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Phylogeography of the Pygmy Rain Frog (*Pristimantis ridens*) across the lowland wet forests of isthmian Central America

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ABSTRACT

We used a phylogeographic approach to elucidate the evolutionary history of a lineage of frogs, known as *Pristimantis* (formerly *Eleutherodactylus*) *ridens* (Anura: Brachycephalidae), restricted to the wet forests occurring along the Caribbean versant of isthmian Central America as well as the disjunct wet forest on the Pacific slope of Costa Rica. We placed our phylogeographic study of *P. ridens* within a larger molecular phylogenetic analysis of Central American *Pristimantis*. All phylogenetic inferences were based on a 1455 base pair fragment of mitochondrial DNA, containing the complete ND2 gene and five flanking tRNA genes. Our reconstruction of the intraspecific phylogeny of *P. ridens* yielded a basal trichotomy dating to an estimated 12+ million years ago (Ma), consisting of central Panama, western Panama, and Costa Rica plus Honduras. Thus, the presence of *P. ridens* appears to predate the completion of the Isthmus 3.1 Ma. Using a parametric bootstrap (SOWH) test, we evaluated four *a priori* zoogeographic hypotheses for the origin and spread of *P. ridens*. This analysis suggested that the *P. ridens* populations on the Caribbean versant of Costa Rica were established by Pacific versant ancestors only recently, in contrast to the very old lineages found in Panama. Our results support a model of Miocene colonization, long-term geographic stasis, followed by rapid dispersal across the Caribbean lowlands during the Pliocene or Pleistocene.

1. Introduction

Phylogeographic studies play an essential role in elucidating the mechanisms by which lineages diversify and generate the biodiversity we observe today in distinctive geographical regions. With molecular data, we can now apply the principles of biogeography to studies of single species, as well as make direct connections between historical biogeography and demography (Bermingham and Avise, 1986; Avise et al., 1987). Research on New World fossil mammals from the Pliocene and Pleistocene has made famous the role of the Isthmus of Central America as a land bridge and a driver of expansion, extinction, and diversification of lineages moving between continents (Marshall et al., 1982; Simpson, 1940; Webb and Rancy, 1996). However, mounting evidence suggests that isthmian Central America itself has a rich biotic history and hosts many endemic species (e.g., Bermingham and Martin, 1998; Ibáñez and Crawford, 2004; Myers, 2003; Smith and Bermingham, 2005; Reeves and Bermingham, 2006). Even at the intraspecific level, widespread Neotropical plants and animals may harbor more genetic diversity within Panama alone than within much larger continental areas (e.g., Dick et al., 2003; Weigt et al., 2005).

The pivotal biogeographic role of the Central American Isthmus on its ecological communities has been studied in a variety of taxa including sea urchins and shrimp (Lessios, 1979; Bermingham and Lessios, 1993; Knowlton and Weigt 1998), fresh water fishes (Bermingham and Martin, 1998; Perdices et al., 2002; Reeves and Bermingham, 2006), and insects (Zeh et al., 2003). However, there have been few such studies of terrestrial vertebrates. Frogs, in particular, are useful for studies inferring geological and environmental history because they are terrestrial, intolerant of salt water, incapable of flight, and often locally abundant (Beebee, 2005). In terms of bioclimatic variables, anuran species show remarkable niche conservatism (Smith et al., 2005; Wiens et al., 2006), and many frogs are restricted to a particular habitat type, such as tropical wet forest (e.g., Savage 2002). Therefore, by studying the history of forest-restricted frog species, we can infer the history of the forest as well as its inhabitants (Marshall, 1988).

Here we present our analysis of the phylogeographic history of *Pristimantis ridens* (Anura: Brachycephalidae; Frost et al., 2006), the Pygmy Rain Frog. Standard length in this species is a diminutive 21–25 mm in females and 16–19 mm in males (Savage, 2002). *Pristimantis ridens* belongs to a genus of largely South American

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species of direct-developing frogs that were until recently included in the genus *Eleutherodactylus* (Heinicke et al., 2007). While *P. ridens* occurs in isthmian Central America, the species group that contains it is believed to be of South American origin (Lynch and Duellman, 1997; Savage, 2002; see below). The geographic distribution of *P. ridens* tracks the low- and mid-elevation wet forests of isthmian Central America. These forests, like *P. ridens*, show a disjunct distribution pattern with a possible connection between Caribbean and Pacific slopes at the mountain pass of Fortuna (Fig. 1, inset; Jungfer, 1988). They range along the entire Caribbean coast but are limited to only one isolated location on the Pacific coast: the Golfo Dulce region of southwestern Costa Rica (McDiarmid and Savage, 2005). The Golfo Dulce region is separated from the Caribbean versant by the >2000 m high Talamanca mountains, and is bounded to the northwest and southeast by lowland dry forest (Holdridge, 1967; Tosi, 1969). Other vertebrates besides *P. ridens* track this disjunct distribution, most notably certain species of birds (Ridgely et al., 2003), snakes (Köhler, 2003), and other frogs (Savage, 2002).

The phylogeographic origins of this disjunct geographic distribution have been investigated only once previously. Crawford et al. (2007) studied another wet forest frog, *Craugastor crassidigitus*. Although *C. crassidigitus* and *P. ridens* are both eleutherodactyline frogs and show very similar geographic distributions (IUCN et al., 2004), the genus *Pristimantis* originated in South America whereas the genus *Craugastor* is of Central American origin (Crawford and Smith, 2005). In *C. crassidigitus*, populations separated by dry forest show >4 million years divergence, suggesting that the wet versus dry forest habitat heterogeneity of isthmian Central America was in place prior to the final completion of the Isthmus of Panama (Coates and Obando, 1996; Coates et al., 2004; Crawford et al., 2007; Graham and Dilcher, 1995). If so, we asked how and when a wet forest species such as *P. ridens*, whose ancestors came from South America, managed to colonize isthmian Central America despite the hypothesized dry forest barriers. To answer these questions, we inferred the spatial and genealogical history of *P. ridens* and compared our results to previous work as well as the environmental and geological history of isthmian Central America.

