we may summarize our current state of knowledge as follows. *Eleutherodactylus* almost certainly originated in South America because this continent is home to all species of all other genera in the subfamily Eleutherodactylinae and most species in all other subfamilies of Leptodactylidae (Duellman, 1999a; Lynch, 1971). Not surprisingly then, the bulk of species diversity of *Eleutherodactylus* falls within the primarily South American subgenus of the same name. Of the approximately 400 species in this group, 16 occur in Central America. The genus *Eleutherodactylus* contains four other subgenera (Hedges, 1989). The subgenus *Craugastor* ranges from the southwestern United States to northwestern South America (Lynch, 1986) and contains just over 100 species. Two subgenera, *Euhyas* and *Pelorius*, are found in the Greater Antilles and collectively contain fewer than 100 species. The subgenus *Syrrhophus* includes two dozen species and ranges from Texas to Belize and Guatemala. Within Central America, therefore, one may find *Eleutherodactylus* from three subgenera.

Darst and Cannatella (2004) found molecular phylogenetic evidence that the genus *Eleutherodactylus* may be polyphyletic. Although taxonomic sampling was limited, their optimal reconstructions suggested that the sister group to *Craugastor* could be *Brachycephalus* from the southern Atlantic rainforests of Brazil, and the Andean genus *Phrynopus* could be the sister group to either the subgenus *Euhyas* or to the subgenus *Eleutherodactylus*. However, placement of these two South American genera relative to *Eleutherodactylus* was not statistically significant in either a Bayesian or a parsimony framework. Unfortunately, these other genera are not included in this study, so we are unable to evaluate these hypotheses.

The following biogeographic scenario for the origins of *Eleutherodactylus* in Central America follows Duellman (2001, pp. 807 and 809) and is based on a mix of data and informed speculation. The three lineages of Central American *Eleutherodactylus* are thought to have arrived there through two (Savage, 1982) or possibly three (Hedges, 1989) separate dispersal events. The genus *Eleutherodactylus* (including *Craugastor*) first entered Central America from South America in the late Cretaceous to early Paleocene via an hypothesized proto-Antillean land bridge (Savage, 1966, 2002) formed at the leading edge of the Caribbean tectonic plate as it moved east between North and South America (Burke, 1988). Among these South American frogs came the ancestor of *Craugastor*, according to this model. With the break-up of the land bridge, the ancestral *Euhyas* came to occupy the area that would become Cuba (Hedges, 1989; Hedges et al., 1992). These West Indian *Eleutherodactylus* diverged from their mainland counterparts an estimated 77–63 million years ago (mya) (Hedges, 1996), coinciding with the land-bridge model. Second, *Syrrhophus* is thought to have originated in northern Mesoamerica 40–30 mya through the dispersal from the Greater Antilles by an ancestral member of the *Euhyas* lineage (Hass and Hedges, 1991; Hedges, 1989). Third, with the gradual formation of a new land bridge (Coates et al., 2004) and the reconnection of Central and South America just prior to 3 mya (Coates and Obando, 1996), species of the subgenus *Eleutherodactylus* entered lower Central America (Savage, 2002; Vanzolini and Heyer, 1985) and today extend as far north as Honduras (McCranie and Wilson, 2002).

Eleutherodactylus are notorious for their high phenotypic variability within populations (Savage and Emerson, 1970) and scant morphological divergence among species (Campbell and Savage, 2000; Savage, 1981). Few morphological characters are informative for parsimony analysis of interspecific relationships within this diverse genus (Lynch, 1986, 2000; Lynch and Duellman, 1997). Besides the three samples included in the study by Darst and Cannatella (2004), the only previous molecular phylogenetic studies of Central American *Eleutherodactylus* used allozyme data to investigate relationships within species groups (Miyamoto, 1983, 1984, 1986), and the conclusions we feel were hampered by

inadequate sampling within species or within populations. Therefore, further DNA sequence data are crucial to advancing our understanding of this group.

Based on the above taxonomy and biogeographic scenario, we investigate the following questions. Does the subgenus *Craugastor* represent a monophyletic group? Was the initial radiation of the ancestral *Craugastor* lineage rapid or adaptive? Are *Syrrhophus* and *Euhyas* sister taxa, as suggested by allozyme, immunological, and some morphological data (Hedges, 1989; Hass and Hedges, 1991)? Do the members of the subgenus *Eleutherodactylus* in lower Central America represent a single invasion with subsequent speciation, or did speciation pre-date dispersal? We also look in detail at two widespread Central American species groups within *Craugastor*, the *rhodopis* and *gollmeri* groups, to test their monophyly and to investigate their geographic origins. Hedges' hypothesis represents an unusual biogeographic scenario, a continental radiation having been derived from an island ancestor, which has been greeted with some skepticism (e.g., Duellman, 2001, p. 807). Here we use mitochondrial and nuclear DNA sequence data to investigate these phylogenetic and biogeographic questions. Finally, we investigate absolute times of origination of species groups within *Craugastor* under the proto-Antilles model and compare these divergence times with the geological history of Central America.

2. Materials and methods

2.1. The study group

The approximately 100 species of *Craugastor* are divided currently into nine (Savage, 2002) or 11 (Lynch, 2000) species groups, and most species are found only in Central America. Most of these species groups are phenetic, lacking synapomorphies. We can divide Savage's nine groups into three sets according to their geographic ranges [a concise overview of *Eleutherodactylus* taxonomy and *Craugastor* distributions is found in Duellman (2001, pp. 806–811)]. Species in the three northern groups, *augusti* (formerly known as the genus *Hylactophryne*, 2 species), *alfredi* (13 spp.), and *milesi* (12 spp.) groups reach as far south as Mexico, Guatemala, and Honduras, respectively. The two uniquely southern groups are the *fitzingeri* (13 spp.; Savage et al., 2004) and *bufoniformis* (2 spp.) groups, and the northern extent of their ranges reaches just over the Nicaragua–Honduras border in the former group but stops in southeastern Costa Rica in the latter group. One small group, the *biporcatus* group (6 spp.), is largely southern, but with a disjunct species in Guatemala. The preceding three are the only groups within *Craugastor* that have representatives in northwestern South America. The final three species groups are widespread but still confined to Central America, each one ranging from Mexico to central Panama: the *rugulosus* (33 spp.), *gollmeri* (10 spp.), and *rhodopis* (16 spp.) groups. All nine species groups (*sensu* Savage, 2002) except the *bufoniformis* group were represented by at least one specimen in the present study (Table 1). The wide-ranging *gollmeri* and *rhodopis* groups were investigated here in detail.

2.2. Taxonomic sampling

We obtained DNA sequence data from 55 frogs representing 45 species, including two outgroup taxa, four undescribed taxa and one unidentified specimen from Brazil (Table 1). Four of five subgenera of *Eleutherodactylus* are represented among the samples, as are eight of nine species groups (*sensu* Savage, 2002) within the subgenus *Craugastor*. Mitochondrial DNA (mtDNA) sequences were obtained from 43 samples, and single-copy nuclear DNA (scnDNA) sequences were obtained from 40 samples. Thus, both loci were sequenced for a subset of 28 samples. All samples are supported by corresponding voucher specimens available in public research collections, and all genetic data are available in GenBank (Table 1).

2.3. 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 PCR amplified using the primers L4437 and H5934 (Macey et al., 1997a). Cycle sequencing utilized D-Rhodamine dye-terminator chemistry, and products were analyzed on an ABI Prism 377 automated DNA sequencer (Applied Biosystems). Both heavy and light strands were completely sequenced using many internal primers (Table 2). Sequences were aligned using Sequencher 3.0 (Gene Codes) and by eye with the same program and with GeneDoc (Nicholas and Nicholas, 1997). Homology of DNA characters was inferred from the nucleotide sequences, the inferred amino acid sequences, and transfer RNA (tRNA) secondary structure models (Kumazawa and Nishida, 1993; Macey et al., 1997c). These protocols yielded a mtDNA fragment of approximately 1460 base pairs (bp) containing the following five complete and two partial genes, plus a stem–loop structure, listed here in order from the 5′ to the 3′ end: a fragment of the $tRNA^{MET}$ gene, the complete NADH dehydrogenase subunit 2 (*ND2*) gene, $tRNA^{TRP}$, $tRNA^{ALA}$, $tRNA^{ASN}$, the origin of light strand replication (O_L), $tRNA^{CYS}$, $tRNA^{TYR}$, and a fragment of the cytochrome oxidase I (*COI*) gene. This mtDNA fragment is referred to as the *ND2*-WANCY region. For those samples that deviated from this standard gene order, the duplicated genes, rearranged genes,

Table 1
Institutional voucher numbers, locality information, and GenBank accession numbers for sampled taxa (FMNH = Field Museum of Natural History, Chicago; UTA = University of Texas at Arlington; SIUC = Southern Illinois University at Carbondale; MVZ = Museum of Vertebrate Zoology, University of California, Berkeley; CH = Círculo Herpetológico de Panamá; USNM = National Museum of Natural History, Washington, DC; SMF = Senckenberg Museum, Frankfurt am Main, Germany; ZUFRJ = Departamento de Zoologia, Universidade Federal do Rio de Janeiro, Brazil)

