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Multilocus molecular phylogenetic analysis of the montane *Craugastor podiciferus* species complex (Anura: Craugastoridae) in Isthmian Central America

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ABSTRACT

The *Craugastor podiciferus* complex is a group of phenotypically polymorphic direct-developing frogs that inhabit the Talamancan highlands of Costa Rica and Panama. The montane distribution of this group creates natural allopatry among members and offers an excellent opportunity to explore geographic models of speciation. Using a multilocus approach, we obtained data from one nuclear (c-*myc*) and three mitochondrial (12S, 16S, and COI) gene regions from 40 individuals within the *C. podiciferus* complex. Molecular phylogenetic analyses revealed a basal split that placed samples from western Panama as sister to Costa Rican (CR) samples, corroborating a previous suggestion that the former lineage may represent an undescribed species. Within the CR clades we found six distinct haplogroups whose distributions largely corresponded to geographic features and included instances of sympatry. Divergence estimates were used to develop a preliminary evolutionary timeframe for the diversification of the *C. podiciferus* complex. Based on collective evidence, we hypothesize that movement of the CR haplogroups has occurred between currently isolated areas of suitable habitat via second order climatic fluctuations during the Pleistocene. The levels of genetic differentiation within the *C. podiciferus* complex are remarkable given the relatively small geographic area (ca. 8000 km^2) of occurrence. This diversity emphasizes the need for further study and taxonomic revision to aid in conservation planning for this complex which, like many amphibians, has experienced recent population declines.

1. Introduction

Tropical montane forests are known to harbor large numbers of species relative to their temperate analogs and are considered hotspots of biodiversity. In addition to acting as more effective barriers to organismal dispersal (Janzen, 1967; Ghalambor et al., 2006), tropical mountains have been shown to contain the highest levels of species richness for a wide range of taxa (Kessler and Kluge, 2008). Elucidating various aspects of this diversity, such as cryptic species and the degree of endemism, is critical in the understanding and formation of geographic models of speciation for these montane tropical regions. In Central America (here defined as south of Mexico and northwest of Colombia), studies of genetic diversity among widely distributed montane taxa are available for most vertebrate groups (e.g., mammals, Arellano et al., 2003; reptiles, Castoe et al., 2005; birds, Cadena et al. 2007; amphibians; Wiens et al., 2007). However, there are few well-sampled molecular studies on the phylogenetic relationships of vertebrates endemic to particular regions and specifically the mountains of Isthmian Central America (here defined as Costa Rica and Panama) (e.g., Hafner, 1991; García-París et al., 2000).

The highlands of Costa Rica and western Panama, i.e., the Tilarán, Central, and Talamancan mountain chains, are known to contain unique floral and faunal assemblages (Olson et al., 2001). These Cordilleran uplands share a dynamic and recent geologic history, which has created an archipelago-like separation of habitat in this ecoregion (Talamancan montane forest [TMF] Fig. 1; Denyer et al., 2000; Olson et al., 2001). While the TMF currently covers elevations of 700–3000 m, evidence from paleoecological studies of montane oak species (*Quercus*) from Panama indicate that the location of this ecoregion has shifted throughout time (Colinvaux, 1991). These historical shifts have been driven by climatic fluctuations lowering components of the ecoregion as much as 1000 m during glacial periods in which local temperatures decreased by 4 °C (Colinvaux et al., 1996; Piperno and Pearsall, 1998). In light of this, distributional patterns of many biota endemic to the mountains of Central America are thought to have been driven by glacial

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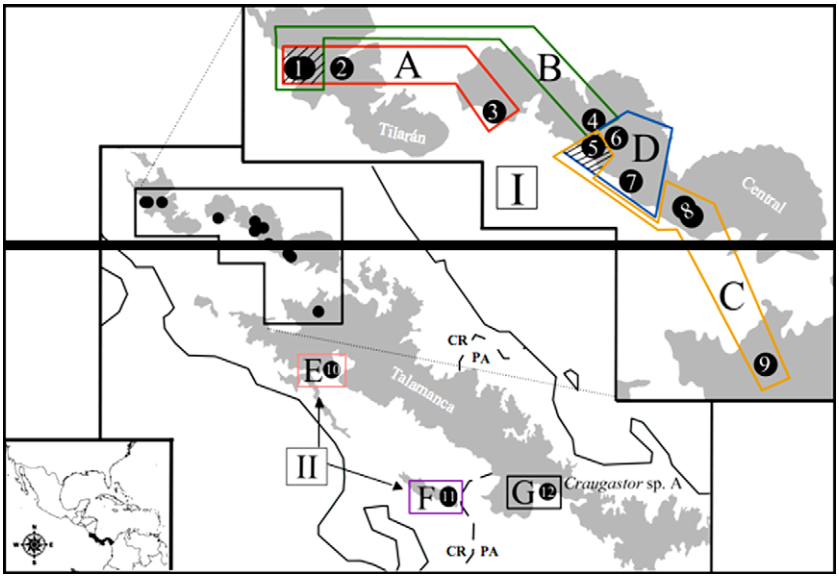

Fig. 1. Geographic locations of sampling sites (locality ID; Table 1) and simplified overlay of *Craugastor podiciferus* complex cladogram in Costa Rica and Panama. Barred areas represent zones of sympatry between Clades A–G. Geopolitical boundary between Costa Rica (CR) and Panama (PA) is indicated by a dashed line. Shaded areas represent a variety of montane habitats ranging in elevation from 750–3000 m. which are collectively referred to as the Talamancan montane forest ecoregion.

cycles where presently isolated segments of habitat became temporarily connected during periods of global cooling (Savage, 2002).

Among vertebrates found in this ecoregion, amphibians offer ideal systems for studying phylogeography on a small spatial scale in that: (1) they occur in a relatively heterogeneous environment created by variable elevations and fragmented topography and (2) they are generally poor dispersers, often locally abundant, and easily studied (Crawford, 2003a; Beebee, 2005). Although amphibian species composition in these Isthmian mountains is relatively well known (Campbell, 1999; Savage, 2002) our understanding of the phylogeographic relationships within these species is not. Previous molecular studies on montane bolitoglossine salamanders from this region have revealed extremely high levels of genetic diversity across a relatively small geographic area (García-París et al., 2000; Wiens et al., 2007) hinting that levels of diversity in these uplands may mirror the high genetic diversity described for lowland amphibians in the region (e.g., Crawford, 2003a; Weigt et al., 2005; Crawford et al., 2007; Wang et al., 2008). To our knowledge, no molecular study has extensively examined the intraspecific relationships of any anuran species that is restricted to the highlands of Costa Rica and western Panama, despite >20 frog and toad species sharing this distribution (Savage, 2002).

The Cerro Utyum robber frog, *Craugastor podiciferus* (Anura: Craugastoridae; Hedges et al., 2008), is a diminutive (21–40 mm) locally abundant member of the leaf litter fauna found at elevations of 1090–2650 m throughout the TMF ecoregion (Savage, 2002). Owing to many isolated populations and phenotypic polymorphism, the species is thought to be polytypic (Savage, 2002) and we therefore refer to it as the *C. podiciferus* complex. Considering its distribution and abundance, this complex represents a unique opportunity to characterize biogeographic patterns in an ecoregion with a dynamic history of geologic uplift and climatic fluctuation. A concomitant motivation to study the phylogenetics of the *C. podiciferus* complex is that, like most highland amphibians of Central America, it has recently experienced unprecedented population declines (Pounds and Crump, 1994; Lips, 1999; Puschendorf et al., 2006) and is red-listed for conservation by The World Conservation Union (IUCN, 2006). This necessitates a strengthened taxonomic understanding of the group to aid with future conservation and management decisions. The goals of this study were as follows: (1) assess the levels of genetic differentiation within the *C. podiciferus* complex, (2) evaluate the phylogeography of its members by examining individuals from geographically distinct regions using mitochondrial (mtDNA) and nuclear (scnDNA) DNA markers, and (3) suggest a possible historical framework for vicariance and dispersal mechanisms among lineages within this species complex.