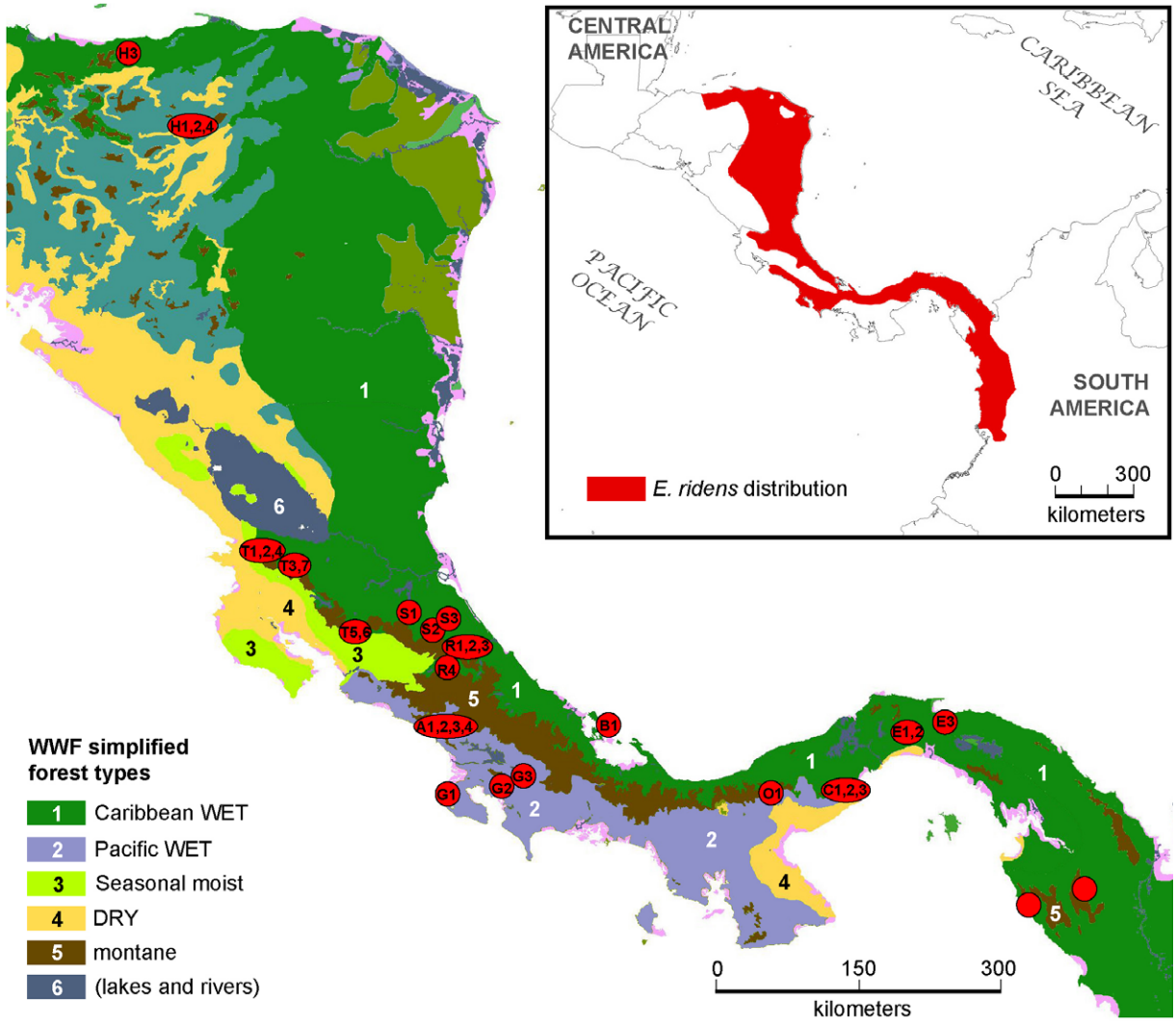

Fig. 1. World Wildlife Fund ecoregions covering Honduras, Nicaragua, Costa Rica and Panama, simplified as follows. "Caribbean WET" combines three WWF moist forest ecoregions: Central American Atlantic, isthmian-Atlantic, and Chocó-Darién moist forests. "Montane" combines three WWF ecoregions: Central American, Talamancan and eastern Panamanian montane forests. "DRY" combines two ecoregions: Central American and Panamanian dry forests. "Pacific WET" is officially known as isthmian-Pacific moist forest. Letters and numbers in red circles denote collecting localities of *P. ridens* and outgroups (see Table 1 for details). Localities are grouped into sampling regions (Fig. 2) by the first letter used in each sample code. Empty red dots are located in the Darien province of Panama and indicate collecting regions of additional outgroup taxa, including samples easily confused with and morphologically very similar to *P. ridens*. Inset map shows range of *P. ridens* on a political map of isthmian Central America, according to the Global Amphibian Assessment database (IUCN et al., 2004).

We used phylogenetic analyses and parametric bootstrap tests of *a priori* hypotheses to reveal the history of isolation and expansion that has led to the current disjunct distribution of this species. We examine the historical expansion of *P. ridens* across the Isthmus by using DNA sequence data to test unique genealogical predictions made by each of four geographic models for the expansion of *P. ridens* across its range from the southeast to the northwest (see Section 2). We test two models involving the expansion of *P. ridens* up both versants of the Isthmus simultaneously (Bicoastal Routes 1 and 2), and we posit two other models (Pacific Route and Caribbean Route) involving independent colonization of each versant (Fig. 2). Each of these four hypotheses implies an historical scenario that is informative about *P. ridens* as well as the wet forests that support these frogs and constrain their geographic distribution. By comparing the phylogeography of *P. ridens* with previous work, we can also gain insights into general evolutionary processes that give rise to ecological communities and shape the geographic distributions of species we observe today.

2. Materials and methods

2.1. Study system

Pristimantis ridens occurs only in wet forest habitat from low- to mid-elevations (Savage, 2002) along the Caribbean coast of Panama (Ibáñez et al., 2001) and through large portions of Costa Rica, Nicaragua, and eastern Honduras (Savage, 2002; Köhler, 2001; McCranie and Wilson, 2002; Fig. 1). This species has also been reported from the Chocó biogeographic region on the Pacific coast of Colombia (Lynch and Suárez-Mayorga, 2004; Ruiz-Carranza et al., 1996). Our own attempts to find *P. ridens* in the Darien portion of eastern Panama, however, have to date resulted in collections of morphologically similar species that are genetically quite distant from the rest of our *P. ridens* samples (see Section 3).

Pristimantis ridens is a member of the *P. cruentus* group of Savage (2002) and the much larger *P. unistrigatus* group of Lynch and Duellman (1997). Members of the *P. cruentus* group, as well as *P. cerasinus,* are regarded as recent invaders from South America into Central America whose arrival coincided with the formation of the isthmian land bridge (Duellman, 2001; Savage, 2002; Vanzolini and Heyer, 1985). In our attempt to root the phylogeographic tree of *P. ridens* and identify our collection of *P. ridens*-like individuals from the Darien, we present an expanded DNA sequence-based phylogenetic study of most of the members of the *P. cruentus* group, including several undescribed species from the Darien Province of eastern Panama plus the only Central American member of the *P. cerasinus* group (Lynch and Duellman, 1997; Savage, 2002; Table 1), *P. cerasinus*, another species that can potentially be confused with *P. ridens* or *P. cruentus*, especially as juveniles (Savage, 1981). A recent molecular phylogenetic study with broad coverage of eleutherodactyline frogs included only two *Pristimantis* samples from Central America, one *P. ridens* and one *P. cruentus* (Heinicke et al., 2007). Thus, the relationships among Central American *Pristimantis* remain unclear. From the *P. cruentus* group we