Species and taxonomy[a]	Institutional voucher #	Collection locality[b]	Geographic coordinates	GenBank Accession No.	
				mtDNA	c-*myc*
Subfamily Leptodactylinae					
Genus *Leptodactylus*					
Species group *melanonotus*					
L. melanonotus	UTA A-53817	San Marcos, GT	14°52′N, 091°54′W	AY273099	Y337266
Species group *fuscus*					
L. labialis	UTA A-48666	Puerto Barrios, Izabal, GT	15°36′N, 088°43′W	AY273100	None
Subfamily Eleutherodactylinae					
Genus *Eleutherodactylus*					
Subgenus *Eleutherodactylus*					
Species group *unistrigatus*					
E. ridens	FMNH 257746	Las Cruces, Puntarenas, CR	08°47′N, 082°59′W	AY273101	AY211306
E. museosus	SIUC H-06970	El Copé, Coclé, PA	08°40′N, 080°36′W	AY273103	None
E. pardalis	FMNH 257675	Fortuna, Chiriquí, PA	09°45′N, 082°13′W	AY273102	AY211305
Species group *conspicillatus*					
E. gaigei	SIUC H-06965	El Copé, Coclé, PA	08°40′N, 080°36′W	None	AY211290
E. sp. (Brazil)	ZUFRJ 7861	Pernambuco, BR	07°16′S, 035°23′W	None	AY211305
Species group *discoidalis*					
E. ibischi	UTA A-55243	Sud Cinti, Chuquisaca, BO	20°51′S, 064°19′W	None	AY211288
Subgenus *Euhyas*					
Species group *gossei*					
E. pantoni	USNM 327872	St. Andrew Parish, JM	18°05′N, 076°43′W	AY273104	AY211282
Subgenus *Syrrhophus*					
Species group *pipilans*					
E. pipilans	UTA A-51050	Nenton, Huehuetenango, GT	15°46′N, 091°51′W	AY273105	None
Species group *nitidus*					
E. dilatus	UTA A-55248	Omiltemi, Guerrero, MX	17°33′N, 099°41′W	None	AY337268
Subgenus *Craugastor*					
Species group *milesi*					
E. trachydermus	UTA A-48500	Livingston, Izabal, GT	15°43′N, 089°14′W	AY273106	AY211300
E. daryi	UTA A-55251	Xucaneb, Alta Verapaz, GT	15°39′N, 089°47′W	AY273107	AY211316
Species group *augusti*					
E. augusti 1	UTA A-54930	Chilpancingo, Guerrero, MX	17°37′N, 099°05′W	AY273108	AY211289
E. augusti 2	MVZ 226839	Roswell, New Mexico, US	33°22′N, 104°15′W	AY273109	None
Species group *alfredi*					
E. bocourti	UTA A-55235	Purulha, Baja Verapaz, GT	15°15′N, 090°10′W	AY273110	AY211301
E. xucanebi	UTA A-51369	Huehuetenango, GT	15°52′N, 091°14′W	None	AY211298
Species group *biporcatus*					
E. megacephalus	FMNH 257714	La Selva, Heredia, CR	10°25′N, 084°02′W	AY273111	AY211296
Species group *rugulosus*					
E. ranoides	MVZ 207277	Volcán Cacao, Guanacaste, CR	10°55′N, 085°27′W	AY273112	AY211287
Species group *fitzingeri*					
E. crassidigitus 1	FMNH 257676	Fortuna, Chiriquí, PA	09°45′N, 082°13′W	AY273113	None
E. crassidigitus 2	FMNH 257693	Nusagandí, San Blas, PA	09°20′N, 078°59′W	AY273114	None
E. crassidigitus 3	FMNH 257695	Nusagandí, San Blas, PA	09°20′N, 078°59′W	None	AY337269
E. fitzingeri	FMNH 257745	Las Cruces, Puntarenas, CR	08°47′N, 082°59′W	AY273117	AY211297
E. longirostris	CH 4735	Cana, Darién, PA	07°45′N, 077°41′W	AY273116	None
E. talamancae	FMNH 257694	Nusagandí, San Blas, PA	09°20′N, 078°59′W	None	AY337270
E. tabasarae	SIUC H-06964	El Copé, Coclé, PA	08°40′N, 080°36′W	AY273115	None
Species group *gollmeri*					
E. chac	UTA A-55261	Livinatón, Izabal, GT	15°43′N, 089°14′W	AY273130	None
E. gollmeri 1	FMNH 257561	Fortuna, Chiriquí, PA	09°45′N, 082°13′W	AY273124	AY211279
E. gollmeri 2	FMNH 257696	Nusagandí, San Blas, PA	09°20′N, 078°59′W	AY273123	None
E. laticeps	UTA A-55249	El Volcán, Alta Verapaz, GT	15°28′N, 089°52′W	AY273129	AY337267
E. lineatus	UTA A-55250	El Volcán, Alta Verapaz, GT	15°28′N, 089°52′W	AY273126	None
E. mimus	FMNH 257605	La Selva, Heredia, CR	10°25′N, 084°02′W	AY273125	AY211281
E. noblei 1	FMNH 257616	El Copé, Coclé, PA	08°40′N, 080°36′W	AY273127	AY211280
E. noblei 2	USNM 534195	Quebrada Machin, Colón, HN	15°19′N, 085°17′W	AY273128	AY211285

(*continued on next page*)

Table 1 (*continued*)

Species and taxonomy[a]	Institutional voucher #	Collection locality[b]	Geographic coordinates	GenBank Accession No.	
				mtDNA	c-*myc*
Species group *mexicanus* (species group *rhodopis* prior to this analysis)					
E. mexicanus	UTA A-55233	Guelatao, Oaxaca, MX	17°26′N, 096°29′W	AY273118	AY211312
E. omiltemanus	UTA A-55240	Xochipala, Guerrero, MX	17°40′N, 99°49′W	None	AY337271
E. pygmaeus 1	UTA A-55241	Nueva Dehli, Guerrero, MX	17°26′N, 100°11′W	None	AY211309
E. pygmaeus 2	UTA A-55246	Río Salado, Oaxaca, MX	16°12′N, 097°06′W	AY273119	AY211313
E. saltator	UTA A-55239	Nueva Dehli, Guerrero, MX	17°29′N, 100°12′W	AY273122	AY211311
E. sartori	UTA A-51105	La Fraternidad, San Marcos, GT	14°56′N, 091°53′W	AY273121	AY211308
E. sp. nov. A	UTA A-55247	Miahuatlán, Oaxaca, MX	16°10′N, 097°00′W	AY273120	AY211310
Species group *rhodopis*					
E. rhodopis 1	UTA A-55245	Yaxchilán, El Petén, GT	17°11′N, 091°06′W	AY273133	AY211315
E. rhodopis 2	UTA A-55231	Jacaltepec, Oaxaca, MX	17°52′N, 096°14′W	AY273131	AY211294
E. rhodopis 3	UTA A-54811	Chimalapa, Oaxaca, MX	16°45′N, 094°45′W	AY273132	None
E. loki	UTA A-54820	Los Tuxtlas, Veracruz, MX	18°22′N, 095°06′W	AY273134	None
Species group *bransfordii* (species group *rhodopis* prior to this analysis)					
E. bransfordii	FMNH 257700	Nusagandí, San Blas, PA	09°20′N, 078°59′W	AY273140	AY211304
E. lauraster	SMF 79760	Selva Negra, Matagalpa, NI	12°00′N, 085°55′W	AY273138	None
E. persimilis	FMNH 257566	CATIE, Cartago, CR	09°54′N, 083°39′W	AY273141	AY211299
E. podiciferus 1	FMNH 257653	Las Cruces, Puntarenas, CR	08°47′N, 082°59′W	AY273135	AY211319
E. podiciferus 2	FMH 257595	Tapantí, Cartago, CR	09°45′N, 083°47′W	None	AY211307
E. polyptychus	FMNH 257555	Isla Colón, Bocas del Toro, PA	09°23′N, 082°17′W	AY273139	AY211322
E. stejnegerianus 1	FMNH 257803	Rincón de Osa, Puntarenas, CR	08°42′N, 083°31′W	AY273137	None
E. stejnegerianus 2	FMNH 257801	Rincón de Osa, Puntarenas, CR	08°42′N, 083°31′W	None	AY211320
E. sp. nov. B	FMNH 257689	Fortuna, Chiriquí, PA	09°45′N, 082°13′W	None	AY211318
E. sp. nov. C	FMNH 257760	Río Claro, Puntarenas, CR	08°45′N, 083°03′W	AY273136	AY211303

Numerals following conspecific names match numbers used in Figs. 1–3.

[a] Taxonomy follows Lynch and Duellman (1997) and Frost (2004), except where noted.

[b] Countries abbreviated by their ISO 3166 two-letter codes: BO, Bolivia; BR, Brazil; CR, Costa Rica; GT, Guatemala; HN, Honduras; JM, Jamaica; MX, Mexico; NI, Nicaragua; PA, Panama; and US, United States.

and pseudogenes were replaced with ambiguities (*N*'s) for purposes of this study. Description and analysis of these unusual features will be discussed elsewhere.

A fragment of the cellular myelocytomatosis (c-*myc*) gene was amplified using the primers cmyc1U and cmyc3L (Table 2). PCR products were sequenced directly and in both directions. The c-myc genes of three problematic samples (*Eleutherodactylus laticeps*, *Leptodactylus melanonotus*, and *Syrrhophus dilatus*) were sequenced using a protocol differing from that used for the mitochondrial fragment. PCR products were obtained on a gradient thermal cycler at annealing temperatures of 54, 55.6, and 57.5 °C. PCR products were cloned into plasmids (Topo TA, Invitrogen) that were isolated using the QIAprep Spin Miniprep Kit (Qiagen). Sequencing reactions were prepared using SequiTherm EXCEL II DNA Sequencing Kit-LC (Epicentre Technologies) and the products were analyzed with a LI-COR 4200 Dual Laser Long-read automated sequencer. Genic structure of the c-*myc* fragment was determined from consensus splice sites and by alignment with an mRNA sequence from *Xenopus* (King, 1991). These protocols yielded approximately 1340 bp sequences that included roughly 540 bp of exon 2, 500 bp of intron 2, and 300 bp of exon 3.

2.4. Phylogenetic inference

Because all models of DNA sequence evolution used in this study assume that nucleotide frequencies are constant over time and among lineages, we first tested the mitochondrial and nuclear data sets for significant departure from this assumption of stationarity using a χ^2 test implemented in *PAUP** 4.0b10 (Swofford, 1998). To evaluate potential phylogenetic heterogeneity between the two data sets we used the incongruence length difference (ILD) permutation test (Farris et al., 1995) with 6800 random partitions of the combined data set as implemented in *PAUP**.

Prior to likelihood-based phylogenetic analyses we identified a model of DNA sequence evolution that required a minimum number of parameters yet was adequate to explain the data. This evaluation was conducted on neighbor-joining (NJ) trees (Saitou and Nei, 1987) based on JC69 distances (Jukes and Cantor, 1969) using *Modeltest* version 3.06 (Posada and Crandall, 1998). The model and the parameter estimates were chosen by Akaike's minimum theoretical information criterion, or AIC (Akaike, 1974), and were then used in maximum likelihood (ML) analyses (Felsenstein, 1981). These results were also used to choose the appropriate model for the Bayesian analyses.