2. Materials and methods

2.1. Study system

A recent taxonomic revision (Hedges et al., 2008) has divided the nominal genus *Eleutherodactylus* (and related genera) into several families that collectively form a clade now known as Terrarana. The genus *Craugastor* is within this clade and contains >110 described species of direct-developing anurans ranging from the southwestern United States to northern South America (Crawford and Smith, 2005). The *C. podiciferus* complex is contained within the *C. podiciferus* species group (Hedges et al., 2008) which is renowned for extreme phenotypic polymorphism within species and within populations (Savage and Emerson, 1970). Due in part to the shared color pattern polymorphisms, systematic work on the *C. podiciferus* species group has been limited (Cope, 1875; Taylor, 1952; Savage and Emerson, 1970; Miyamato, 1983). Recent studies have supported the hypothesis that the *C. podiciferus* complex may contain multiple taxa; Chen (2005) noted significant chromosomal variation within *C. podiciferus*, and Crawford and Smith (2005) documented significant mitochondrial and nuclear sequence divergence between just two populations. While both studies provided evidence of cryptic diversity, the small number of samples and localities included ($N \leqslant 4$) were insufficient to characterize the true geographic and phylogenetic extent of the potential diversity within the *C. podiciferus* complex.

2.2. Taxon sampling

Tissue samples were collected from 40 *C. podiciferus* complex frogs from 12 sites in Costa Rica and Panama (Table 1). This sampling includes representatives from the Talamanca, Tilarán,

Table 1
Institutional voucher numbers, locality information, and GenBank accession numbers for sampled taxa.

Taxon and institutional voucher[a]	Locality ID	Collection locality[b]	Geographic coordinates/ approximate location	Elevation (m)	GenBank accession number			
					12S	16S	COI	*c-myc*
Genus *Craugastor* *podiciferus* group								
C. cf. *podiciferus*								
1. UTA A-52449	1	Puntarenas, CR	(10°18′N, 84°48′W)	1520	EF562312	EF562365	None	EF562417
2. MVZ 149813	2	Puntarenas, CR	(10°18′N, 84°42′W)	1500	EF562319	EF562373	EF562386	EF562430
3. FMNH 257669	1	Puntarenas, CR	(10°18′N, 84°47′W)	1500	EF562320	EF562372	EF562380	EF562432
4. FMNH 257670	1	Puntarenas, CR	(10°18′N, 84°47′W)	1500	EF562317	EF562336	EF562376	EF562421
5. FMNH 257671	1	Puntarenas, CR	(10°18′N, 84°47′W)	1500	EF562314	EF562374	EF562409	None
6. FMNH 257672	1	Puntarenas, CR	(10°18′N, 84°47′W)	1500	EF562318	None	EF562382	None
7. FMNH 257673	1	Puntarenas, CR	(10°18′N, 84°47′W)	1500	EF562311	EF562343	EF562392	None
8. UCR 16361	3	Alejuela, CR	(10°13′ N, 84°22′W)	1930	EF562321	EF562371	EF562375	EF562431
9. UCR 16353	4	Heredia, CR	(10°12′N, 84°09′W)	1500	EF562313	EF562349	None	EF562420
10. UCR 16354	4	Heredia, CR	(10°12′N, 84°09′W)	1500	EF562315	EF562363	None	EF562418
11. UCR 16355	4	Heredia, CR	(10°12′N, 84°09′W)	1500	EF562316	EF562366	None	EF562419
12. UCR 18062	6	Heredia, CR	(10°10′N, 84°06′W)	1900	EF562302	EF562342	EF562395	None
13. UCR 17439	5	Heredia, CR	(10°09′N, 84°09′W)	2000	EF562298	EF562341	EF562387	EF562427
14. UCR 17441	5	Heredia, CR	(10°09′N, 84°09′W)	2000	EF562299	EF562345	EF562388	EF562429
15. UCR 17442	5	Heredia, CR	(10°09′N, 84°09′W)	2000	EF562300	EF562337	EF562385	EF562422
16. UCR 17443	5	Heredia, CR	(10°09′N, 84°09′W)	2000	EF562301	EF562340	EF562384	EF562428
17. UCR 17462	5	Heredia, CR	(10°09′N, 84°09′W)	2000	EF562309	EF562355	EF562406	EF562440
18. UCR 17469	5	Heredia, CR	(10°09′N, 84°09′W)	2000	EF562310	EF562334	EF562405	EF562414
19. MVZ 164825	7	Heredia, CR	(10° 05′N, 84° 04′W)	2100	EF562303	EF562346	EF562381	EF562423
20. UCR 16357	8	San José, CR	(10°02′N, 83°57′W)	1600	EF562306	EF562339	EF562400	EF562433
21. UCR 16358	8	San José, CR	(10°02′N, 83°57′W)	1600	EF562307	EF562370	EF562412	EF562415
22. UCR 16356	8	San José, CR	(10°01′N, 83°56′W)	1940	EF562308	EF562329	None	None
23. UCR 16359	10	San José, CR	(9°26′N, 83°41′W)	1313	EF562297	EF562369	EF562396	None
24. UCR 16360	10	San José, CR	(9°26′N, 83°41′W)	1313	EF562296	EF562368	None	EF562434
25. FMNH 257595	9	Cartago, CR	(9°44′N, 83°46′W)	1600	EF562304	EF562338	EF562408	None
26. FMNH 257596	9	Cartago, CR	(9°44′N, 83°46′W)	1600	EF562305	EF562335	None	EF562416
27. FMNH 257550	11	Puntarenas, CR	(8°47′N, 82°59′W)	1350	EF562294	EF562330	EF562393	EF562443
28. FMNH 257651	11	Puntarenas, CR	(8°47′N, 82°59′W)	1350	EF562291	EF562367	EF562402	EF562435
29. FMNH 257652	11	Puntarenas, CR	(8°47′N, 82°°59′W)	1350	EF562288	EF562364	EF562390	None
30. FMNH 257653	11	Puntarenas, CR	(8°47′N, 82°59′W)	1350	EF562292	EF562354	EF562392	EF562438
31. FMNH 257755	11	Puntarenas, CR	(8°46′N, 82°59′W)	1410	EF562289	EF562344	EF562379	None
32. FMNH 257756	11	Puntarenas, CR	(8°46′N, 82°59′W)	1410	EF562290	EF562347	EF562377	EF562413
33. FMNH 257757	11	Puntarenas, CR	(8°46′N, 82°59′W)	1410	EF562293	EF562352	EF562383	EF562437
34. FMNH 257758	11	Puntarenas, CR	(8°46′N, 82°59′W)	1410	EF562295	EF562348	EF562397	EF562436
Craugastor sp. A								
35. USNM 563039	12	Chiriquí, PA	(8°48′N, 82°24′W)	1663	EF562284	EF562356	EF562389	EF562445
36. USNM 563040	12	Chiriquí, PA	(8°48′N, 82°24′W)	1663	EF562285	EF562350	EF562391	EF562439
37. AJC 0890	12	Chiriquí, PA	(8°48′N, 82°24′W)	1663	EF562282	EF562351	EF562398	EF562444
38. MVUP 1880	12	Chiriquí, PA	(8°48′N, 82°24′W)	1663	EF562283	EF562358	EF562399	EF562442
39. FMNH 257689	12	Chiriquí, PA	(8°45′N, 82°13′W)	1100	EF562287	EF562353	EF562407	EF562446
40. FMNH 257562	12	Chiriquí, PA	(8°45′N, 82°13′W)	1100	EF562286	EF562357	EF562410	EF562441
C. underwoodi								
41. USNM 561403	N/A	Heredia, CR	(10°24′N, 84°03′W)	800	EF562323	EF562361	EF562378	None
42. UCR 16315	N/A	Alejuela, CR	(10°13′N, 84°35′W)	960	EF562322	EF562362	EF562394	None
C. stejnegerianus								*
43. UCR 16332	N/A	San José, CR	(9°18′N, 83°46′W)	900	EF562325	EF562360	EF562411	AY211320
C. bransfordii								*
44. MVUP 1875 fitzingeri group	N/A	BDT, PA	(9°24′N, 82°17′W)	50	EF562324	EF562359	None	AY211304
C. tabasarae								
45. MVUP 1720	N/A	Coclé, PA	(8°40′N, 80°35′W)	800	EF562326	EF562332	EF562401	EF562424
C. cf. *longirostris*								
46. FMNH 257561	N/A	Chiriquí, PA	(8°45′N, 82°13′W)	1100	EF562327	EF562331	None	EF562426
47. FMNH 257678	N/A	Chiriquí, PA	(8°45′N, 82°13′W)	1100	EF562328	EF562333	EF562404	EF562425