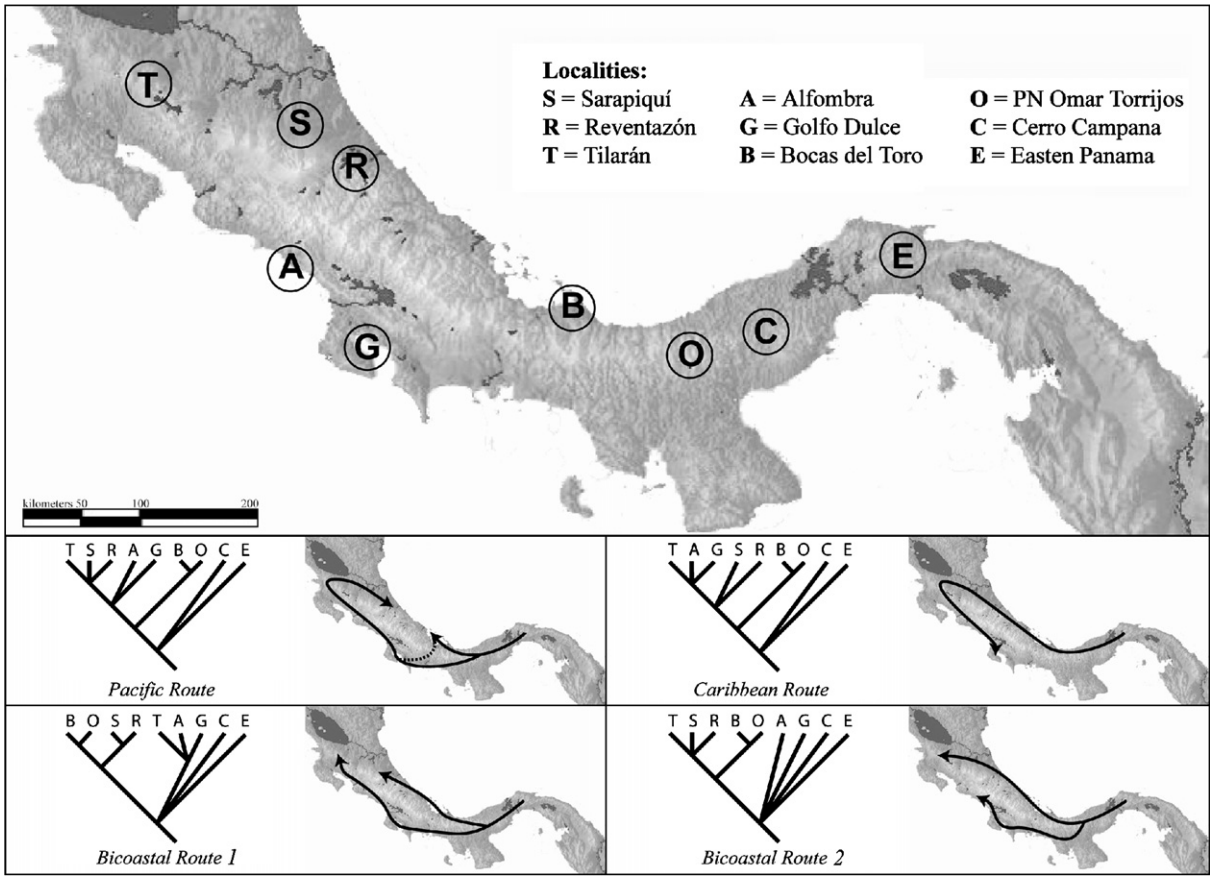

Fig. 2. Map illustrating the nine regions (circled letters) which summarize our collecting regions of *P. ridens* in isthmian Central America (Fig. 1) for purposes of generating hypotheses and testable predictions. Below the regional map are four *a priori* hypothesized dispersal scenarios for the expansion of *P. ridens* across the landscape, presented as arrows. Each scenario makes a unique prediction regarding the expected phylogenetic relationships among sampling regions. Cladograms were used as constraint trees in parametric bootstrap tests. Note, a model of vicariance due to uplift of the central mountain range (the Talamanca mountains) would make the same predictions as the Bicoastal dispersal hypotheses.

Table 1
Sample codes, species names, institutional voucher numbers, field tag numbers, locality information, and GenBank accession numbers for the 46 frog samples used in this study