Table 2
Primers used in this study for amplifying and sequencing the mitochondrial *ND2*-WANCY gene region and intron 2 plus partial flanking exons of the nuclear gene, c-*myc*

	Nucleotide sequence (5′–3′)
mtDNA primer (lab name)	
L4437 (METf.6)[a]	AAGCTTTCGGGCCCATACC
H5934 (COIr.1)[a]	AGRGTGCCAATGTCTTTGTGRTT
H4996 (ND2r.564s)[b]	AGTATGCTAAGAGTTTTC
H4980 (ND2r.6)[a]	ATTTTTCGTAGTTGGGTTTGRTT
H4980 (ND2r.B)	AATTTTCGAATTTGTGTTTGGTT
H4980 (ND2r.D)	ATTTTCCGAACTTGTGTTTGATT
H4980 (ND2r.C)	AGTTTACGAATTTGAGTTTGGTT
H4980 (ND2r.U1)	ATTTTTCGGGTTTGAGTTTGATT
H4980 (ND2r.G1)	ATTTTTCGAATTTGTGTTTGATT
H4980 (ND2r.L)	ATTTTTCGGACTTGTGTTTGGTT
H4980 (ND2r.M)	AGTTTACGAACTTGTGTTTGGTT
H4980 (ND2r.H)	ATTTTTCGTAGCTGTTTTTGGTT
H4980 (ND2r.G2)	ATTTTTCGAATCTGTGTTTGATT
H4980 (ND2r.G3)	ATTTTTCGAAGTTGTGTTTGGTT
L4811 (ND2f.7)	GGCATTGCCCCMTTYCACTTCTG
L4882 (ND2f.15)[a]	TGACAAAAACTAGCACC
L4882 (E-ND2f.15)	TGACAAAAACTTCCACC
L4976 (ND2f.0533)	AATTGTAGGCGGCTGAGGAGGC
L5038 (ND2f.590s)	GCTCACCTTGGCTGAAT
L5002 (ND2f.5)[b]	AACCAAACCCAACTACGAAAAAT
L5002 (ND2f.C)	AACCAAACTCAAATTCGTAAAGT
L5002 (E-ND2f.5)	AATCAAACTCAACTACGAAAACT
L5002 (ND2f.B)	AACCAAACTCAAGTTCGAAAAAT
L5002 (ND2f.U1)	AATCAAACTCAAACCCGAAAAAT
L5002 (ND2f.k)	AACCAAACACAAATTCGAAAAKT
L5002 (ND2f.M1)	AACCAAACACAACTTCGAAAGAT
L5002 (ND2f.G1)	AATCAAACACAAATTCGAAAAAT
L5002 (ND2f.U4)	AACCAAACTCAAGTCCGAAAACT
L5002 (ND2f.L)	AACCAAACACAAACACGAAAAAT
L5002 (ND2f.M3)	AACCAAAAACAGCTACGAAAAAT
L5002 (ND2f.M2)	AATCAAACACAACTTCGAAAAAT
L5002 (ND2f.G2)	AATCAAACACAGATTCGAAAAAT
H5465 (ND2r.1413)	GGCGAGAAAGAGTGAGAGA
H5687 (ANSr.2)[c]	GCGTTTAGCTGTTAACTAAA
H5687 (E-ANSr.2)	GTTCTTAGCTGTTAATTAAG
H5686 (ANSr.A)	GTATTTAGCTGTTAATTAA
H5686 (ANSr.B)	GTTTTTAGCTGTTAACTAA
H5686 (ANSr.C)	GTTTTTAGCTGTTACCTAA
H5575 (ALAr.2)	CGCAAGTCTTACAGAAAC
H5586 (ALAr.1)	GGTTAGTGTCCCGCAAGT
L5551 (TRPf.5)[b]	GACCAAAGGCCTTCAAAGCC
L5551 (TRPf.A)	AACCCTGAGCCTTCAAAGCT
L5551 (E-TRPf.5)	AACCCTGGGCCTTCAAAGCC
L5551 (TRPf.C)	AACCCCGAGCCTTCAAAGCT
L5551 (TRPf.D)	GACCAAAAGCCTTCAAAGCT
L5603 (ALAf.1)	AAGACTTGCGGGACACTAACC
L5603 (ALAf.B)	AAGACCTGCAGGATATTAACC
c-myc primer (location)	
cmyc1U[d] (exon 2)	GAGGACATCTGGAARAARTT
cmyc3L[d] (exon 3)	GTCTTCCTCTTGTCRTTCTCYTC
cmyc5L (exon 2)	ATGGGTGGYGTTTCCATRTT
cmyc5Lp (exon 2)	ATGGGCGGCGTGTCCATATT
cmyc3U[d] (exon 2)	TCTTTCCTTACCCGTTGAATGATRC
cmyc3Up (exon 2)	TTCCCTTACCCGTTGAATGA
cmyc4U (exon 2)	TATGGAAACRCCACCCATCAG
cmyc6L[d] (intron 2)	CAAAAGCCAGMCATTGGAAGATAA
cmyc6Lg (intron 2)	CCAGCCATCGAAAGATAA
cmyc6U (intron 2)	CGGCACGCTTTCTAAGAA
cmyc4La (exon 3)	CTTGGATGCGGTATATCKTTT
cmyc4Lb (exon 3)	TTGGCTGCGGTATATCKTTTYTC
cmyc7L (intron 2)	AATGCATACAAGTTAGTAAT
cmyc7La (intron 2)	AAGGCATACGAGTTAGTAAT
cmyc7Lg (intron 2)	AATACATATGCGTTAGTAAT
cmyc7Lv (intron 2)	AATGCATACAAGTTAGTAAA
cmyc8L (intron 2)	GCGTCGCTGCCCTAAACTAYC
cmyc5U (exon 3)	TATACCGCATCCARGAAAA

The first two primers listed for each genome were used for amplifying and sequencing. The other primers were used for sequencing only. Mitochondrial primers are designated by their 3′ ends corresponding to the homologous position in the human genome (Anderson et al., 1981). In parentheses are indicated the laboratory names of mtDNA primers and the priming location of c-*myc* primers.

[a] Indicates primers previously published in Macey et al. (1997a).
[b] Indicates primers previously published in Macey et al. (1997b).
[c] Indicates an unpublished primer from J. R. Macey.
[d] Indicates primers previously published in Crawford (2003a).

Bayesian phylogenetic analyses (Rannala and Yang, 1996) were conducted on separate and combined data sets using *MrBayes* version 3.0b4 (Huelsenbeck and Ronquist, 2001). Four Monte Carlo Markov chains (MCMC; Yang and Rannala, 1997), one cold and three heated, were run simultaneously for one million generations (Metropolis-coupled MCMC). Trees were sampled every 100 generations. Burn-in was evaluated by examination of the plateau in log-likelihood over generations. Burn-in occurred in fewer than 60,000 generations in all cases, so the first 1000 trees were always excluded, leaving 9000 trees for estimating the marginal posterior distribution of topologies and parameter values. Default priors and conditions were used in all cases.

Using *PAUP** 4.0b10, maximum parsimony (MP) (Camin and Sokal, 1965) and weighted parsimony (WP) analyses were conducted on mtDNA, scnDNA, and combined data sets, whereas ML analyses were limited to the separate mt- and scnDNA data sets. MP reconstructions employed Fitch parsimony (Fitch, 1971) with gap sites excluded, while WP reconstructions utilized a tri-level weighting scheme (Benabib et al., 1997; Flores-Villela et al., 2000) with gaps coded as a fifth base. Tri-level weighting incorporates three different levels of information on the structure and inferred function of nucleotide substitutions. Under this WP scheme, transitions have a weight of 1, transversions are weighted 2, and any nucleotide substitution that is inferred to cause an amino-acid substitution is weighted +1 more. MP and WP employed ACCTRAN optimization of character state changes. Tree searching was conducted with 500 random addition sequence replicates, while ML searches were initiated from a NJ tree. All searches used the tree-bisection-regrafting method of proposing new topologies. MP and WP bootstrap analyses (Felsenstein, 1985) involved 2000 pseudoreplicates with 2–10 random addition sequences each.

We employed likelihood ratio tests (LRTs) to evaluate whether either data set, *ND2*-WANCY or c-*myc*, was significantly unlikely under the assumption of constancy of rates of molecular evolution (Felsenstein, 1981). Significance of the LRTs was evaluated assuming that the expectation of twice the absolute value of the difference in support (Ln) under the clock versus the non-clock model was χ^2_{n-2} distributed, where n equals number of sequences in the data set (Felsenstein, 1981).

For testing the relative support of alternative topologies we used the paired-sites test of Shimodaira and Hasegawa (1999, SH test) as implemented in *PAUP**. This test uses bootstrap resampling and corrects critical values for multiple comparisons. For a given topological test, we constrained only the node/s in question and performed a new ML search, as above. The magnitude of the difference in likelihood support for the ML tree (H_1) and the constrained tree (H_0) was evaluated by RELL sampling with 1000 bootstrap replicates (Kishino and Hasegawa, 1989).

2.5. Estimating divergence times

Rather than assume an externally calibrated rate of molecular evolution to estimate divergence times, we assumed the proto-Antillean biogeographic model and then estimated divergence times and evolutionary rates. We then compared these results with a paleogeological model and previously published information on rates of molecular evolution. We employed a MCMC approach to estimate posterior probability distributions of absolute divergence times, as developed by Thorne et al. (1998; Kishino et al., 2001) and implemented for multigenic data (Thorne and Kishino, 2002) in Thorne's software package "multidistribute" version 05/Aug/03 (ftp://statgen.ncsu.edu/pub/thorne/). Divergence times were estimated using all sequences of both genes, assuming the Bayesian consensus topology based on the combined data (see Section 3). Some assumptions about divergence times are required to facilitate the decoupling of rate from time. Therefore, we temporally constrained two nodes on the tree, one basal and one nested. For our basal constraint we began with the proto-Antilles model and restricted the origin of *Craugastor* to the interval 80–60 mya (Savage, 1966, 1982), corresponding to a likely time interval for the hypothesized formation and break-up of a land connection between Nuclear Central America and northern South America (Burke, 1988; Iturralde-Vinent and MacPhee, 1999). For our nested constraint, we bounded the divergence time of *E. bransfordii* and *E. polyptychus* to the interval 13–7 mya based on Crawford (2003a), whose temporal results were based on a recalibration of rates estimated from toads (Macey et al., 1998). The MCMC analysis used default protocols, and multiple analyses were run to check for convergence of the posterior distributions from independent MCMC runs. We also conducted an additional analysis using just the *ND2*-WANCY data and assuming the Bayesian consensus topology based on just those data.