[a] UTA, University of Texas at Arlington; UCR, Universidad de Costa Rica; USNM, National Museum of Natural History, Smithsonian Institution; FMNH, Field Museum of Natural History; MVZ, Museum of Vertebrate Zoology; MVUP, Museo de Vertebrados de la Universidad de Panamá; AJC, Andrew J. Crawford.
[b] CR, Costa Rica; PA, Panama; BDT, Bocas del Toro.
* Sequence obtained from Crawford and Smith (2005) via GenBank.

and Central mountain ranges and therefore spans the known range of this species complex. Frogs were collected in the field, photographed, and euthanized with dilute Chloretone or 10% benzocaine gel. Fresh liver samples were stored in either 95% ethanol or a NaCl-saturated buffer containing 0.25 M EDTA and 20% dimethyl sulphoxide (DMSO; Seutin et al., 1991). Corresponding voucher specimens were fixed in 10% formalin, stored in 70% ethanol (Pisani, 1973), and deposited in biodiversity collections at the following public research collections: University of Texas at Arlington (UTA), the Smithsonian Institution's National Museum of Natural History (USNM), Field Museum of Natural History (FMNH), Museum of Vertebrate Zoology (MVZ), Universidad de Costa Rica (UCR), and Museo de Vertebrados de la Universidad de Panamá (MVUP). All *C. podiciferus* complex samples were collected at eleva-

tions between 1100 and 2600 m. The closely related lowland species *C. bransfordii*, *C. stejnegerianus*, and *C. underwoodi* were also included as ingroup taxa based on reports of their close affiliation with the *C. podiciferus* complex (Lynch, 2000; Savage, 2002; Crawford and Smith, 2005). We used three individuals belonging to the *C. fitzingeri* species group as outgroup taxa (one *C. tabasarae* and two *C.* cf. *longirostris*).

2.3. DNA amplification and sequencing

We investigated the *C. podiciferus* complex using DNA sequences from four genes. Nucleotide sequences from the mitochondrial 12S ribosomal gene (12S), mitochondrial 16S ribosomal gene (16S), mitochondrial Cytochrome Oxidase I gene (COI) and intron two of the nuclear cellular *myelocytomatosis* gene (c-*myc*) were identified as phylogenetically informative and incorporated as both separate and concatenated data sets in our analyses. Genomic DNA was extracted from liver and thigh muscle using a Qiagen Dneasy kit (QIAGEN®, Valencia, California). DNA fragments were amplified using published primer sets and primers designed for this study (Table 2). Polymerase Chain Reaction (PCR; Saiki et al., 1988) amplification was performed using AmpliTaq Gold™ (Applied Biosystems, Foster City, California) and variable thermal cycling profiles depending on the target region (detailed below). Reactions were performed in 20 μL reaction volumes containing 2–4 μL of template DNA and 1 μL of 0.1% Bovine Serum Albumin (BSA). Thermal cycling was performed on either a PTC-100 or PTC-200 Peltier Thermal Cycler.

The gene regions 12S, 16S, and COI were all PCR amplified using the following parameters: An initial cycle of 95 °C (11 min) followed by 45 cycles of 95 °C (30 s) denaturing, 50 or 45 °C (30 s) annealing, and 72 °C (2 min) extension. A final phase of 72 °C (10 min) followed. A region of c-*myc* including portions of exon 2 and intron 2 was amplified using touchdown PCR (Don et al., 1991) with the following parameters: 95 °C (11 min) followed by 20 cycles of 95 °C (30 s) denaturing, 58 °C (30 s) annealing and 72 °C (1 min) extension followed by 3 cycles of 95 °C (30 s) denaturing, 58 °C (−1 °C per cycle) (30 s) annealing, and 72 °C (1 min) extension followed by 20 cycles of 95 °C (30 s) denaturing, 55 °C (30 s) annealing and 72 °C (1 min) +5 s per cycle extension; a final phase of 72 °C (10 min) followed. All PCR experiments contained a positive and negative control. PCR products were quantified on either a 1% or 2% TAE agarose gel and cleaned using the AMPure magnetic bead system (Agencourt® Bioscience, Beverly, Massachusetts). Sequencing reactions were performed using the BigDye terminator kit (Applied BioSystems, Foster City, California) and were cleaned using Sephadex® G-50 powder (Sigma–Aldrich, St. Louis, Missouri). DNA sequencing was performed on a multi-capillary genetic analyzer (Spectrumedix®, State College, Pennsylvania). Both directions of PCR product were sequenced directly and their consensus sequence submitted to GenBank (Accession numbers listed in Table 1).

2.4. Phylogenetic analysis

Contiguous sequences from overlapping fragments were assembled in Sequencher 4.1 (GeneCodes) and multiple sequence alignments were constructed using Clustal X (Thompson et al., 1997) and checked by eye. For c-*myc* fragments, heterozygosity was assumed if automated sequencing chromatograms contained strong and equal double peaks on both strands or when alternately dominant peak height was observed from the two chromatograms (Hare and Palumbi, 1999). Genic structure of the COI fragment was determined via alignment to the *Xenopus laevis* mitochondrial genome (GenBank Accession No.: NC_001573; Roe et al., 1985) where it corresponded with bases 8138–8645. Sequences were analyzed by individual gene region and as a combined data set. To test for significant phylogenetic heterogeneity between pairs of gene regions, we used an Incongruence Length Difference (ILD) test (Farris et al., 1994) with 2000 iterations as implemented in PAUP* 4.0b10 (Swofford, 2002). Distance and parsimony-based analyses were also conducted using PAUP* 4.0b10. Neighbor joining (NJ; Saitou and Nei, 1987) trees were constructed using uncorrected distances. Maximum Parsimony (MP) analysis was conducted under heuristic search criteria using TBR branch swapping and 10 random addition sequence replicates with all characters weighted equally and gaps considered missing data. Using parsimony criterion, non-parametric bootstrapping was performed for 2000 replicates (Hedges, 1992) and partitioned Bremer support values (Baker et al., 1998) were generated using TreeRot 2b (Sorenson, 1999).