Sample code	Species	Institutional voucher number[a]	Field collection number[b]	Collection locality[c]	Longitude	Latitude	GenBank Accession No.
A1	*ridens*	UCR 16456	AJC 0376	Alfombra, San José, CR, 900m.	−83.77203	9.31228	EU443157
A2	*ridens*	UCR 16435	AJC 0390	Dominical, San José, CR, 130m.	−83.84980	9.31956	EU443187
A3	*ridens*	UCR 16458	AJC 0378	Alfombra, San José, CR, 900m.	−83.77203	9.31228	EU443158
A4	*ridens*	UCR 16468	AJC 0388	Tinamaste, San José, CR, 550m.	−83.76663	9.29505	EU443179
B1	*ridens*	Not catalogued	AJC 0878	I. Bastimentos, Bocas del Toro, PA, 75m.	−82.1031	9.3056	EU443169
C1	*ridens*	FMNH 257833	AJC 0336	Cerro Campana, Panama, PA, 900m.	−79.92738	8.68564	EU443159
C2	*ridens*	FMNH 257837	AJC 0340	Cerro Campana, Panama, PA, 900m.	−79.92738	8.68564	EU443160
C3	*ridens*	FMNH 257552	AJC 0216	Cerro Campana, Panama, PA, 900.	−79.927	8.685	EU443183
E1	*ridens*	Not catalogued	AJC 0973	Cerro Azul, Panama, PA, 600m.	−79.40327	9.22175	EU443163
E2	*ridens*	MVUP 1889	AJC 0972	Cerro Azul, Panama, PA, 600m.	−79.40327	9.22175	EU443162
E3	*ridens*	FMNH 257697	AJC 0211	Nusagandi, Panama, PA, 400m.	−78.98330	9.3167	EU443164
G1	*ridens*	UCR 16460	AJC 0506	Peninsula Osa, Puntarenas, CR, 120m.	−83.66675	8.6718	EU443174
G2	*ridens*	FMNH 257768	AJC 0126	Río Claro, Puntarenas, CR, 70m.	−83.04935	8.68658	EU443189
G3*	*ridens*	FMNH 257746	AJC 0103	Las Cruces, Puntarenas, CR, 60m.	−82.97500	8.78333	AY273101
H1*	*ridens*	UTA A-57017	ENS 10727	Agalta, Olancho, HN, 1080m.	−86.148	14.959	EU443154
H2*	*ridens*	UTA A-57014	ENS 10722	Agalta, Olancho, HN, 1310m.	−86.139	14.933	EU443155
H3*	*ridens*	USNM 514547	LDW 10706	Atlantida, Olancho, HN, 940m.	−86.80	15.63	EU443175
H4*	*ridens*	UTA A-57016	ENS 10726	Agalta, Olancho, HN, 1080m.	−86.148	14.959	EU443153
O1	*ridens*	MVUP 1787	KRL 0692	PN Omar Torrijos H., Coclé, PA, 800m.	−80.592	8.667	EU443165
R1	*ridens*	UCR 16268	AJC 0404	Guayacan, Limón, CR, 530m.	−83.53555	10.0052	EU443173
R2	*ridens*	UCR 16467	AJC 0526	Volcán Turrialba, Limón, CR, 700m.	−83.717	10.125	EU443180
R3	*ridens*	UCR 16272	AJC 0415	Guayacán, Limón, CR, 500m.	−83.54863	10.0433	EU443167
R4	*ridens*	FMNH 257579	AJC 0248	Turrialba, Cartago, CR, 540m.	−83.650	9.892	EU443161
S1	*ridens*	UCR 16461	AJC 0522	EB La Selva, Sarapiquí, CR, 75m.	−84.0070	10.4303	EU443170
S2	*ridens*	UCR 16451	AJC 0356	Guápiles, Limón, CR, 320m.	−83.83	10.18	EU443168
S3	*ridens*	UCR 16454	FB 2674	Guácimo, Limón, CR, 200m.	−83.7184	10.2140	EU443166
T1	*ridens*	MF 6063	DL 677	Volcán Cacao, Alajuela, CR, 1100m.	−85.4667	10.9225	EU443182
T2	*ridens*	UCR 18074	FB 4331	Volcán Cacao, Alajuela, CR, 1100m.	−85.47	10.9225	EU443172
T3	*ridens*	UCR 16464	AJC 0494	Upala, Alajuela, CR, 760m.	−85.04077	10.7137	EU443156
T4	*ridens*	UCR 17648	FB 4076	North Tilarán, Alajuela, CR, 350m.	−85.37	10.92	EU443178
T5	*ridens*	UCR 16789	FB 2623	San Ramón, Alajuela, CR, 960m.	−84.59690	10.2188	EU443177
T6	*ridens*	UCR 16455	AJC 0395	MHN La Paz, Alajuela, CR, 1230m.	−84.55855	10.1822	EU443171
T7	*ridens*	UCR 16466	AJC 0496	Upala, Alajuela, CR, 670m.	−85.14215	10.8113	EU443181
*	*museosus*	SIUC H-06970	KRL 8881	PN Omar Torrijos H., Coclé, PA, 800m.	−80.5917	8.6667	AY273103
*	*cruentus*	UCR 16443	AJC 0463	San Ramón, Alajuela, CR, 960m	−84.59698	10.22100	EU443186
*	*cruentus*	UCR 16448	AJC 0458	San Ramón, Alajuela, CR, 960m	−84.59698	10.2188	EU443176
*	*cruentus*	Not catalogued	AJC 0603	Cana, PN Darién, PA, 1600m	−77.7167	7.7711	EU443188
*	sp. nov. A	Not catalogued	AJC 0922	Piñas, Darién, PA, 800m	−78.20217	7.68200	EU443191
*	sp. nov. A	Not catalogued	AJC 0924	Piñas, Darién, PA, 800m	−78.20217	7.68200	EU443192
*	sp. nov. B	Not catalogued	AJC 0580	Cana, PN Darién, PA, 1300m	−77.72225	7.76358	EU443193
*	*cerasinus*	FMNH 257713	AJC 0071	EB La Selva, Sarapiquí, CR, 75m.	−84.0070	10.4303	EU443194
*	*pardalis*	FMNH 257675	AJC 0188	Fortuna, Chiriquí, PA, 1000m	−82.22	8.75	AY273102
*	*altae*	UCR 16472	AJC 0398	MNH La Paz, San Ramón, Alajuela, CR, 1230m	−84.55855	10.1822	EU443185
*	*pirrensis*	CH 5641	AJC 0594	Cana, PN Darién, PA, 1600 m	−77.68405	7.75607	EU443190
*	sp. C	Not catalogued	AJC 0601	Cana, PN Darién, PA, 500 m	−77.68405	7.75607	EU443184
*	*Craugastor noblei*	SIUC H-06971	KRL 8921	PN Omar Torrijos H., Coclé, PA, 800m.	−80.5917	8.6667	EU443195

All species are members of the genus *Pristimantis*, except for the outgroup, *Craugastor noblei*. Sample codes correspond to those used in the gene tree (Fig. 3) and the capital letter in a code refers to the collecting region (Fig. 2). An asterisk (*) in the Sample column indicates that this sample was not included in the SOWH tests. Four specimens are still awaiting accession into institutional natural history collections.

[a] CH, Círculo Herpetológico de Panamá, Panama City, Panama; FMNH, Field Museum of Natural History, Chicago, USA; MF, Michael Forstner collection at southwest Texas State University; MVUP, Museo de Vertebrados de la Universidad de Panamá, Panama City, Panama; SIUC, southern Illinois University at Carbondale, USA; USNM, National Museum of Natural History, Washington, DC, USA.

[b] AJC, Andrew J. Crawford; DL, David Laurencio; ENS, Eric N. Smith; FB, Federico Bolaños V. (Robert Puschendorf and Gerardo "Cachí" Chaves, collectors); KRL, Karen R. Lips; LDW, Larry David Wilson (Randy McCranie, collector).

[c] EB, Estación Biológica; MHN, Monumento Histórico Natural; PN, Parque Nacional; PA, Republic of Panama; CR, Costa Rica; HN, Honduras. Final numbers indicate elevation in meters (m).

sampled *P. altae*, *P. cruentus*, *P. museosus*, *P. pardalis*, *P. pirrensis*, plus three undescribed species (*P.* sp. A aff. *cerasinus*, *P.* sp. B aff. *cruentus* Panama, and *P.* sp. C aff. *ridens* Panama), in addition to *P. ridens*. We used *Craugastor noblei* as an outgroup (Crawford and Smith, 2005; Frost et al., 2006). Species distributions may be viewed online at http://www.globalamphibians.org/servlet/GAA#-name (IUCN et al., 2004). All species in the present study had been in the subfamily Eleutherodactylinae, a taxon recently synonymized with Brachycephalidae (Frost et al., 2006).

2.2. Sampling frogs and genes

Frogs were collected in the field, photographed, and euthanized with diluted Chloretone™, in compliance with Smithsonian Institutional Animal Care and Use protocols. Fresh liver samples were stored in a NaCl-saturated storage solution containing 0.25 M EDTA and 20% DMSO (Seutin et al., 1991). Each specimen was fixed in 10% formalin and stored in 70% ethanol (Pisani, 1973). Collections were made in Panama, Costa Rica, and Honduras (Figs. 1 and 2, Table 1). Genomic DNA was extracted from liver tissues using a standard phenol–chloroform protocol with ethanol precipitation. A 1455 base pair (bp) fragment of mitochondrial DNA containing a partial $tRNA^{MET}$ gene fragment, the complete ND2 gene, partial COI gene fragment and the complete intervening "WANCY" region of five tRNA genes was amplified using primers L4437 and H5934 of Macey et al. (1997a), also know as MET.f6 and COI.r1, respectively. Amplified fragments were sequenced using PCR plus internal primers (Crawford and Smith, 2005) with Big Dye 3.1 dye-terminator reaction chemistry and analyzed on an ABI 3100 automated sequencer. DNA sequences were aligned with Sequencher 4.2 (Gene Codes Corporation Inc.) and manually adjusted using the inferred amino acid sequence and tRNA secondary structure (Kumazawa and Nishida, 1993; Macey et al., 1997b). Gapped sites in our alignment were removed from all analyses, as were base pairs from tRNA loops in which the aligned base pairs contained gaps.