2.6. Paleogeography

Our effort in phylogeny reconstruction and divergence time inference presented in this paper would be incomplete without a biogeographic context. Herein we present a revised consensus model of the complex paleogeography of Central America and the Caribbean from 75 to 18 mya. In our paleogeographic summary we focused on land configuration (subaerial), instead of plate-tectonics, and tried to incorporate information on elevation where possible. For northern Central America we based most of our paleogeography on Smith (2001) and references therein. Kuenzi et al. (1979) and Rogers (2000) provided additional insights on the elevational configuration of this region throughout the Cenozoic. For the Antillean region we followed Iturralde-Vinent and MacPhee (1999). Regarding the southern Central American region, we have incorporated ideas from Lloyd (1963), Coates and Obando (1996), and Orvis and Horn (2000). However, the diversity of mammalian fossils of strictly North American affinity collected from a mid-Miocene formation in central Panama suggests that continuous land stretched from North America to Panama 19–16 mya (Ferrusquía-Villafranca, 1975; MacFadden, 2005; Whitmore and Stewart, 1965). For northern South America we followed Hoorn (1993), Díaz de Gamero (1996), Marshall and Lundberg (1996), Vergara (1997), Iturralde-Vinent and MacPhee (1999), Audemard and Audemard (2002), and Donato et al. (2003). We present the paleogeographic reconstruction in Fig. 5.

3. Results

3.1. DNA sequence alignments

IIn addition to gapped sites, the following gene regions were removed from all samples in the analysis due to ambiguities in the alignment. In the *ND2*-WANCY region we removed 119 consensus sites from the 3′ end of *ND2* following a completely conserved tryptophan amino acid site and continuing through to the 2 bases shared by *ND2* and $tRNA^{TRP}$. This portion of *ND2* was highly variable in both inferred amino acid sequences and in length. Prior to analyses, we also removed from the alignment 7 nucleotide sites of the $tRNA^{TRP}$ D-loop and 8 of the T-loop, 13 sites of the O_L loop structure, 8 of $tRNA^{CYS}$ T-loop, and in $tRNA^{TYR}$ we removed 7 sites of the T-loop, 10 of the D-loop and 5 nucleotide sites between the T-stem and AC-stem. Thus, the mtDNA analysis consisted

of 1288 sites, of which 318 were constant and 854 were parsimony-informative. In the c-*myc* alignment we removed 148 aligned sites from the 5′ end of intron 2 due to the prevalence of gaps, and 217 sites from around the intron 2/exon 3 boundary due to a stretch of 8–23 pyrimidines in intron 2 followed by 13–26 trinucleotide $(GAR)_n$ repeats in phase with exon 3. Thus, the c-*myc* data set included 905 sites, of which 630 were constant and 156 were parsimony-informative. Our alignment is available from TreeBASE at www.treebase.org (study accession number S1249, matrix accession number M2177), and includes DNA sequence annotation and a complete list of all sites excluded from the analyses. Complete annotation of tRNA secondary structures is available from the authors.

3.2. Stationarity, compatibility, and parameter estimation

While the c-*myc* data show no signs of departure from equal nucleotide frequencies among taxa ($p = 1$), the mtDNA data set reject strongly the hypothesis of stationarity among samples whether we applied the χ^2 test to all the data, to the ingroup, or to just the subgenus *Craugastor* ($p < 0.000001$). To examine heuristically the effects of this departure from stationarity, we compared NJ trees based on LogDet distances (Lake, 1994; Lockhart et al., 1994) versus an JC69 NJ tree, a BioNJ (Gascuel, 1997) tree using TrN93 distances (Tamura and Nei, 1993), and our MP, ML, and Bayesian trees (see below). On only one basal node (i.e., among subgenera or among species groups) does the LogDet mtDNA tree disagree with the other NJ trees, the ML tree, or the Bayesian consensus tree based on the mtDNA data. Only the LogDet tree recovers a sister-group relationship between the *gollmeri* group and the *rhodopis* + *bransfordii* clade. This relationship, however, does not appear in any of our reconstructions based on the c-*myc* data. Insofar as paralinear distances are resistant to biases caused by heterogeneity of nucleotide frequencies, non-stationarity does not appear to have hindered substantially our estimate of relationships among the deeper nodes. No evidence of incongruence between data sets was found by the ILD test ($p = 0.989$).

For likelihood-based analyses of the mtDNA multigene fragment, the general time reversible model (Tavaré, 1986) was found by the AIC to be the best fit model of sequence evolution, with the distribution of rates of evolution among sites described by the gamma density shape parameter, α (Yang, 1994), and a proportion of invariable sites, I (Hasegawa et al., 1987), aka, the GTR + Γ + I model. A limited case of the preceding model was selected by the AIC for the c-*myc* data, i.e., equal base frequencies and equal transition rates (aka, TVMef + Γ + I). As this model is a special case of the GTR but not the HKY model, the former was used in the Bayesian analysis of the c-*myc* data. For both data sets, the models and parameter values obtained by the AIC using *Modeltest* were adopted for ML topology searches and branch-length estimation. Parameter values estimated for both genes by *Modeltest* and *MrBayes* are presented in Table 3. Because of the differences in parameter estimates, no ML analysis was conducted on the combined data set. A Bayesian analysis of the combined data was conducted using *MrBayes* version 3.0b4, in which all parameters except one were estimated independently in each data partition. Because the estimates of the Γ-distribution shape parameter, α, derived from *Modeltest* were similar in both data sets, only one α parameter was estimated across the combined data set.

3.3. Phylogenetic reconstructions

Inferred phylogenies and nodal support based on MP, WP, and Bayesian analyses of *ND2*-WANCY and

Table 3
Parameter estimates obtained from *Modeltest* and *MrBayes* for the models of evolution assumed in ML and Bayesian phylogenetic analyses of separate mitochondrial and nuclear data sets

	ND2-WANCY		c-*myc*	
	Modeltest	*MrBayes*	*Modeltest*	*MrBayes*
π_A	0.3458	0.3435 (0.3282–0.3599)	0.25	0.2479 (0.2240–0.2743)
π_C	0.3248	0.3260 (0.3123–0.3403)	0.25	0.2592 (0.2348–0.2851)
π_G	0.0813	0.0823 (0.0766–0.0885)	0.25	0.2543 (0.2296–0.2800)
π_T	0.2481	0.2482 (0.2359–0.2602)	0.25	0.2387 (0.2143–0.2652)
α	0.6682	0.6685 (0.5957–0.7448)	0.6664	0.6491 (0.4421–0.9628)
I	0.1654	0.1633 (0.1300–0.1942)	0.4555	0.4235 (0.3149–0.5182)
A-C	0.5132	0.5573 (0.4259–0.6949)	1.4156	1.6271 (1.0529–2.4675)
A-G	6.5057	7.0618 (5.7369–8.8227)	2.9437	2.9734 (1.9555–4.2380)
A-T	0.5896	0.6589 (0.4917–0.8656)	0.3860	0.5175 (0.2789–0.8640)
C-G	0.6885	0.7457 (0.5395–1.0274)	0.6427	0.7281 (0.4125–1.1518)
C-T	3.1106	3.4121 (2.6978–4.2332)	2.9437	3.8876 (2.6921–5.4432)

Using the Akaike information criterion, the following models were selected for ML analyses: GTR + Γ + I for the *ND2*-WANCY data and TVMef + Γ + I (i.e., equal transition rates and equal base frequencies) for the c-*myc* data. *MrBayes* analyses assumed a GTR + Γ + I model for both data sets. π_N, estimated nucleotide frequencies. Substitution rates are relative to G-T = 1.