Since several models of DNA sequence evolution used herein assume constant frequency over time and between lineages, likelihood-based analyses were preceded with a $\chi^2_{3(n-1)}$ test for heterogeneity in base frequency executed in PAUP* 4.0b10. We employed a Bayesian Information Criterion (BIC) in *Modeltest* 3.7 (Posada and Crandall, 1998) to select an appropriate model of DNA sequence evolution for each gene individually and the four gene concatenated dataset. In estimating these BIC models, we used the total number of nucleotides in each alignment as a surrogate for sample size (Posada, 2006). For the concatenated dataset, Bayesian Markov chain Monte Carlo (MCMC) analyses (Yang and Rannala, 1997) were conducted in MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003) with independent models applied to each data partition (Table 3). Two parallel MCMC runs were conducted over 10 million generations, with sampling occurring every 1000 generations. In order to identify topological convergence in our searches, we used the online program AWTY (Wilgenbusch et al., 2004; Nylander et al., 2008) to compare the MCMC runs. Both runs contained four Metropolis-coupled MCMC chains with a default temperature parameter of 0.2. For comparison, we also conducted a non-partitioned Bayesian MCMC analysis with the concatenated data set.

Due to the large number of mtDNA sites relative to scnDNA sites used in this study along with an overall slower mutational rate in

Table 2
Primers used in the amplification (amp) and sequencing (seq) of *Craugastor* genes.

Locus	Primer	Use	Reference	Sequence (5′–3′)
12S	12SF	both	Bossuyt and Milinkovitch (2000)	AAACTGGGATTAGATACCCCACTAT
12S	12SR	both	Liu et al. (2000)	ACACACCGCCCGTCACCCTC
16S	16SAR	both	Kessing et al. (1989)	CGCCTGTTTAYCAAAAACAT
16S	16SBR	both	Kessing et al. (1989)	CCGGTCTGAACTCAGATCACGT
COI	COIF	both	Kessing et al. (1989)	CCTGCAGGAGGAGGAGAYCC
COI	COIR	both	Kessing et al. (1989)	AGTATAAGCGTCTGGGTAGTC
c-*myc*	cmyc1U	amp	Crawford (2003a)	GAGGACATCTGGAARAARTT
c-*myc*	cmyc3L	amp	Crawford (2003a)	GTCTTCCTCTTGTCRTTCTCYTC
c-*myc*	cmycseqF	seq	this study	TTCTGAATGCATTGACCCTTCGG
c-*myc*	cmycseqR	seq	this study	TTCTTCCTCATCTTCGTCTTCATCC

Table 3
Likelihood ratio test (LRT) statistics for combined mtDNA and four individual gene regions in *Craugastor*. Note that while several genes reject a molecular clock when applied to all samples (significant values indicated with an *), all genic regions fail to reject rate homogeneity when applied to the *C.* cf. *podiciferus* complex alone. Description of evolutionary models can be found in Posada and Crandall (1998).

	BIC model selection				Likelihood ratio testing		
Locus	*n*	Model	-lnL	BIC	χ^2	df	*p*-value
Total data set (all taxa)							
12S	380	SYM + G	1880.3461	3796.3333	91.6466	45	0.0000498*
16S	357	GTR + I	1456.9196	2966.7388	44.7136	44	0.44168
COI_{TOTAL}	507	HKY + I + G	2963.5994	5964.5698	65.97378	36	0.0016833*
$COI_{position1}$	169	K80 + G	580.5992	1171.4581			
$COI_{position2}$	169	TrN	372.6541	770.9576			
$COI_{position3}$	169	GTR + G	1712.8069	3471.7830			
c-*myc*	414	K80 + I	1123.6691	2259.3899	44.45174	34	0.10825
$Total_{4\text{-gene}}$	1658	SYM + I + G	7757.1875	15566.2686			
Craugastor cf. *podiciferus* + *Craugastor* sp. A *(only focal taxa)*							
12S	380	TrNef + G	1145.1263	2308.0732	52.266	38	0.061564
16S	357	HKY + G	887.4183	1804.2253	29.56436	37	0.80867
COI_{TOTAL}	507	HKY + G	1935.4519	3902.0464	41.2516	31	0.10317
c-*myc*	414	JC	868.0661	1736.1322	25.61098	29	0.64619

the nuclear locus, it is feasible that the relationship of nuclear alleles may be overwhelmed by phylogenetic signal from the mtDNA data. To examine this potential issue we constructed a 95% plausible parsimony network from c-*myc* haplotypes for focal taxa included in the *C. podiciferus* complex using the program TCS 1.21 (Clement et al., 2000). Additionally, to better assess the levels of c-*myc* diversity we used DnaSP 4.9 (Rozas et al., 2003) to calculate per site nucleotide diversity ($\hat{\pi}$).

2.5. Estimating divergence times

Prior to estimating divergence times, we tested each locus for a molecular clock (homogeneity of branch lengths) in (1) a global context using all samples and (2) a local context using a subset including only *C. podiciferus* complex frogs (focal taxa). Testing was achieved via the use of likelihood ratio tests (Felsenstein, 1981) assuming those evolutionary models selected under BIC in *Modeltest* 3.7.

We chose to estimate divergence dates generated from (1) a molecular clock and (2) previously published dates for *Craugastor* (Crawford, 2003b; Crawford and Smith, 2005; Heinicke et al., 2007) in an attempt to generate confidence intervals around estimated divergence times. To evoke a broad sense molecular clock we applied a sequence divergence rate of 0.75% per million years (my) per lineage since the mean rate across vertebrate mtDNA is generally estimated to be between roughly 0.50–1.0% per my per lineage (Klicka and Zink, 1997; Macey et al., 2001). To estimate divergence times using previously suggested dates, we calibrated our phylogeny using two published divergence times for the Time to Most Recent Common Ancestor (TMRCA) between the *C. podiciferus* complex and *C.* cf. *longirostris*. Heinicke et al. (2007) used a combination of fossil dates and insular emergence in the West Indies to obtain a TMRCA of *C. podiciferus* and *C.* cf. *longirostris* (*C. fitzingeri* group) at 27.63 to 13.85 million years ago (Ma). Crawford and Smith (2005) used previously calibrated substitution rates and a proto-Antilles biogeographic model for Central America to obtain a TMCRA of 58–38 Ma. For each of these estimates, we ran a relaxed clock analysis calibrated with the respective TMRCA of *C. podiciferus* and *C.* cf. *longirostris*. Since no published estimate for the TMRCA between the *C. podiciferus* complex and *C.* cf. *longirostris* exceeds 60 Ma, we limited the root height of the tree at this point for all analyses.