2.3. Phylogenetic and population genetic analyses

Using PAUP* version 4.0b10 (Swofford, 2001), we performed maximum parsimony (MP) analyses through heuristic searches using 100,000 random addition sequence replicates involving tree bisection and reconnection (TBR) branch swapping. We assessed statistical support for clades with nonparametric bootstrap analysis (Felsenstein, 1985) using 5000 bootstrap replicates, each having 20 random addition sequence replicates and TBR branch swapping.

For maximum-likelihood (ML) analyses (Felsenstein, 1981), we used Modeltest version 3.6 (Posada and Crandall, 1998) to evaluate among 56 potential models of DNA sequence evolution. The appropriate model was chosen by the Akaike information criterion, or AIC (Akaike, 1974). We used heuristic searches to estimate the ML phylogenetic tree starting from a neighbor-joining (NJ) tree (Saitou and Nei, 1987) and using TBR branch swapping.

We conducted a Bayesian MCMC phylogenetic analysis (Rannala and Yang, 1996; Yang and Rannala, 1997) using MrBayes version 3.0b5 (Huelsenbeck and Ronquist, 2001). We conducted two independent runs, one with 3 million generations sampled every 500 generations and one with 8 million generations sampled every 1000 generations, both with four Metropolis-coupled MCM chains using default heating ($T = 0.2$) and default prior distributions of model parameters. We checked burnin and discarded all trees obtained before each run achieved a stationary state, and combined the remaining trees into one dataset after confirming that both runs achieved stationary states at similar −Ln-likelihood values and resulted in similar levels of support for topological bipartitions. We estimated the marginal posterior probability (mpp) distribution of topologies, branch lengths and parameter values from the combined 9802 samples of post-burnin trees.

The molecular phylogeny and the SOWH test results (see below) suggested one clade may represent the product of a recent expansion. We explored this inference further by using a haplotype network to infer the ancestor and we applied a population genetic test of demographic expansion. We used the computer program TCS version 1.21 (Clement et al., 2000) to calculate a parsimony network that represented a 95% set of plausible solutions based on coalescent theory (Hudson, 1989), thus not connecting divergent haplotypes whose true genealogy could be obscured by homoplasy (Templeton et al., 1992). We tested for demographic expansion using the R_2 statistic, which compares the average pairwise number of mutations versus singleton mutations in a population sample relative to the expectation under the standard neutral model (Ramos-Onsins and Rozas, 2002). Point estimates and 95% confidence intervals based on 10,000 coalescent simulations were obtained using dnaSP version 4.10.4 (Rozas et al., 2003).

2.4. Hypothesis testing

We generated phylogeographic hypotheses from distributional data for *P. ridens* and the lowland wet forest to which it is endemic (Köhler, 2001; McCranie and Wilson, 2002; Savage, 2002; Fig. 1), from geological data on the formation of the Isthmus and Central American mountain ranges (Coates and Obando, 1996; Coates et al., 2004; Denyer et al., 2000), and from existing biogeographical research on eleutherodactyline frogs (Crawford et al., 2007). *Pristimantis ridens*, the *P. cruentus* species group, and the genus *Pristimantis* are all thought to have origins in South America (Duellman, 2001; Heinicke et al., 2007; Savage, 2002; Vanzolini and Heyer, 1985). Today *P. ridens* is distributed on both coasts of isthmian Central America north to Honduras. Mountain ranges in Central America provide a significant dispersal barrier between the Caribbean and Pacific coasts for a lowland endemic. Therefore, to achieve its present distribution we posit that the ancestor of *P. ridens* likely had one of four dispersal histories. It may have dispersed along the Caribbean coast (Caribbean Route) and invaded the Pacific coast after migrating far enough northward that the central mountain range was low enough to pass. Second, it may have dispersed along the Pacific coast (Pacific Route) and invaded the Caribbean coast. Finally, it may have dispersed simultaneously along both coasts (Bicoastal Routes 1 and 2; Fig. 2). Alternatively, *P. ridens* might have entered Central America prior to the formation of the Isthmus 3.1 Ma (Coates and Obando, 1996) and before the uplift of the central ranges 5.4 Ma (Denyer et al., 2000). In this case, dispersal through Central America could have occurred ubiquitously, and the current distribution would have instead resulted vicariantly from the uplift of the Talamanca range; however, the topological predictions of this model are congruent with the predictions of either Bicoastal Routes 1 and 2 (Fig. 2).

Since we had no explicit expectation or interest in whether the Honduran region was more closely related to the Tilarán (T) region or to the Sarapiquí (S) region (Fig. 2), we simplified the analysis by removing the four Honduran haplotypes from our hypothesis testing. This left us with 29 of the original 33 *P. ridens* samples (Table 1). We found that we could distinguish among our four *a priori* hypotheses by focusing on two important questions concerning the Costa Rican samples. (1) Do Costa Rican samples form a monophyletic clade relative to Panamanian samples? If so, we would infer that dispersal occurred between Caribbean and Pacific versants of Costa Rica. If not, we would infer that at least one versant of Costa Rica was colonized independently from Panama. (2) Are the Tilarán samples (region T in Fig. 2) more closely related to the Pacific versant samples (regions A and G) or the Caribbean versant samples (regions S and R)? While progressive colonization of

regions in the order R, S, T, A, G would be expected to yield the topology (R(S(T(AG)))), we relaxed this prediction to the minimally resolved constraint tree (SR(TAG)) because our hypothesis was not concerned explicitly with the relationship between S and R. Similarly, the reverse direction of colonization suggests the minimally resolved constraint tree ((SRT)AG). Therefore, the important distinction between these two hypotheses is the position of T, the Tilarán haplotypes (Fig. 2).

Using a version of the SOWH test (Swofford et al., 1996; Goldman et al., 2000) we performed a parametric bootstrap analysis of the topological predictions made by each of our four *a priori* models for the expansion of *P. ridens* across isthmian Central America (Fig. 2). The parametric bootstrap method tests whether a ML phylogenetic tree estimated under a topological constraint is significantly worse than the optimal (unconstrained) tree. The probability of the observed difference in log-likelihood scores between the optimal tree (H_1) and the constrained tree (H_0) is evaluated by comparison against a distribution of differences in tree scores obtained from simulated datasets. Simulations used the ML model of evolution (see below) and the topology obtained under the null hypothesis constraint (Huelsenbeck et al., 1996; Goldman et al., 2000). The parametric bootstrap is more powerful and performs better than other available tests of topology (Goldman et al., 2000; Shi et al., 2005), but may be sensitive to model misspecification (Buckley, 2002; Felsenstein, 2004).

Because our dataset was prohibitively large to conduct full ML optimizations, we assumed fixed parameter values for ML tree searches in the original simulated datasets. Because of the aforementioned sensitivity to model misspecification (Buckley, 2002), we used an iterative process to obtain the most appropriate model of DNA sequence evolution independently for each of our four tests. We employed Modeltest and used the AIC to choose among alternative models. We then obtained a ML tree by assuming the new model and fixing the parameter values. These values were calculated as an AIC-weighted average among all 56 models (Posada and Buckley, 2004). We used the resulting ML topology as the default tree in a second round of Modeltest, which allowed us to refine our parameter estimates. In cases where the newly chosen model differed from the model selected for the actual data (without topological constraint), we then calculated a new −Ln score for the unconstrained ML tree under the new model. The difference, δ, between −Ln for constrained versus unconstrained trees was used as our test statistic for a given topological test.