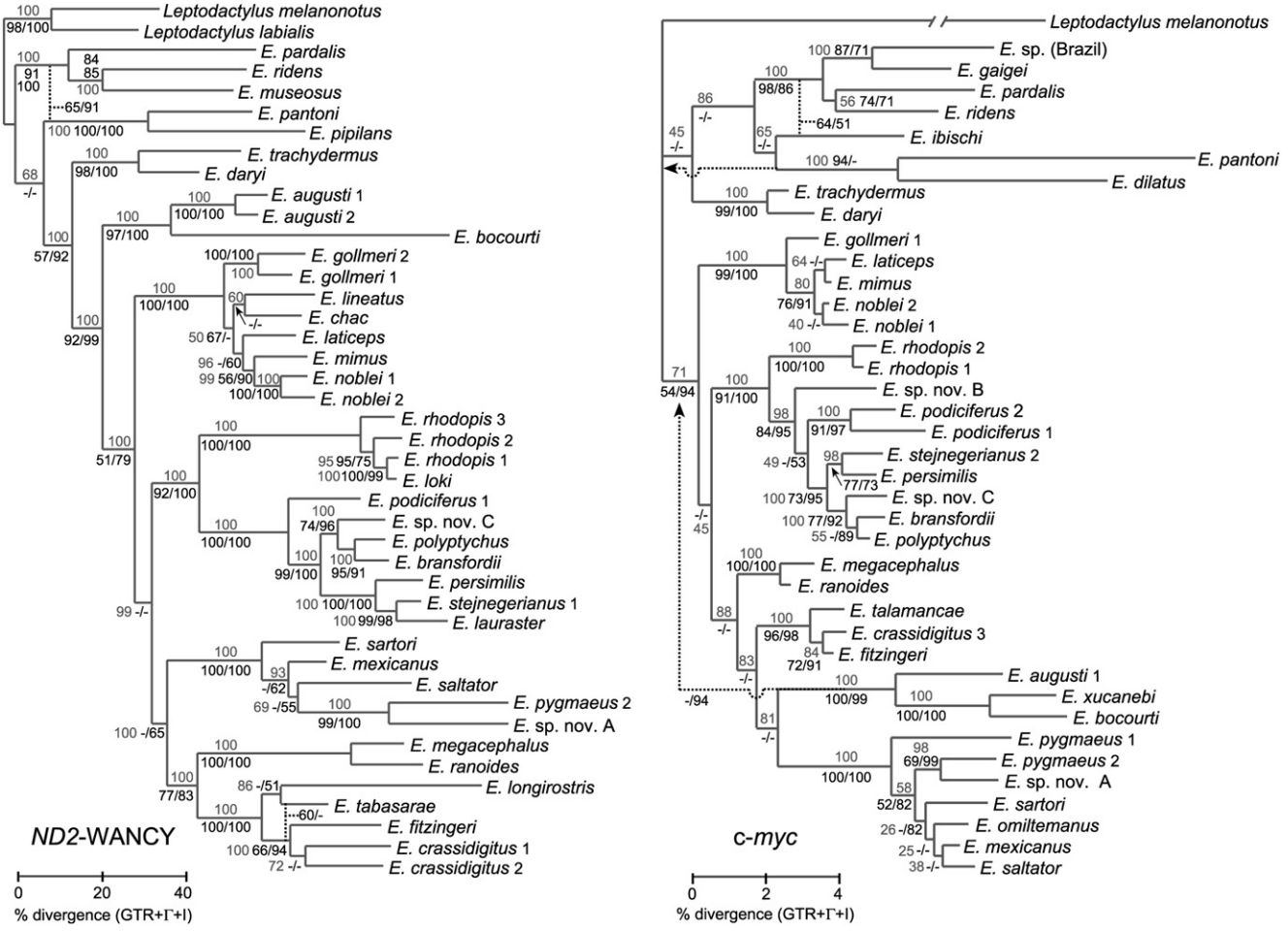

Fig. 1. Gene trees for the *ND2*-WANCY mitochondrial genes (left) and c-*myc* gene fragment (right) resulting from Bayesian MCMC phylogenetic analyses. Support for each node is indicated by three numbers. The upper or first, lighter-colored number shows Bayesian marginal posterior probabilities. The lower or trailing paired black numbers show the percent support obtained from parsimony bootstrap analyses, in both unweighted (before the slash) and weighted (after the slash) analyses. Bootstrap values <50% are indicated with a hyphen. Those branches inferred from parsimony bootstrap analyses that conflicted with the Bayesian topologies are indicated by the dotted lines. Maximum-likelihood topologies were identical to the Bayesian consensus trees shown here, except for the position of (*E. trachydermus*, *E. daryi*) in the c-*myc* tree (see Section 3).

c-*myc* data separately (Fig. 1) and combined (Fig. 2) are concordant in their support for the monophyly of species groups, but show some discrepancies in their estimation of relationships among species groups and among subgenera. These discrepancies tend to involve clades not strongly supported by one or both data sets or optimality criteria, with one notable exception deep in the phylogeny. Bayesian and MP analyses of separate mtDNA and c-*myc* data sets recover a sister-group relationship between the subgenus *Euhyas* and the subgenus *Syrrhophus*, as represented by *Eleutherodactylus pantoni* + *E. pipilans* or *E. dilatus*, respectively. The discrepancy lies in the placement of this clade. Based on the mtDNA data, this clade could be either the sister group to *Craugastor*, as weakly suggested by Bayesian inference (0.68 marginal posterior probability, or mpp; Fig. 1), or the sister group to the subgenus *Eleutherodactylus* clade, as supported by WP analysis (91% bootstrap support, or bss; Fig. 1). The c-*myc* data are equivocal on this issue. In the combined data set, however, Bayesian inference gives strong support (0.98 mpp; Fig. 2) to a sister-group relationship between *Craugastor* and all other sampled *Eleutherodactylus*, while parsimony methods give ambiguous results (64% MP bss, 58% WP bss; Fig. 2).

Bayesian consensus topologies for the *ND2*-WANCY and c-*myc* data sets (Fig. 1) are identical to those of their respective ML trees (not shown), with one small exception, again deep in the phylogeny. Unlike the c-*myc* Bayesian consensus tree, the c-*myc* ML tree places the *milesi* group (*E. trachydermus* + *E. daryi*) as the sister lineage to the rest of the *Craugastor* taxa, as found in the mtDNA and combined data estimates (see Fig. 2). However, the branch length is non-significant for this internode on the ML c-*myc* tree.

Parsimony analyses among data sets yield the following hypotheses of relationships. Two MP trees of length

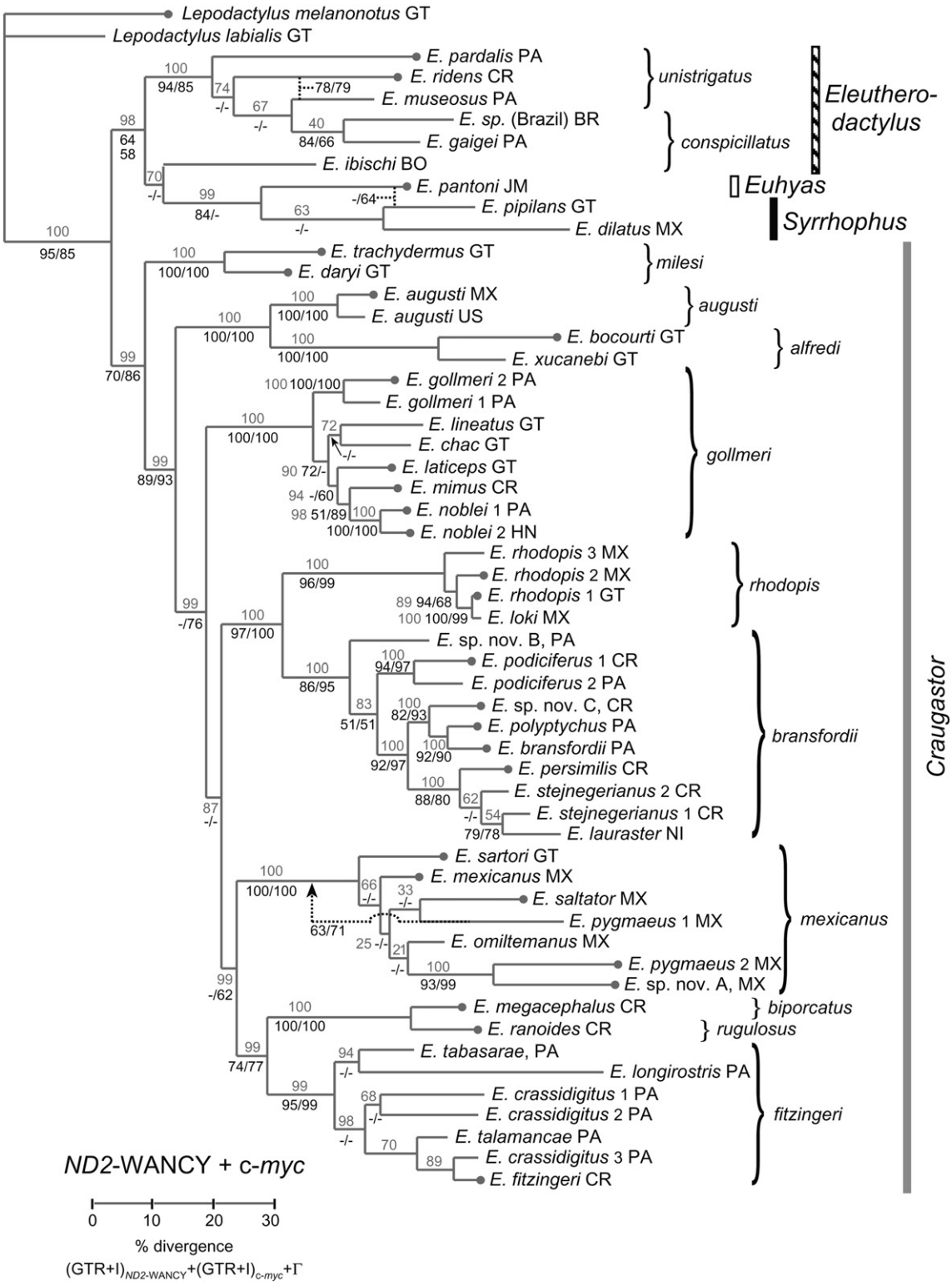

Fig. 2. Bayesian phylogenetic inference of relationships among all sampled taxa based on combined mtDNA and c-*myc* data sets. Those taxa represented in both data sets are indicated with a circle at the tip of their respective terminal branches. See Fig. 1 for explanation of the three nodal-support values and the black dotted lines. The origin of each sample is indicated by two-letter ISO 3166 country codes to the right of each taxon name. Previously recognized subgenera are indicated by vertical bars. We recommend here that *Craugastor* be recognized as a separate genus. Species groups are indicated by brackets and follow taxonomic changes recommended in this paper.

7009 were inferred from the mtDNA data, differing only within the *fitzingeri* group. The homoplasy index excluding uninformative characters (HIeuc) is 0.7468, and the rescaled consistency index (RC) is 0.1238. The MP trees place the subgenus *Craugastor* as the sister lineage to all other ingroup taxa. The WP tree infers the same relationships among subgenera. The shortest WP tree differs from the MP trees only among taxa within the *gollmeri* group. With weighting, the relative homoplasy is reduced slightly: HIeuc = 0.7032 and RC = 0.1724. In the MP analysis of the c-*myc* data, the 500 random-addition sequence replicates recovered 4600 shortest trees with length 597 steps. All of these trees put *E. pantoni* + *E. dilatus* as the sister group to all other ingroup samples, suggesting the following relationships among subgenera: (((*Euhyas*, *Syrrhophus*) *Eleutherodactylus*) *Craugastor*). Unlike the Bayesian c-*myc* consensus tree (Fig. 1), however, all the MP c-*myc* trees place the *milesi* group (*E. trachydermus* + *E. daryi*) as the sister lineage to all other *Craugastor* species groups (in agreement with the mtDNA and combined-data Bayesian results). MP analysis of the combined data sets and all taxa, including those with missing data, resulted in 10,000 shortest trees of length 7609, all of which agree with the mtDNA rather than the c-*myc* MP topologies in their relationships among subgenera and species groups. The 156 parsimony-informative sites in the c-*myc* data set are evidently overwhelmed by the 854 contained in the *ND2*-WANCY data.