All divergence estimates were generated using the computer program BEAST 1.4.7 (Drummond and Rambaut, 2007). This program uses a Bayesian MCMC algorithm and allows users to estimate rates and dates using both strict and relaxed molecular clock approaches. In conducting these divergence estimates we used identical parameter settings to those from the MrBayes 3.1.2 analysis using the BIC model selected for the entire alignment. Because the relatedness of many taxa in this study is above the species level, a constant lineage birth rate Yule tree prior was used along with constant rate models (in strict molecular clock analyses) and uncorrelated lognormal models (in relaxed molecular clock analyses). Following the completion of parallel runs in BEAST 1.4.7 the consensus topologies were examined to confirm congruence with the previously generated Bayesian phylogeny. Convergence of these runs was checked using TRACER 1.4 (Rambaut and Drummond, 2004) and all parameter sampling exceeded an effective sample size of 200. Given the suboptimal condition of a single calibration point and a desire to be conservative in our divergence estimation, we used a combination of relaxed and strict rate methods when enforcing the Crawford and Smith (2005) and Heinicke et al. (2007) constraint schemes. After applying a constraint scheme, we sampled divergence rates (per my per lineage) across the Bayesian phylogeny using relaxed clock analysis (Drummond et al., 2006) in BEAST 1.4.7 and then subsequently in a second BEAST 1.4.7 analysis (with no constraints) applied the sampled mean rate as a molecular clock to segments of our topology that failed to reject rate homogeneity.

3. Results

3.1. Sequence analyses and model selection

Homology assessment was not obvious for small portions of the aligned ribosomal DNA sequences (12S and 16S) and the intron region of the scnDNA (c-*myc*) so these variable regions, which ranged in size from 19 to 114 bases, were excluded from all phylogenetic analysis. The majority of c-*myc* sequences across all taxa were found to be homozygous. Those identified as heterozygous did not share alleles with any other samples included in this study. Heterozygosity was limited to 1–2 bases per heterozygous individual that were coded as degenerate sites prior to analysis. In total, the final alignment contained 380 bp of the 12S region, 357 bp of 16S, 507 bp of COI, and 414 bp of c-*myc* (intron 2) for a total of 1658 sites. This alignment is available from TreeBASE at www.treebase.org (Study No.: S2444; Matrix No.: M4647).

No locus showed any departure from equal nucleotide freq uencies among taxa as evidenced by the following χ^2 statistics: 12S ($\chi^2_{[138]} = 36.73$, $p = 1.0$), 16S ($\chi^2_{[135]} = 15.14$, $p = 1.0$), COI

($\chi^2_{[111]}$ = 44.35, p = 1.0), and c-*myc* ($\chi^2_{[105]}$ = 6.10, p = 1.0). The ILD test found that the separate data partitions did not produce significantly different topologies (p = 0.17). Therefore, the data were analyzed phylogenetically as a single contiguous alignment. Within this concatenated data set 1111 sites were constant and of the variable sites 140 were variable but parsimony uninformative and 407 parsimony informative. Evolutionary models selected by BIC and used in the partitioned and non-partitioned Bayesian MCMC analyses are listed in Table 3. Partitioned Bayesian analysis of the total data set was conducted using six partitions (12S, 16S, $COI_{position1}$, $COI_{position2}$, $COI_{position3}$, and c-*myc*, respectively; Table 3), each with independent parameters, which is relatively complex when compared to the employment of the SYM + I + G model ($Total_{4\text{-}gene}$; Table 3) across all sites. We present the results of the partitioned analysis below since it has been suggested that phylogenetic accuracy is more vulnerable to models which are under-parameterized rather than those which are over-parameterized (Huelsenbeck and Rannala, 2004). Based on AWTY visualization, topological convergence of MrBayes runs occurred at ca. 7,000,000 generations, so the first 7000 trees from each run were discarded as burn-in, leaving a combined 6000 samples to estimate marginal posterior probabilities of topologies, branch lengths and parameter values.

3.2. Phylogenetic reconstructions

Phylogenies generated by NJ, MP, and partitioned and non-partitioned Bayesian MCMC analyses for the combined data are concordant in their support of a series of monophyletic groups within the *C. podiciferus* complex. Disagreement between topologies was confined to terminal nodes with little statistical support in all analyses. The distinct clades (haplogroups) within the complex are best described by the geographic origins of their constituent taxa (Fig. 1).

'Clade A' includes frogs from the southern Tilarán range and the northern Central range (localities 1–3; Fig. 1). 'Clade B' also includes frogs from the southern Tilarán range and northern Central range (localities 1 and 4, respectively; Fig 1.). 'Clade C' includes frogs from the Central range and the extreme northern portions of the Talamancan range (localities 5, 8, and 9; Fig. 1). 'Clade D' includes frogs from the Central range (localities 5–7; Fig. 1). 'Clade E' contains frogs from the Pacific slope of the Talamancan range (locality 10; Fig. 1). 'Clade F' includes frogs from a low disjunct mountain range on the Pacific slope of the Talamancas known as the Fila Costeña (locality 11; Fig. 1). 'Clade G' is comprised of individuals from montane western Panama (locality 12; Fig. 1). Clades A–F form a well-supported monophyletic group endemic to Costa Rica (Fig. 1 and 2). We define this clade as *C.* cf. *podiciferus* pending further systematic revision. Within the *C.* cf. *podiciferus* clade there is limited but significant branch support for a northern group containing Clades A–D and a southern group containing clades E and F which we label I and II, respectively (Fig. 2). 'Clade G' from western Panama was recovered as sister to the lowland samples (*C. bransfordii* and others) on our optimal trees, although with relatively poor statistical support (Fig. 2). Thus, rather than include Clade G in the *C. podiciferus* complex, we refer to the lineage as *Craugastor* sp. A. The lowland taxa used in this study (*C. bransfordii* and others) form a clade that is phylogenetically consistent with previous findings for these species (Crawford and Smith, 2005; Hedges et al., 2008).

For the 95% plausible c-*myc* parsimony network of the *C.* cf. *podiciferus* clade (Fig. 3), nuclear sequences were trimmed to 331 bp so that any sequence length heterogeneity was confined to a small number of internal indels. The network contained a total of 14 unique haplotypes excluding degenerate sites. The network supported our Bayesian phylogeny in recovering the northern and southern groups (I and II, respectively). No clades identified by mtDNA (A–F) shared c-*myc* haplotypes; thus, Clades A–F are monophyletic at both mtDNA and scnDNA markers. The southern group (II) showed a greater $\hat{\pi}$ value among nuclear haplotypes than

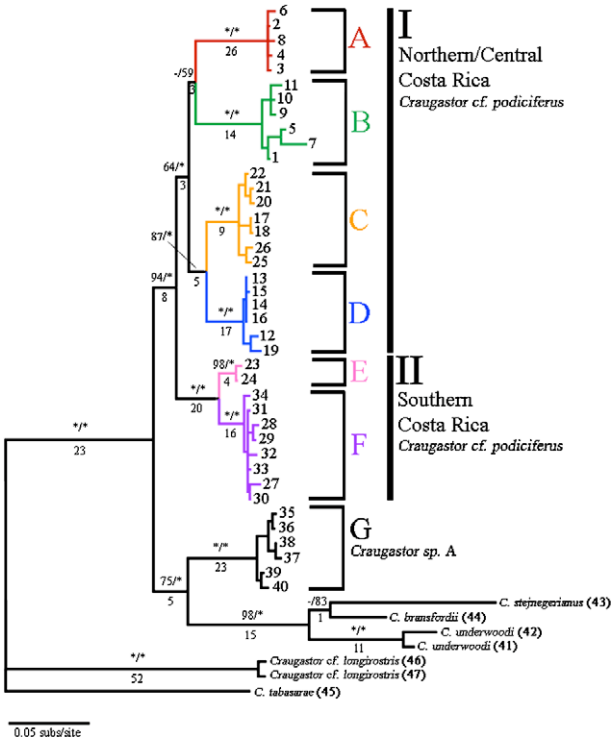

Fig. 2. Bayesian phylogeny from mixed-model multilocus dataset depicting relationships within the *Craugastor podiciferus* complex. Numbers found at the end of terminal branches correspond with taxon and institutional voucher numbers from Table 1. Posterior probabilities resulting from Bayesian Markov chain Monte Carlo analysis and non-parametric bootstrap values generated from 2000 replicates appear above branches. Partitioned Bremer support values (decay indices) appear below branches. An asterisk (*) represents values of 100. See text for description of clade and group designations (A–G and I and II, respectively).