Five hundred simulated datasets were generated using the ML model of sequence evolution obtained for the given constraint tree. Any polytomies in the H_0 topology were first converted to dichotomies with zero-length branches, using TreeEdit version 1.0 alpha 10 (Rambaut and Charleston, 2001). Simulations were performed using Seq-Gen version 1.3.2 (Rambaut and Grassly, 1997). For each of the 500 simulated datasets, two ML trees were inferred, one without and one with the null constraint enforced, by heuristic searches using PAUP*, starting from NJ trees and using TBR branch swapping. The probability of correctly rejecting any null hypothesis was obtained by comparing the observed δ to the distribution of δ obtained from the 500 simulated datasets.

2.5. Divergence time estimation

We employed a likelihood ratio test (LRT) to evaluate whether the DNA sequence data were significantly unlikely under the assumption of rate constancy of molecular evolution (Felsenstein, 1981). Significance of the LRT was evaluated assuming that the expectation of twice the absolute value of the difference in support (−Ln) under the clock versus the nonclock model is χ^2_{n-2} distributed, where n equals number of sequences in the dataset (Felsenstein, 1981). For the LRT test, $n-2$ degrees of freedom comes from the $2n-3$ independent branch lengths on the unconstrained tree minus the $n-1$ independent branch lengths on the clock-enforced tree. To ensure that the two models (with and without clock) were truly nested hierarchically the topology we inferred from the unconstrained ML tree search was used as a complete constraint tree during the searches under the enforced clock model.

To estimate divergence times across the phylogeny we applied one molecular clock method (utilizing only the genetic distance information) and one relaxed-clock approach (relying upon the phylogenetic tree). First, we applied a 1.91% rate of total divergence per million years for model-corrected amphibian (Anura: Hyloidea) mtDNA sequence divergence (Crawford, 2003a,b). This rate was obtained by applying a model-based correction to the ND2 data used in Macey et al. (1998) to estimate a molecular clock in toads based on a 10 million year old calibration point (see also Macey et al. (2001) for a comparison of rates among vertebrates). This recalibrated rate was then applied to model-corrected genetic distances (plus/minus two standard errors), because uncorrected genetic distances may bias divergence time estimates towards the calibration point (Arbogast et al., 2002). Genetic distances were calculated as net divergences, i.e., total divergence between two clades minus the mean within-clade polymorphism (Nei and Li, 1979), and standard errors calculated by bootstrap with 500 replicates using MEGA version 3.0 (Kumar et al., 2004). Second, we employed nonparametric rate smoothing (NPRS; Sanderson, 1997) to create an ultrametric tree using TreeEdit version 1.0 alpha 10 (Rambaut and Charleston, 2001), which we then calibrated by assuming that the most recent common ancestor (MRCA) of *Pristimantis* and *Craugastor* diverged either 33.4 or 62.4 Ma, thus covering the 95% confidence interval estimated by Heinicke et al. (2007) using mitochondrial and nuclear gene sequences. Note, Heinicke et al. (2007) did not include either *P. ridens* or *P. cruentus* in their divergence time analysis, so we could not use a more recent calibration point.

3. Results

3.1. Sequence analysis

We sequenced and aligned a total of 1455 base pairs (bp) from the ND2 gene and adjoining WANCY tRNA region in 33 *P. ridens* individuals, 7 other described species, 3 undescribed species, and one outgroup (Table 1). We infer that the sequences represented functional mitochondrial DNA based on the following observations. The inferred ND2 amino acid sequence contained no premature stop codons. The light strand shows strongly biased nucleotide frequencies as observed previously in animal mtDNA (e.g., Macey et al., 1998; Sperling and Hickey, 1994). The inferred secondary structure of the five completely sequenced tRNA genes appeared functional. However, the $tRNA^{CYS}$ gene from ingroup samples lacked the D-stem, as has been reported previously in certain lineages of squamate reptiles (Macey et al., 1997b,c). We discarded 45 sites including sites with gaps and adjacent sites in tRNA loops containing gaps, leaving a fragment of 1410 bp that was used in all phylogenetic analyses. These data included a total of 838 (59.4%) variable sites and 692 (49.1%) parsimony informative sites. DNA sequence alignment and ML tree (Fig. 3) are available at TreeBASE (http://www.treebase.org/) under submission number SN3785.

3.2. Phylogenetic analyses

Estimated phylogenetic relationships among species and among geographic regions within *P. ridens* were consistent across inference methods. The MP analysis yielded nine equally parsimonious trees of 2595 steps, which differed only at nodes connecting indi-