The most fundamental difference among the phylogenetic results involves the placement of the *E. pantoni* + *E. pipilans* clade, representing the subgenera *Euhyas* and *Syrrhophus* in our analysis. On the one hand, the combined data analyses, as well as the parsimony analyses of the mtDNA data, all support *Craugastor* as the sister group to all other sampled *Eleutherodactylus*, especially in the Bayesian combined analysis (Fig. 2) and the WP mtDNA analysis (Fig. 1). Bayesian and ML analyses of the mtDNA data places the *E. pantoni* + *E. pipilans* clade as the sister group to subgenus *Craugastor*, albeit with a low mpp of 0.68 (Fig. 1). Therefore, using the SH test, we asked whether the mtDNA significantly reject the hypothesis of *Craugastor* as the sister group to all other sampled *Eleutherodactylus*. We found no significant difference in likelihood support between the ML tree (−28254.8239) and one constrained to contain a clade of all non-*Craugastor* ingroup taxa (−28256.4542; $p = 0.409$). We adopt the combined data Bayesian topology (Fig. 2) as our single tree used in the estimates of divergence rates and times, below.

3.4. Evolutionary rates and divergence times

Both the mitochondrial and nuclear DNA sequence data are significantly inconsistent with a model of rate constancy using a LRT ($p < 0.001$ in both cases). Therefore, we employ the MCMC approach to Bayesian estimation of the "rate of evolution of the rate of molecular evolution" (Thorne and Kishino, 2002; Thorne et al., 1998) using the combined mitochondrial plus nuclear data sets, and assuming the combined data Bayesian tree (Fig. 2) and the proto-Antilles model (Fig. 3). The rates of evolution in the two genes show a similar degree of departure from clock-like behavior. For the *ND2*-WANCY gene region, $v = 0.1700$ (0.0901–0.2944), and $v = 0.2498$ (0.0808–0.5364) for c-*myc*, where $v = 0$ implies a perfect molecular clock. However, the direction or magnitude of rate evolution among branches is not significantly correlated between genes (rank correlation coefficient of 0.4093, $p = 0.135$). The higher estimate and 95% confidence interval for the nuclear gene probably reflect the much smaller absolute number of mutations observed in the c-*myc* data.

Absolute rates of divergence vary widely among branches for both genes, but the central tendencies are roughly consistent with previous studies (e.g., Macey et al., 2001). Among the 105 branches, the median estimated rate of divergence for *ND2*-WANCY is 0.8410% per million years (my) (SD = 0.4958%). These rates are per lineage, and the *multidistribute* software package (Thorne and Kishino, 2002) employs the F84 + Γ model of DNA sequence evolution. Similarly for the c-*myc* gene fragment, the median is 0.0380%/my (SD = 0.0249%). To estimate the relative rate of evolution in the mitochondrial versus nuclear genes, we plotted the estimated mtDNA divergence versus scnDNA divergence for each branch of the phylogeny. The squared Pearson's correlation coefficient (r^2) between rates is 0.0360 and the slope is 17.521 with the regression line forced through the origin (Fig. 4). This relative rate is similar to a previous result of 16 based on silent-site divergences estimated within species groups (Crawford, 2003b).

Given the basal and nested node constraints, the Bayesian MCMC estimation of divergence times provided the following results based on simultaneous analysis of the mtDNA and c-*myc* data (Fig. 3). The Caribbean subgenus *Euhyas* (represented here by *E. pantoni*) shared a common ancestor with the subgenus *Syrrhophus* (*E. pipilans* and *E. dilatus*) 46–24 mya. The width of this 95% credibility interval probably reflects the missing data among *Syrrhophus* samples (Table 1), but the mean estimate of 35 mya matches well the 40–30 mya interval estimated previously from immunological and allozyme data (Hass and Hedges, 1991; Hedges, 1989). Within *Craugastor*, most species groups arose even earlier, principally during the Eocene. Within the well-sampled species groups (*gollmeri*, *bransfordii*, *mexicanus*, and *fitzingeri*), group members share a most recent common ancestor (MRCA) that probably

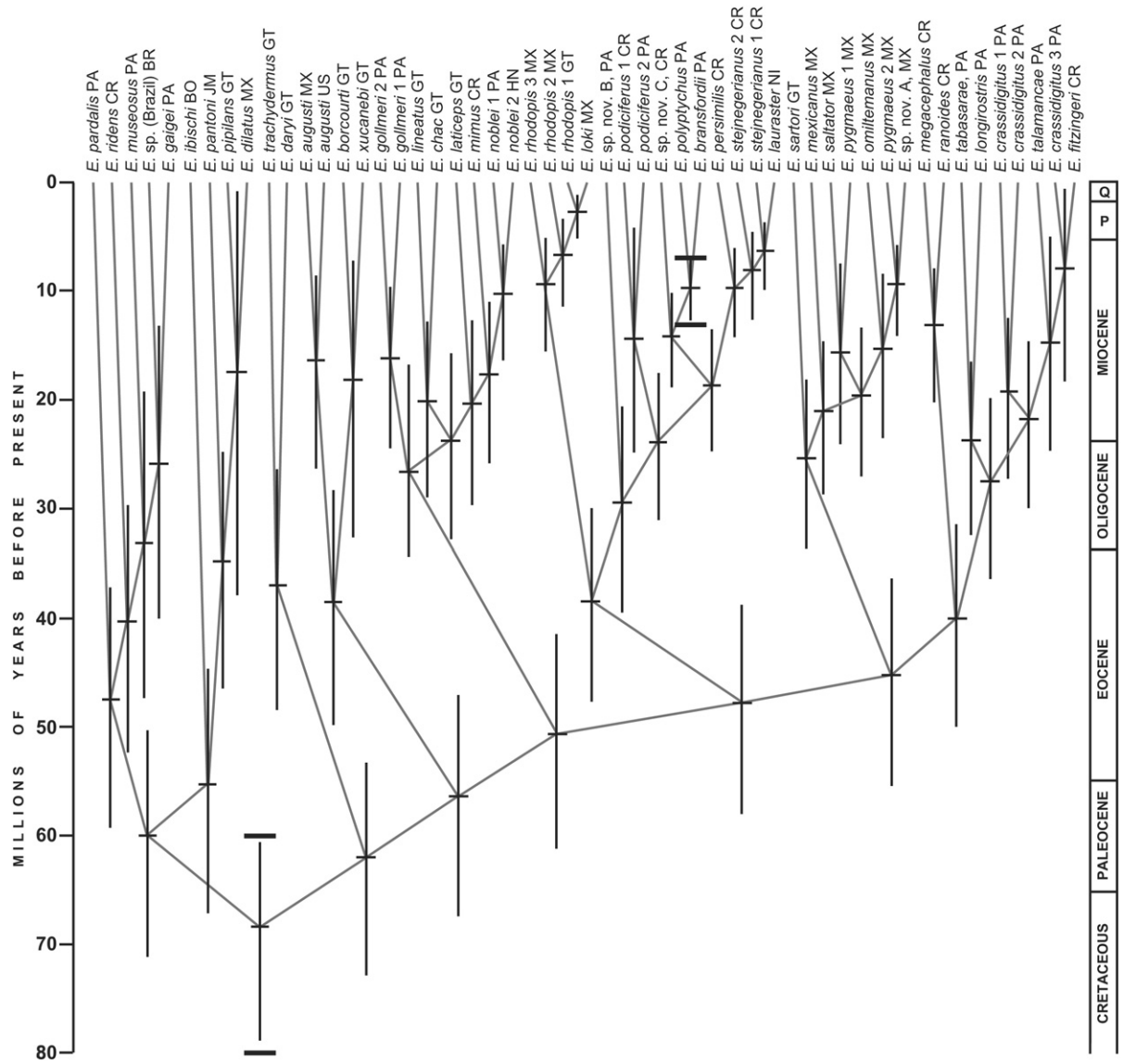

Fig. 3. Absolute divergence times of each node estimated using the Bayesian MCMC method of Thorne and Kishino (2002), based on the topology shown in Fig. 2, incorporating both mtDNA and c-*myc* sequence data, and assuming the proto-Antillean land-bridge model. For each node, the horizontal line indicates the point estimate and the vertical line indicates the extent of the central 95% of the posterior distribution of divergence times. The two pairs of horizontal heavy black lines indicate temporal constraints placed on the divergence times of two nodes prior to the analysis. The vertical bar on the right indicates geological periods and epochs where Q, Quaternary and P, Pliocene.

lived during the Oligocene or possibly the Early Miocene. Species of *Eleutherodactylus*, too, can be quite old. According to this analysis, five of the eight sets of conspecific samples show >95% probability of having diverged prior to the Pliocene (Fig. 3). Again, these figures are based on the proto-Antilles model. We find that using wider priors and lowering the minimum age of the *bransfordii–polyptychus* divergence to 5 mya has no appreciable effect on our estimates of divergence times. Removing the nuclear data from the analysis results in an increase of ~10% in the mean age of species groups.

4. Discussion

4.1. The proto-Antilles assumption

Estimating absolute divergence time requires an assumption of either rates or dates. Because our data preclude the application of a rate calibration or the assumption of an incontrovertible vicariance event, we are evaluating a controversial biogeographic scenario. We assume the proto-Antilles model and then explore the implications of this model in terms of divergence dates and rates. Under this model, land continuity or

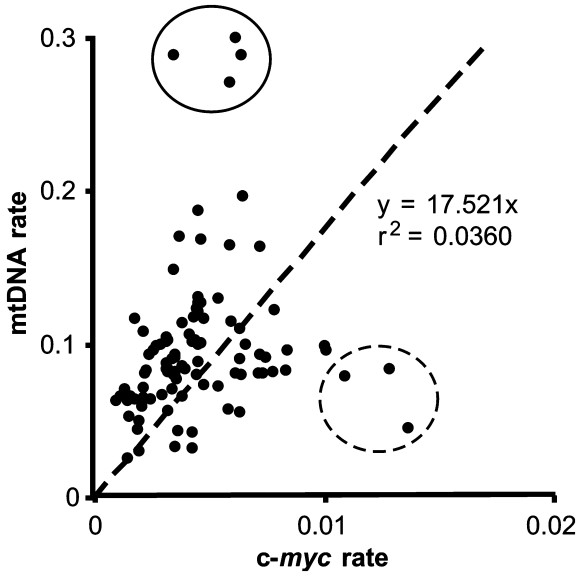

Fig. 4. Relative rates of DNA sequence evolution in the mtDNA fragment versus the c-*myc* gene fragment. Each point represents one branch on the phylogeny in Fig. 3 and shows the rate of evolution at each gene on that branch, as estimated by the Bayesian MCMC approach of Thorne and Kishino (2002). Dashed line equals the linear regression forced through the origin. Points in the solid circle represent branches corresponding to *E. longirostris*, *E.* sp. nov. A, *E. pygmaeus* 2, and the MRCA of the latter two taxa. Points in the dashed circle represent *E. pantoni*, the MCRA of *E. pantoni* and (*E. pipilans* + *E. dilatus*), and the MCRA of all non-*Craugastor* taxa. Note that some taxa were missing data for one gene (Table 1). The median ratio of mtDNA to c-*myc* divergence rates was 25.616 (SD = 14.134).

proximity between northwestern South America and Central America allowed faunal exchange between the two areas during the end of the Cretaceous and the beginning of the Paleocene (e.g., Guyer and Savage, 1987, 1992; Rosen, 1975, 1985; Savage, 1982). Some paleotectonic reconstructions of the Caribbean region refer to a proto-Antilles island chain in the area now occupied by lower Central America (e.g., Burke, 1988; Pindell et al., 1988; Ross and Scotese, 1988), yet the evidence for a land connection between the two continents would have long since disappeared due to geologic processes, insomuch as "the geography of the proto-Antilles probably has nothing to do with the geography of the existing islands [Greater Antilles]" (Iturralde-Vinent and MacPhee, 1999). However, evidence for low sea levels during the Maastrichtian (71–65 mya) have been identified globally (Hallam, 1984; Haq et al., 1987; Vail and Hardenbol, 1979) and in northwest South America in particular (Vergara, 1997). These recorded periods of low sea levels coincide with the timing of the proto-Antilles and make a land-pass between Central America and South America more likely. Unfortunately, we cannot demonstrate its existence with the data at hand. We can, however, explore the implications of this model for the ages of clades and the rates of molecular evolution.