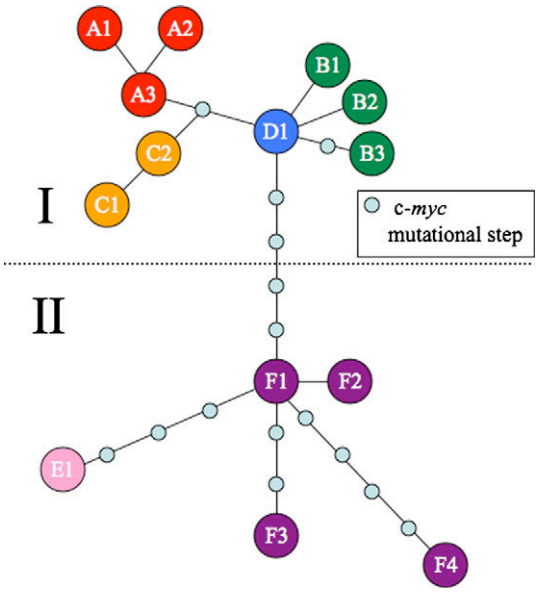

Fig. 3. Parsimony network featuring 14 nuclear c-*myc* haplotypes recovered from the Costa Rican focal taxa included in this study. Circles represent unique haplotypes. Colored circle shading and labeling (A–F) corresponds to the clades in Fig 2. Description of group designation (I and II) can be found in text.

did the northern group (I) ($2N = 6$, $\hat{\pi} = 0.00784$ vs. $2N = 18$, $\hat{\pi} = 0.00694$, respectively).

3.3. Estimation of evolutionary rates and divergence

A summary (by gene region) of the χ^2 statistics resulting from the LRT tests is included in Table 3. In the analysis including all samples, two loci rejected the constant rate model of a molecular clock ($p < 0.002$) and two failed to reject it ($p > 0.05$). In the local clock analyses of just *C.* cf. *podiciferus* plus *C.* sp. A (Clade G) all regions failed to reject a molecular clock suggesting that the majority of rate heterogeneity is confined to the lowland taxa, such as *C. stejnegerianus* (Fig. 2).

Estimated mean rate assuming the temporal calibration based on Heinicke et al. (2007) was 0.57% sequence divergence per my per lineage (95% highest posterior density [HPD] = 0.27–0.92;) which contrasts with the rate of 0.22% (HPD = 0.14–0.30) obtained when assuming the Crawford and Smith (2005) calibration. Divergence estimates from the three analyses generated disparate divergence times for this group of taxa. Relaxed clock dates (collected during rate sampling) had older ages for internal nodes relative to those dates recovered when using the sampled mean divergence rate as a molecular clock. The inverse situation occurred for basal nodes where relaxed clock dates had younger ages relative to the molecular clock analyses (Table 4).

4. Disscussion

4.1. Phylogenetic relationships

Cryptic diversity in terraranan lineages has been observed on multiple occasions (e.g., Miyamato, 1983; Crawford 2003a; Crawford and Smith, 2005; Elmer et al., 2007; Crawford et al., 2007; Wang et al., 2008; Padial and De la Riva, 2009). However, the *C. podiciferus* complex is atypical when compared to previous examples in that many of the divergent lineages described herein are syntopic with one another (Fig. 1). The levels of sequence divergence within the complex (Table 5) are notable given the small geographic range of ca. 8000 km^2 that it is known to occupy (IUCN, 2006). Patterns that are evident in both the mitochondrial and nuclear data include the restriction of the *C.* cf. *podiciferus* clade to Costa Rica (CR) and a northern and southern division (groups I and II, respectively; Fig. 2) within CR. As reported previously (Crawford and Smith, 2005; Chen, 2005), *C. podiciferus* complex frogs from western Panama (Clade G, aka *Craugastor* sp. A) fall well outside the Costa Rican taxa. Our Bayesian analysis and Crawford and Smith (2005) recovered *C. podiciferus*-like animals as paraphyletic with respect to the lowland taxa, although the statistical support for these relationships was weak in both studies. Although we are unable to make firm conclusions regarding the phylogenetic position of the Panamanian portion of the *C. podiciferus* complex at this time, clearly *Craugastor* sp. A is a distinct evolutionary entity. *Craugastor* sp. A is directly referable to "C. sp. B" of Crawford and Smith (2005) as their study and ours both included the sample, FMNH 257689, from western Panama.

The distribution of nuclear haplotypes (Fig. 3) within the *C.* cf. *podiciferus* clade appears to mirror the deeper phylogenetic relationships recovered in the multi-locus Bayesian analysis. Although our sampling provides no evidence of shared nuclear haplotypes among clades identified from the predominately mtDNA concatenated dataset, Clades B and D are not reciprocally monophyletic at the c-*myc* marker (Fig. 3). Lack of nuclear monophyly in these mtDNA clades could be due to introgression or incomplete lineage sorting. Range overlap among mtDNA lineages suggests introgression may be possible, while the large effective population sizes and incomplete lineage sorting observed at the c-*myc* gene in other members of the *C. podiciferus* species group (Crawford 2003a) suggest that lack of monophyly could also reflect segregating ancestral polymorphisms. The greater $\hat{\pi}$ value for nuclear haplotypes in the

Table 4
Time to most recent common ancestor (TMRCA) in millions of years (my) obtained using three separate dating/rate criteria under molecular clocks. Also presented are dates corresponding to estimated rates of sequence divergence acquired in the analogous relaxed clock analyses for constraint schemes (1) Heinicke et al. (2007) and (2) Crawford and Smith (2005). TMRCA values below the black line belong to focal taxa in this study. Approximate rates are presented per million years per lineage.