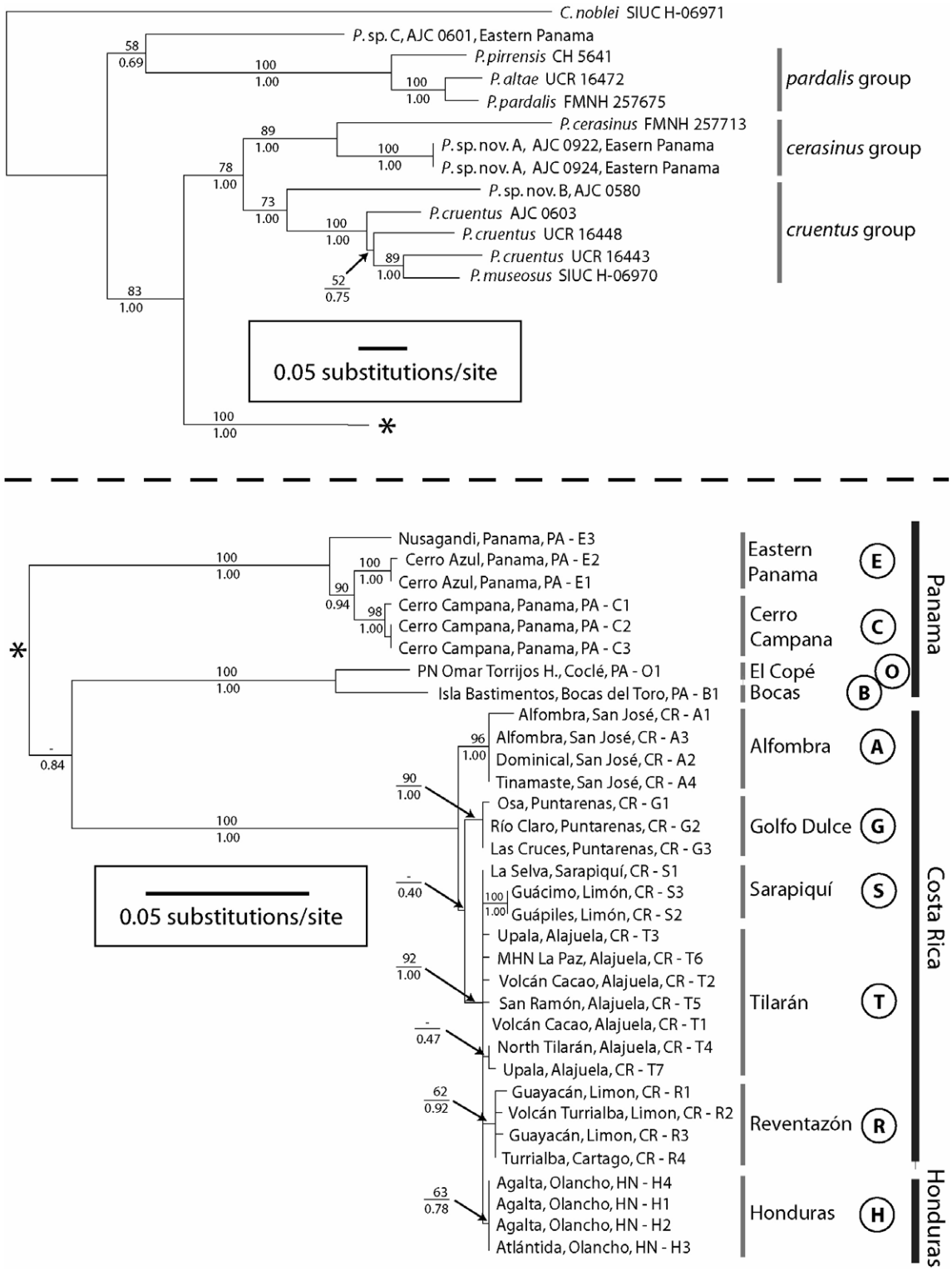

Fig. 3. Inferred phylogenetic maximum-likelihood tree for relationships among species and among haplotypes within *P. ridens* generated from analysis of 1410 bp of the ND2 gene and adjoining WANCY region of tRNA genes. Numbers below branches represent posterior probabilities; numbers above branches represent MP bootstrap support values. The * indicates the node at which the interspecific gene tree joins the *P. ridens* portion of the tree. Note the different scale bars for the separate portions of the tree above versus below the horizontal dashed line.

viduals within populations. The GTR+I+Γ model of evolution (Hasegawa et al., 1987; Tavaré, 1986; Yang, 1994) was selected as the optimal model for our dataset and was used in ML and Bayesian analyses. ML analysis yielded a tree that was topologically congruent with the inferred MP tree and Bayesian consensus tree (Fig. 3). MP bootstrap support and mpp values from the Bayesian analysis were consistently high for most nodes (Fig. 3).

Our inferred phylogeny for the *P. cruentus* group placed the Central American representative of the *P. cerasinus* group nested within our ingroup taxa and sister to *P.* sp. nov. A. We recognize this

undescribed species as a member of the *P. cerasinus* group, making it the second Central American representative of this group (Crawford et al., in prep.). Our molecular phylogeny also established the mtDNA monophyly of a group comprised of *P. altae*, *P. pardalis*, and *P. pirrensis*. *Pristimantis* sp. C (AJC 0601) from the Darien Province of eastern Panama, a specimen much like *P. ridens* in morphology, appears as the sister lineage to the *P. pardalis* group in optimal trees, but this result was not statistically supported. *Pristimantis ridens* formed the sister lineage to the remaining samples of the *P. cruentus* group plus *P. cerasinus* samples. *Pristimantis cruentus* was found to be paraphyletic with respect to *P. museosus*, supporting the idea that *P. cruentus* may represent a complex of species (F. Bolaños, pers. comm.; R. Ibáñez, pers. comm.).

Our results suggest that *P. ridens* may not occur in eastern Panama. We sampled along two elevational transects in the Darien province (empty red dots in Fig. 1) and found specimens morphologically and ecologically similar to *P. ridens* (*P.* sp. C aff. *ridens* and *P.* sp. nov. A) at either site. However, these samples were phylogenetically very distant from *P. ridens*. Pending molecular data from Colombian samples, we posit that *P. ridens* may be a Central American endemic.

Within *P. ridens*, we encountered a basal trichotomy of three highly divergent and statistically well-supported lineages (Fig. 3). The eastern lineage was comprised of samples from either side of the Panama Canal (regions C and E in Fig. 2). The central lineage contained one sample from El Copé (region O) in central Panama and one from Bocas del Toro (region B) on the Caribbean coast of Panama near the Costa Rican border. The third lineage contained all Costa Rican samples from both coasts plus all Honduran samples. Average pairwise genetic distance between these three conspecific lineages was 14% uncorrected or 25% corrected divergence (Table 2).

Within the Costa Rica + Honduras lineage we find a basal trichotomy comprised of two lineages from the Golfo Dulce region of southwestern Pacific Costa Rica (regions A and G in Fig. 2) plus one lineage containing all samples from the Tilarán (T) mountain range, the Caribbean coast of Costa Rica (S and R) and Honduras (H). Net genetic divergence among these three lineages averaged 1.5% (Table 2).

The Tilarán plus northern Caribbean coast clade (regions TSRH in Fig. 2) represented the largest geographic extent of any clade, yet the mean divergence among haplotypes was <0.5%. The parsimony network united all TSRH haplotypes to the exclusion of all other samples, and identified sample T1 from Tilarán (Fig. 2) as the ancestral haplotype (Fig. 4). Minimal genetic divergence coupled with a wide geographic distribution suggests that this clade could represent a continuous or recently expanded population. In support of this inference, the R_2 test statistic for population expansion rejected the equilibrium neutral model for the TSRH clade (R_2 = 0.0736; 95% C.I. = 0.08394–0.20371; p = 0.0053).

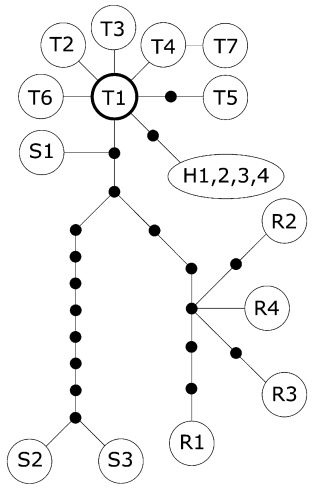

Fig. 4. Haplotype network based on 95% connection probability using parsimony criterion for *P. ridens* samples from northern and Caribbean Costa Rica and Honduras (regions TSRH in Fig. 2). Other haplotypes could not be reliably positioned in this network due to their large genetic divergence. Haplotypes represent 1410 base pairs of the ND2 gene and adjoining tRNA genes. Haplotype T1 was inferred as the ancestral haplotype, suggesting that demographic expansion may have proceeded eastward from Tilarán (Fig. 2).

3.3. Tests of alternative topologies

The tree inferred from our phylogenetic analyses (Fig. 3) matched most closely the prediction set forth by the 'Pacific Route' model for the relationships among Costa Rican samples (Fig. 2). The topology is consistent with this hypothesis and is supported by bootstrap values >95%. Using the SOWH test, we failed to reject the 'Pacific Route' hypothesis while soundly rejecting the other three hypotheses (Fig. 5). The 'Caribbean Route' hypothesis is rejected because the Tilarán samples grouped with the Caribbean samples from the S and R regions and not with the Golfo Dulce samples (A and G). The strongly supported monophyly of Costa Rican samples clearly rejects the predictions of both Bicoastal Route models (Fig. 5). Because the predictions of the Bicoastal models matched the predictions made by vicariance due to the uplift of the Talamanca mountains, this model was also rejected.