4.2. The origin of the subgenera of Eleutherodactylus

Our Bayesian phylogenetic analysis of the combined data set provides significant support for the novel hypothesis that the subgenus *Craugastor* is the sister lineage to all other *Eleutherodactylus*. Single-gene and combined-data analyses support a sister-group relationship between the subgenera *Syrrhophus* and *Euhyas*. Although statistically significant, these results should be considered tentative due to limited sampling of this enormous genus and subfamily. This study lacks samples of additional eleutherodactyline genera, such as *Phrynopus* or *Brachycephalus*, and, like Darst and Cannatella (2004), this study includes only six species from among the roughly 400 species of the subgenus *Eleutherodactylus*. Keeping in mind, therefore, that all phylogenetic hypotheses are subject to further scrutiny by the addition of more taxa and more characters, we discuss briefly the biogeographic implications in light of the statistical support we find for some relationships among subgenera.

The hypothesis that *Craugastor* could share a sister-group relationship with the rest of *Eleutherodactylus* suggests that the genus *sensu lato* could have arisen in northwestern South America (Fig. 5). Our divergence-time analysis under the proto-Antilles model suggests that the common ancestor of the Greater Antillean subgenera (including *Syrrhophus*) split from a lineage ancestral to the mainland group containing *E. ibischi* 66–45 mya (Fig. 3). This calibration overlaps with Hedges' (1996) estimate of 77–63 mya for the origin of *Eleutherodactylus* in the West Indies. The ancestors of the subgenera *Syrrhophus* and *Euhyas* then diverged 46–25 mya, agreeing with a previous estimate of 37 mya (Hedges et al., 1992) and the fossil record (Holman, 1968; Iturralde-Vinent and MacPhee, 1996).

4.3. Are the frogs really that old?

Independent estimation of the time of origin of *Craugastor* is difficult in the absence of early fossils representing this group. In our Bayesian MCMC estimation of divergence times we constrained its origin to occur 80–60 mya, based on the proto-Antilles model (see above). If this divergence time was a substantial overestimation, then our resulting rates of evolution should err on the slow side. To the contrary, our rates appear to be on the high side of previous estimates of mtDNA divergence. Our resulting mean rate estimate of 0.8410% per lineage per million years is higher than previous estimates for poikilotherms, which range from 0.57% to 0.69% (reviewed in Macey et al., 2001). The absolute level of DNA sequence divergence observed is also particularly high. The *ND2*-WANCY divergences between *Craugastor* and *Eleutherodactylus* subgenera are quite high at 34% uncorrected distance, or an estimated

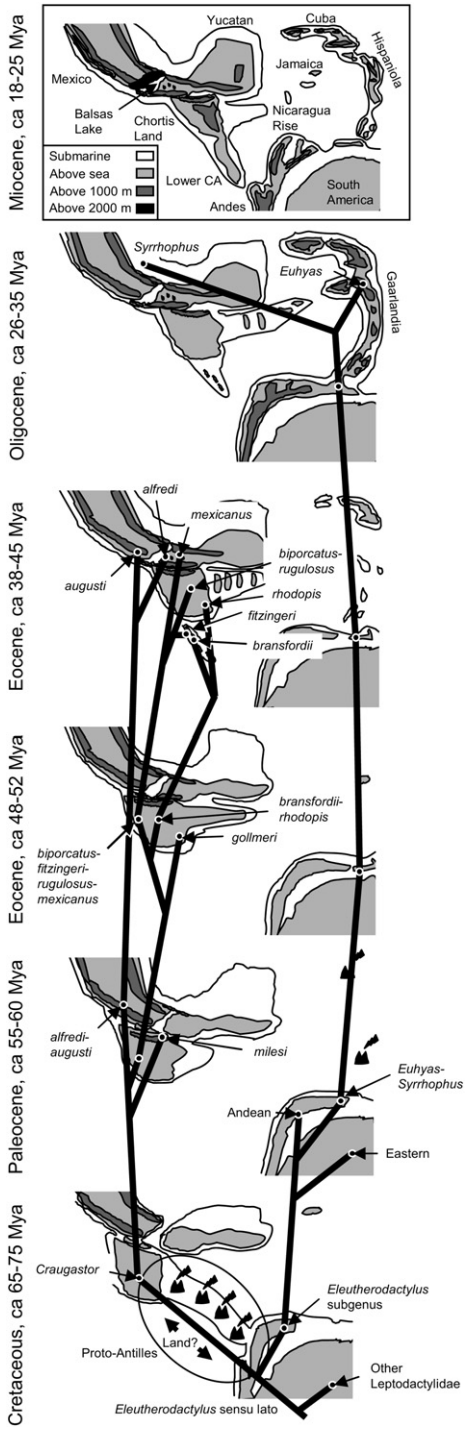

Fig. 5. Cenozoic biogeography of *Eleutherodactylus* in Central America. This figure shows a geographic and temporal hypothesis for the origin of four subgenera and the major species groups within the subgenus *Craugastor*. This hypothesis is based on the present phylogenetic results (Fig. 2) and the proto-Antillean land-bridge model (Fig. 3). The top panel provides a geographic key to the other panels. Circles represent areas where phylogeny and geography coincide in time. Smoking volcano icons represent hypothesized islands of magmatic origin.

138% using the ML model selected by the AIC. At the amino acid level the aligned portion of the inferred ND2 protein sequence shows an average difference of 41% between *Craugastor* and *Eleutherodactylus*. Looking at the c-*myc* data, Crawford (2003b) assumed these same two subgenera diverged 72 mya to estimate divergence rates at silent sites. Once again the estimated rates were not low. Instead they were quite similar to estimates from other nuclear genes in other frog lineages. Thus, the proto-Antilles assumption results in reasonable rates of molecular evolution.

4.4. The origin and diversification of *Craugastor*, the first invasion

The Cretaceous–Paleocene proto-Antillean land bridge or island-arc model can account for the present distribution of *Eleutherodactylus* subgenera (Hedges, 1989; Savage, 1982), and our molecular phylogenetic results appear largely concordant with this proposal. Monophyly of the subgenus *Craugastor* is well supported by Bayesian and WP analyses of the mitochondrial and combined data sets. Therefore, we infer parsimoniously that *Craugastor* originated from a single dispersal northward. This invasion would have represented the first occurrence of a direct-developing frog in Neotropical Laurasia, setting the stage for a potential adaptive radiation if this reproductive mode represented a key innovation in that environmental context (e.g., Vences et al., 2002). Under this hypothesis one predicts rapid cladogenesis relative to divergence, resulting in short internodes and the potential for a hard polytomy (Jackman et al., 1999; Schluter, 2000). Although most basal nodes uniting species groups in *Craugastor* were resolved, particularly using Bayesian inference, these internodes are distinctly shorter than the subtended branches that lead to the basal node of each species group (Fig. 2). Further support for a rapid evolutionary radiation comes from the observation that these short, basal internodes occur both in the phylogeny inferred from fast-evolving mtDNA sequences and in the phylogeny inferred from the slowly evolving nuclear gene (Fig. 1).

Ecological and morphological diversification of *Craugastor* appears to have taken place early in cladogenesis, concomitant with the origination of species groups (cf., Harmon et al., 2003). Body size and habitat preferences vary among groups but are largely constant within groups (Savage, 2002). For example, almost all members of the *milesi* and *rugulosus* groups are riparian, the latter reaching larger adult body sizes. Frogs of the *gollmeri*, *rhodopis*, and *bransfordii* groups are all forest floor species, with *gollmeri* taxa attaining much larger body sizes. Species in the *fitzingeri* group are moderately sized and inhabit low vegetation. Speciation within groups, therefore, did not involve obvious ecological displacement. Consistent with this conclusion, previous ecological studies have found that niche partitioning is scarcely observable among sympatric *Eleutherodactylus* (Lieberman, 1986; Toft, 1980a,b).

Under the proto-Antilles hypothesis, the ancestor of extant *Craugastor* began its potentially rapid diversification around the Paleocene–Eocene transition on the Chortis block (modern day southern Guatemala, El Salvador, Honduras, and northern Nicaragua) and adjacent southern Mexico now north of the Balsas basin (Fig. 5). This hypothesis we base on the observation that within *Craugastor* the species groups with ancestral branches positioned closest to the root node (*milesi* and *augusti* + *alfredi*) are northern Central American endemics. The *gollmeri* group lineage diverged next, also presumably originating in the north, although it contains both southern and northern taxa (cf., Savage, 1987). Subsequently, multiple dispersal events took place from north to south, dividing Chortis block (northern) from lower Central American species groups: the *rhodopis–bransfordii* split and the divergence of the *mexicanus* group from its sister group (Figs. 3 and 5). Based on the proto-Antilles model, we infer that these cladogenic events took place around the Eocene–Oligocene boundary. Some paleogeological reconstructions posit that the highlands of lower Central America were an island at this time (Iturralde-Vinent and MacPhee, 1999). The geographic origins of the *biporcatus* and *rugulosus* groups are unclear, because both groups contain northern and southern species, though we predict they are northern as well.