	Molecular clock rates per million years per lineage (my/l)						Relaxed clock rates per my/l			
	0.75%		0.57%		0.22%		(0.27–0.92%) constraint scheme 1		(0.14-0.30%) constraint scheme 2	
Node (TMRCA)	Mean	(95% HPD)	Mean	(95% HPD)	Mean	(95% HPD)	Mean	(95% HPD)	Mean	(95% HPD)
C. cf. *podiciferus*–C. cf. longirostris	19.28	(16.82, 21.86)	24.72	(21.38, 27.84)	65.37	(56.72, 73.93)	18.79	(10.39, 27.98)	46.78	(36.86, 56.41)
C. bransfordii–*C.* cf. *podiciferus*	12.48	(10.82, 14.28)	16.02	(13.81, 18.18)	42.29	(36.64, 48.35)	15.21	(7.04, 23.64)	38.44	(24.98, 51.48)
C. cf. *podiciferus*–Clade G	7.94	(6.64, 9.08)	10.16	(8.72, 11.81)	26.85	(22.80, 31.01)	15.21	(7.04, 23.64)	38.44	(24.98, 51.48)
C. cf. *podiciferus* (I and II)	5.54	(4.70, 6.40)	7.08	(6.04, 8.18)	18.70	(15.95, 21.65)	11.45	(4.85, 19.08)	29.29	(16.32, 42.11)
Group I (crown age)	4.88	(4.18, 5.76)	6.25	(5.34, 7.21)	16.51	(14.11, 19.30)	9.00	(3.61, 15.37)	23.40	(12.69, 35.63)
Clade A (crown age)	0.44	(0.24, 0.66)	0.55	(0.29, 0.83)	1.48	(0.81, 2.24)	2.05	(0.28, 4.55)	5.10	(0.80, 10.83)
Clade B (crown age)	1.10	(0.60, 1.60)	1.41	(0.82, 2.08)	3.71	(2.11, 5.44)	3.54	(0.80, 6.85)	8.18	(2.50, 15.23)
Clade C (crown age)	0.90	(0.56, 1.24)	1.14	(0.72, 1.59)	3.01	(1.90, 4.13)	3.02	(0.66, 5.94)	7.52	(2.30, 14.01)
Clade D (crown age)	0.70	(0.40, 1.02)	0.90	(0.53, 1.29)	2.40	(1.35, 3.43)	2.38	(0.54, 5.06)	6.21	(1.02, 13.60)
Clade E (crown age)	0.22	(0.04, 0.48)	0.28	(0.03, 0.60)	0.76	(0.08, 1.61)	1.00	(0.02, 3.02)	2.47	(0.05, 7.35)
Clade F (crown age)	0.60	(0.40, 0.84)	0.76	(0.48, 1.05)	2.01	(1.29, 2.84)	2.63	(0.55, 5.29)	6.40	(1.62, 12.12)
Clade G (crown age)	0.94	(0.60, 1.32)	1.21	(0.78, 1.68)	3.23	(1.98, 4.51)	3.93	(0.86, 8.15)	9.18	(2.58, 17.69)

Table 5
Genetic variability partitioned by locus for (1) the maximum amount of sequence divergence within the *C. podiciferus* complex (focal taxon) and (2) the maximum amount of sequence divergence among all taxa included in this study. Distances are based on uncorrected "*p*" values. Also presented is the number of focal taxon haplotypes recovered for each locus.

Locus	# of bases	# of focal taxa	# of haplotypes	Max.% divergence among focal taxa (%)	Max.% divergence with outgroup (%)
12S	380	40	30	11	20
16S	357	39	24	7	16
COI	507	33	26	17	26
c-*myc*	414	31	22	5	8

southern group (II) may be explained by the age of the Cordillera de Talamanca relative to the northern/central mountain ranges of Costa Rica (ca. 14 vs. 3 my, respectively [Denyer et al., 2000; Marshall et al., 2003; MacMillan et al., 2004]) and indicate the presence of ancestral *C. podiciferus* complex frogs in this Cordillera prior to the younger ranges.

4.2. Biogeography of Craugastor podiciferus

Considering the methodology used to obtain divergence estimates and the rejection of the molecular clock by two genes in the global context (Table 3), we primarily restrict our biogeographic commentary to the *C. podiciferus* complex. We use the clock-like rates shared by these focal lineages to identify a range of sequence divergence rates (and hence their respective timeframes) that seem most likely given the geologic and environmental history of montane Costa Rica and western Panama. Geologic times and period boundaries are based on Gradstein et al. (2005). The topology recovered for our focal taxa (Box 1; Fig. 4) contains six unique clades collectively distributed across three mountain ranges. Among these ranges, the oldest radiometric dated deposits discovered thus far originate from the Cordillera de Talamanca and are between 13 and 14 my old (MacMillan et al., 2004). Although Central America may have been a continuous peninsula 20 Ma (Kirby et al., 2008), we assume a boundary condition of 14 Ma for the presence of mountain habitat. We refrain from discussing TMRCA estimates associated with the 0.22% per my per lineage rate in detail since many of the internal node dates predate this boundary condition; however, even though this rate would indicate isolating mechanisms before radiation into the Cordillera de Talamanca, future consideration is warranted as an alternative explanation to the model we present below.

The earliest Cordillera de Talamanca radiation by any frog in this study should coincide with the TMRCA for the *C.* cf. *podiciferus* and *Craugastor* sp. A clades. Based on our divergence results, divergence rates between ca. 0.57% and 0.75% per my per lineage produce a TMRCA estimate between 11.81 and 6.64 Ma for this node (Table 4) which fits our boundary condition. The other cordilleras that the *C. podiciferus* complex is known to inhabit (Cordillera de Central and Cordillera de Tilarán) are volcanically active and substantially younger, having been formed during the late Tertiary and Quaternary (Denyer et al., 2000; Marshall et al., 2003). The primary restriction of group I *C.* cf. *podiciferus* clade taxa (Clades A–D) to these northern/central Cordilleras and the lower $\hat{\pi}$ values in this group (I), suggest that these mountains have played a role in the origin of this group. Divergence rates between ca. 0.57 and 0.75% place the origin of this group (TMRCA, Group I; Table 4) between 7.21 and 4.18 Ma (late Miocene through middle Pliocene). Despite predating the hypothesized origins of the northern/central Cordilleras, these dates overlap the presence of the now extinct Cordillera de Aguacate in northwestern Costa Rica (ca. 5 Ma; Marshall et al., 2003).

For shallower nodes in our phylogeny, there are several issues that complicate our ability to elucidate natural barriers that may have affected dispersal or led to vicariance in the *C. podiciferus* complex. In particular, a recurrent and enigmatic find is the syntopic occurrence of divergent haplogroups at the same locality (localities 1 and 5 in Fig. 1.). Based on our divergence estimates, we explain the origin of diversity with the description of a localized version of the temperature depression model presented by Savage (2002) that is congruent with the observed genetic

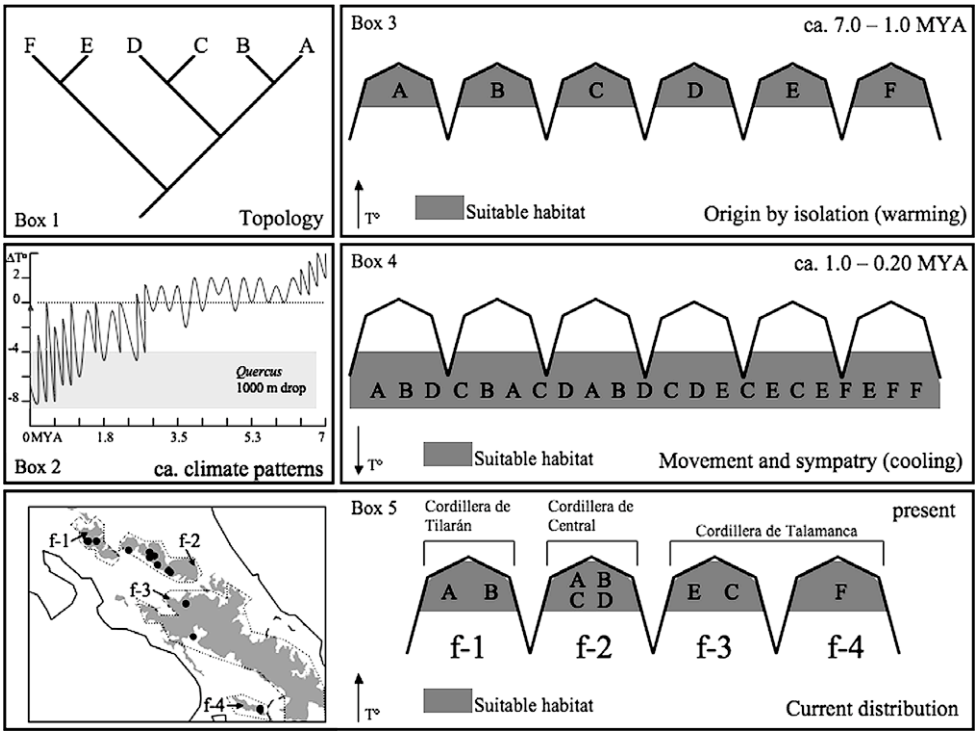

Fig. 4. Model for evolutionary origin and geographic spread of *Craugastor* cf. *podiciferus* clade. Inset boxes (1–5) are described as follows: (1) Cladogram depicting the relationships between *C.* cf. *podiciferus* clade haplogroups. (2) Generalized climate patterns over the last 7 my using change in temperature against time. Note shaded area which corresponds to a 1000 m drop in oak species (*Quercus* spp.) that currently share habitat with the *C. podiciferus* complex. This indicates periods when lowland dispersal corridors were likely available for highland taxa. The dotted line in this graph represents the current temperature. (3) Diagrammatic representation of the origin of each of these haplogroups by isolation during warming periods throughout the late Miocene and early Pleistocene. (4) Diagrammatic representation of sympatric conditions that most likely occurred among haplogroups multiple times during Pleistocene cooling events. (5) Current distribution of haplogroups in four disjunct fragments (f-1 through f-4) of Talamancan montane forest.

variation and geographic distribution of the *C.* cf. *podiciferus* haplotype groups. We employ this model under the hypothesis that opportunities for dispersal and the occurrence of vicariance in the *C. podiciferus* complex have been controlled by habitat continuity; thus, the movement of these frogs has been limited by climatic fluctuations which generated periods of habitat discontinuities between the three mountain ranges in question. In order for this hypothesis to be realistic (given the taxa in this study), three primary assumptions are required: (1) taxa unique to each 'island-like' fragment of TMF (Box 5; Fig. 4) are presently restricted from movement between fragments, (2) anthropogenic interference (e.g., translocation/poor data collection) has not occurred in our sampling of these taxa (Lynch and Duellman, 1997), and (3) the ecological niche of these frogs has been largely conserved through time. Prior to approximately 7 Ma (Zachos et al., 2001), global temperatures were consistently above the current temperature levels indicating that ancestral *C. podiciferus* complex frogs should have been locally isolated during this time. From approximately 7–2 Ma, global temperatures reached similar levels to present and during multiple glaciations dropped below this level (Lisiecki and Raymo, 2005) which would have briefly lowered TMF habitat allowing for expansion and mixing of present day high elevation fauna and flora via the lowlands (Fig 4, Box 4). Following these habitat depressions subsequent interglacial periods would have forced these expanded communities upwards and imposed isolation once again. Using the ca. 0.57–0.75% rates of sequence divergence, the timing of these cycles roughly matches the origin of the *C.* cf. *podiciferus* clade groups (I and II; Table 4) between 8.18 and 4.70 Ma. More recently (1 Ma), a transition to more rapid glacial cycles of 0.01 my with climate fluctuations of 4 °C and lower should have provided the *C.* cf. *podiciferus* clade groups with sufficient time for dispersal and mixing (between fragments) during these cycles (Boxes 3 and 4; Fig 4.). The TMRCA for each haplogroup that would have originated during these periods (Clades A–F) matches this shift in cycle period when using rates of ca. 0.57–0.75% per my per lineage in that these rates produce TMRCA dates between 2.08 and 0.04 Ma. This type of dispersal also resolves the occurrence of the syntopic yet divergent clades (A and B, C and D, respectively; Fig 1) and Group I individuals in northern sections of the Cordillera de Talamanca (Locality 9; Fig. 1) all of which were presumably forced upward and restricted to their current location at the end of the last glacial cycle (Box 5; Fig. 4). Given our hypothesized topology (Box 1; Fig. 4) and the distinction between Clades A–D and Clades E and F (groups I and II, respectively), the putative admixture of haplogroups (Box 4; Fig. 4) may have not been panmictic across lowlands but rather locally confined within groups (I and II) and also species (*C. podiciferus* complex and *Craugastor* sp. A). If supported by future sampling, this further localized admixture would suggest biogeographic breaks between Localities 9 and 10 and Localities 11 and 12 (Fig. 1). While this model is an oversimplification of the mosaic-like structure of temperature and habitat variables that likely drove these events, it does offer a generalized explanation for the observed distributional patterns of *C.* cf. *podiciferus* clade haplogroups that is consistent with hypothesized geologic events. Additionally, there is preliminary evidence that suggests similar elevational shifts in montane amphibian distributions are presently occurring in response to the changing climate (Raxworthy et al., 2008).

4.3. Conclusion and future directions

Although members of the *C. podiciferus* complex are relatively abundant and familiar to most naturalists in Costa Rica, they have been poorly studied given the tendency for their phenotypic polymorphism to confound gestalt-based field identification. This difficulty has plagued the complex since its description in the late 19th century when Cope (1875) noted the high levels of phenotypic variability in *C. podiciferus*: "The colors in this species vary remarkably, more than I have observed to be the case in any other frog". In revisiting our study objectives we report that (1) the *C. podiciferus* complex possesses relatively extensive genetic diversity across its known range, especially on the Pacific versant of the Cordillera de Talamanca; (2) with two notable exceptions, this complex has a phylogenetic history that corresponds with geography; and (3) the two instances of sympatric yet highly divergent haplotypes can be explained via a Pliocene–Pleistocene temperature depression model.

The evidence presented here supports the suggestion of previous authors that the current concept of *C. podiciferus* includes multiple taxa (Savage, 2002; Chen, 2005; Crawford and Smith, 2005). Future molecular sampling, coupled with detailed morphological analyses are necessary to delineate species boundaries within the complex. Currently, an extensive morphological analysis of this group is in progress and preliminary comparisons to this molecular phylogeny appear generally congruent (J.M. Savage, pers. comm.). We recommend that genetic sampling from the Cordillera de Talamanca (particularly from the type locality of Cerro Utyum) be conducted prior to the construction of a revised species level taxonomy for the group. Additionally, the presence and status of *C. podiciferus*-like frogs in the Tabasará range of Panama requires further examination.

The level of genetic divergence in sympatry documented in this examination of the *C. podiciferus* complex provides evidence that climatic fluctuation during the Pleistocene in lower Central America may have contributed to the high levels of lineage and species richness observed in the montane biota of this region, however; more homogeneous sampling of the complex is necessary to test this hypothesis. The rate heterogeneity documented between montane (*C. podiciferus* complex) and lowland (*C. bransfordii* and others) frog species in our study (Fig. 2; Table 3) may be explained by ecological differences related to elevation and should be explored further with comparison to similarly stratified biota in Isthmian Central America. While the levels of diversity in the *C. podiciferus* complex are notable, in order to identify general phylogeographic patterns in the lower Central American Cordilleras, there is a need for analogous studies in additional taxonomic groups (e.g., García-París et al., 2000). The presence of at least 6 distinct clades of *C. podiciferus* complex frogs in Costa Rica emphasizes the need for future conservation plans to maintain as much of the TMF as possible. This would protect not only the remarkable known diversity, but also the potential cryptic diversity that may be uncovered as studies similar to the present one are conducted on additional montane organisms.

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