4.5. The origin of Syrrhophus, the second invasion

We find little doubt that the subgenera *Syrrhophus* and *Euhyas* are sister taxa, as first suggested by Hedges (1989; also Hass and Hedges, 1991). All Bayesian and MP bootstrap analyses showed strong support for this union, though WP analyses involving c-*myc* data were equivocal (Figs. 1 and 2). In addition to the various data sets of Hedges (1989; also Hass and Hedges, 1991), samples from these two clades share an unusual structural feature of their mtDNA molecules. *Eleutherodactylus pantoni* and *E. pipilans* share a 2 bp deletion corresponding to the AA-acceptor bases of $tRNA^{TRP}$ and $tRNA^{ALA}$. Because these two genes are encoded on opposite strands, their corresponding tRNAs are necessarily cleaved from opposite transcripts, and both genes can therefore remain functional.

The Antillean ancestor of *Syrrhophus* could have dispersed to Mexico via the Yucatan Peninsula, but fossil evidence supports a possible alternative hypothesis. Early Miocene *Eleutherodactylus* fossils from Florida (Holman, 1968) suggest that the genus might have crossed from the Caribbean into eastern North America. However, these fossils consist of only two ilial bones and their taxonomy is controversial (Lynch, 1971). Two more recent and less controversial pieces of fossil evidence support the presence of *Eleutherodactylus* in late Pleistocene southern Florida (Emslie and Morgan, 1995). Also, as recently as the Pleistocene *Syrrhophus* occupied a wider range in North America, as evidenced by fossils collected from 300 km north of its present range (Lynch, 1964; Tihen, 1960). Therefore, the ancestral *Syrrhophus* lineage could have dispersed from the Antilles into Florida and expanded west along the southern United States, subsequently becoming confined to central Texas and southwards.

Regardless of the route, dispersal between the Antilles and the mainland may have been much easier during the Oligocene and Miocene than it would appear to be today. There is evidence that the dry land in the Caribbean may have been more extensive due to historical sea-level fluctuations, e.g., the Pleistocene (Olson and Pregill, 1982) and the Oligocene (Iturralde-Vinent and MacPhee, 1999). Periods of low sea level occurred approximately 30, 15, and 5 mya (Haq et al., 1987; Vail and Hardenbol, 1979). The low sea level at about 30 mya may have facilitated the dispersal of the *Syrrhophus* ancestor into the mainland (Fig. 5), as well as the widespread dispersal of *Eleutherodactylus* in the West Indies, through GAARlandia.

4.6. The third invasion

Our data suggest that the third independent origin of *Eleutherodactylus* in Central America involved a wave of species rather than a single dispersal event. The four lower Central American taxa sampled from the subgenus *Eleutherodactylus* apparently diverged from one another long before the Pliocene (Fig. 5) when the Panamanian land bridge formed (Coates and Obando, 1996). Assuming that these frogs, like many other South American lineages, reached Central America recently via this land bridge (Savage, 2002; Vanzolini and Heyer, 1985; Webb and Rancy, 1996), each of our four Panamanian and Costa Rican species in this subgenus represents an independent dispersal event. In addition to our divergence time estimates, we note that the c-*myc* data support a paraphyletic relationship of the lower Central American samples of the subgenus *Eleutherodactylus* with respect to the Brazilian sample. Therefore, the topology alone suggests a minimum of two dispersal events.

4.7. An alternative hypothesis concerning rates and dates

Despite the concordance between previously published rates of evolution and the rates obtained here based on the proto-Antilles model, we still cannot reject the alternative hypothesis that rates of molecular evolution in *Eleutherodactylus*, particularly in their mtDNA, could be higher than those estimated previously in temperate-zone amphibians, reptiles, and tropical marine fishes (reviewed in Macey et al., 2001). Although no evidence has yet been found to support the hypothesis that

tropical organisms in general have higher rates of molecular evolution than temperate-zone species (Bromham and Cardillo, 2003), *Eleutherodactylus* are unusual in two respects. First, among the taxa studied here we uncovered five independent mtDNA gene duplications and rearrangements (details to be presented in a follow-up study), and the rate of rearrangement could be positively correlated with rates of nucleotide substitution (e.g., Shao et al., 2003). Second, *Eleutherodactylus* are unusual relative to other frogs in their wide diversity of karyotypes (DeWeese, 1976), although high rates of chromosomal rearrangements need not imply high rates of DNA sequence evolution.

One alternative hypothesis might be that *Craugastor* is half as old but has a rate of molecular evolution twice as high as found in previous studies of poikilotherms. This "young *Craugastor*" hypothesis has a disadvantage in requiring that the ancestral *Craugastor* rafted to Central America during the late Eocene. Trans-oceanic dispersal by amphibians is an unlikely phenomenon (Darwin, 1859), but it does happen (Hedges et al., 1992; Vences et al., 2003, 2004). One advantage of a younger *Craugastor*, however, is that the southern portion of its biogeographic history would be more easily reconciled with the relatively young age of lower Central America (Coates et al., 2004; Whitmore and Stewart, 1965). Such alternative possibilities highlight our need for more independent rate calibrations for *Eleutherodactylus*, and for more amphibians in general.

4.8. Taxonomic implications

The genus *Craugastor* is clearly monophyletic and has a long history separate from the other subgenera. We propose that the taxonomic rank of the subgenus *Craugastor* be elevated to the status of a genus. This taxonomic change is also supported by the finding that the sister group of *Craugastor* might not even be an *Eleutherodactylus* (Darst and Cannatella, 2004), but rather *Brachycephalus* or another eleutherodactyline genus. Monophyly of *Craugastor* is supported by a morphological synapomorphy involving the jaw musculature (Lynch, 1986, 2000, 2001b), although the same character state appears in the *E. fraudator* group from Bolivia (De La Riva and Lynch, 1997; Köhler, 2000; Lynch and McDiarmid, 1987). The phylogenetic position of the *fraudator* group is unclear at this time.

Other subgenera could probably be elevated, but we leave such changes to future studies of *Eleutherodactylus*, leptodactylid, and hyloid systematics. We refrain from recognizing *Euhyas* or *Syrrhophus* as full genera at this time because our sampling from within these taxa was minimal. Our data suggest that *Hylactophryne* should not be recognized as a genus (e.g., Darst and Cannatella, 2004) unless both the *alfredi* and *milesi* groups are elevated to the generic level, which we do not endorse at this time. Within the *Eleutherodactylus* subgenus, the probable paraphyly of the Central American taxa relative to the Brazilian sample suggests that the Central American species do not form a monophyletic group relative to other South American endemics, in which case the *cruentus* group of Savage (2002) would not be valid (Lynch and Duellman, 1997).

Within *Craugastor*, we found that the southern members of the former *rhodopis* group are quite diverged from the northern species. Although the name *podiciferus* predates *bransfordii*, we propose the name "*bransfordii* group" for the southern clade, as this name is already used commonly among workers in the field. Among the northern species, we propose the name "*mexicanus* group" for the other former *rhodopis*-group clade that is distantly related to the *rhodopis* + *bransfordii* clade (Fig. 2). We found no support for recognizing *Craugastor daryi* in the *gollmeri* group (Lynch, 2000) or for recognizing the *omiltemanus* group of Ford and Savage (1984). Under our organizational scheme, *C. omiltemanus* would now be a member of the *mexicanus* group (cf., Lynch, 2000). We concur with Lynch's (2000) placement of *C. bocourti* in the *alfredi* group. We currently have no molecular data to evaluate the validity or phylogenetic positions of the species assigned by Lynch (2000) to the *andi* and *bufoniformis* groups of *Craugastor*, so we are unable either to support or to reject these hypotheses.

We suspect that the unassigned *Craugastor* taxon *Eleutherodactylus uno* will be related to the *alfredi* group because it inhabits cloud-forest environments in southern Mexico and has the fifth toe longer than the third, expanded and notched pads on the fingers, and lacks tympanic sexual dimorphism. In summary, we propose to use a node-based definition of the new genus *Craugastor* and define it as that crown clade containing the following taxa and their MRCA: *C. augusti*, *C. bocourti* (*alfredi* group), *C. bransfordii*, *C. daryi* (*milesi* group), *C. fitzingeri*, *C. gollmeri*, *C. megacephalus* (*biporcatus* group), *C. mexicanus*, *C. rhodopis*, and *C. ranoides* (*rugulosus* group). If the members of the *bufoniformis* and *fraudator* groups were found to share this same MRCA, then they too would be placed in the genus *Craugastor*.

5. Conclusions

Craugastor has differentiated enormously, and this diversity seems to be associated with the old age of the group and the complex geologic history of Central America during the Cenozoic that promoted numerous dispersal and vicariance events. We find that ecological diversity among species groups within *Craugastor* coincides with short internodes in both the mitochondrial and nuclear phylogenies, suggestive of an early adaptive radiation. Ecological and geographical correlates of spe-

ciation within groups warrant further investigation. We hope that this study has set the genealogical stage for future studies of evolution within species groups.

For improved studies of *Eleutherodactylus* evolution we need to incorporate additional historical events that may be used as internal rate-calibration points for molecular evolution. These events would decrease our dependence on a priori biogeographical hypotheses or estimates of molecular evolutionary rates derived from other taxa. Expanded taxonomic sampling of *Eleutherodactylus* (apart from *Craugastor*) and additional South American genera (Darst and Cannatella, 2004) are required before a clear and inclusive picture of evolution and diversification of these frogs can be achieved. Within *Craugastor*, the high-elevation *alfredi* group of northern Central America appears to be particularly old, yet we have sampled very little of its potential diversity. In the south, molecular data are lacking from the *bufoniformis* group, from Lynch's (2000) *andi* group contained within the *fitzingeri* group of Savage et al. (2004), and from within the *rugulosus* + *biporcatus* clade. Finally, to understand *Craugastor*'s place among the eleutherodactylines, more molecular data are needed from these additional genera, as well as northern Andean *Eleutherodactylus* and the *fraudator* group of Bolivia. We hope that our analysis here contributes positively to our understanding of relationships of at least one twig of the eleutherodactyline tree and promotes further research on these remarkably diverse and locally abundant animals.

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