3.4. Divergence time estimates

The LRT test of rate constancy showed that a molecular clock was not compatible with our data. These results combined with the lack of fossils or vicariance events of known age in the history of *P. ridens* precluded precise estimation of divergence times using a molecular clock. We estimated divergence times, therefore, using

Table 2
Measurement of net genetic divergence (Nei and Li, 1979) between collecting regions

	E	C	O	B	G	A	R	S	T	H
E		0.046	0.146	0.138	0.157	0.158	0.156	0.159	0.157	0.157
C	0.014		0.151	0.139	0.154	0.157	0.154	0.157	0.155	0.154
O	0.232	0.246		0.013	0.148	0.152	0.153	0.155	0.152	0.15
B	0.232	0.239	0.053		0.14	0.144	0.144	0.146	0.144	0.143
G	0.23	0.232	0.263	0.273		0.016	0.013	0.014	0.014	0.014
A	0.234	0.236	0.263	0.271	0.015		0.017	0.017	0.017	0.016
R	0.243	0.246	0.273	0.276	0.018	0.018		0.002	0.003	0.004
S	0.24	0.244	0.267	0.273	0.015	0.018	0.006		0.004	0.004
T	0.237	0.244	0.262	0.267	0.014	0.017	0.005	0.003		0.002
H	0.244	0.252	0.271	0.276	0.015	0.017	0.005	0.004	0.003	

Mean net distances between groups are below the diagonal (lower-left), using a Tamura and Nei (1993) nucleotide substitution model, homogenous pattern among lineages, and different rates among sites with $\alpha = 0.5$. Uncorrected mean net distances between groups are above the diagonal (upper-right), using a p-distance nucleotide substitution model, homogenous pattern among lineages, and uniform rates among sites. Letters correspond to collecting regions defined on Fig. 2 and used throughout.

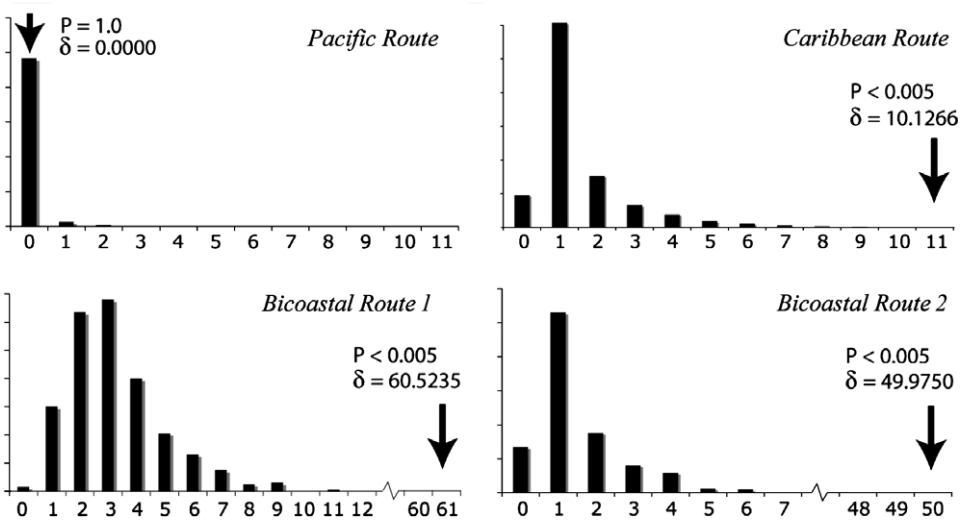

Fig. 5. Results of SOWH test for each of the *a priori* hypotheses shown in Fig. 2. For each of the four hypotheses, the test statistic, δ, equals the difference in −Ln between the ML tree inferred under the constraint topology (Fig. 2) versus the unconstrained ML tree (Fig. 3). The null distribution of δ was obtained through phylogenetic analyses of 500 simulated datasets (see text).

NPRS and the findings of Heinicke et al. (2007), while reporting molecular clock results for comparative purposes. The ages of deeper nodes showed general agreement between NPRS and molecular clock calibration methods, while the more nested or recent nodes appeared older using NPRS relative to the traditional clock method. The average divergence time from the initial radiation (the basal node) of *P. ridens* dated to the Mid- to Early-Miocene 12–22 Ma (or Late to Mid-Miocene 10–16 Ma using the molecular clock method). The divergence time for the radiation of the MRCA of all Costa Rican samples dated to the Pliocene 2.9–5.3 Ma (or Pleistocene 0.66–1.4 Ma by the clock method). The radiation from the Tilarán mountains outward across the Caribbean lowlands dated to the Late Pliocene 1.8–3.4 Ma (or Late Pleistocene 80,000–400,000 years ago by the clock method).

4. Discussion

We found that *P. ridens* has a unique dispersal history that can illustrate much about the environmental history of lower Central America as well as the role of the Isthmus in the diversification of lineages prior to and during the time of the Great American Biotic Interchange (GABI; Stehli and Webb, 1985). Although *P. ridens* sustains a relatively continuous current range along the Caribbean coast and is often locally abundant within wet forest sites across lower Central America, our mtDNA phylogeny reveals very deep genetic divergences over very short geographic distances and between sites that contain no obvious geographic barrier. However, the haplotype network and R_2 test demonstrate that at least one lineage of *P. ridens* is demographically capable of rapid, long-range dispersal. Thus, the history of *P. ridens* displays a mixture of lineages showing long-term geographic stasis (Panama) as well as recent and rapid geographic expansion across the landscape (Costa Rica to Honduras).

4.1. Phylogeny of P. cruentus group

Our phylogeny agrees with that of Miyamoto (1984), based on allozymes, in that we place *P. cruentus* with *P. cerasinus* relative to the other samples. These results conflict with current taxonomy. Both Savage (2002) and Lynch and Duellman (1997) place *P. cerasinus* in a separate *P. cerasinus* species group, while *P. cruentus*, *P. ridens*, and *P. pardalis* are placed together in the *P. cruentus* species group (Savage, 2002) or the *P. unistrigatus* group (Lynch and Duellman, 1997). Both taxonomies place *P. cerasinus* apart based upon the long third toe relative to the length of the fifth toe. Our molecular data suggest that this toe character, one of the few morphological characters available to build our taxonomy of *Pristimantis* and other eleutherodactyline frogs, may be subject to more homoplasy than previously thought. Some of the conflict between traditional taxonomy and our phylogeny we can relieve by recognizing the *P. pardalis* species group as distinct from the *P. cruentus* group (Ibáñez and Crawford, 2004). However, the *P. cruentus* group is still not a monophyletic group unless at least three strictly South American species are included (Heinicke et al. 2007).

Our interspecific phylogenetic results confirm the presence of various cryptic species in the Darien Province of eastern Panama. For example, sample AJC 0601 from central Darien (Cana) is very similar to *P. ridens* in gross morphology, but shows roughly 50% model-corrected genetic distance relative to the other samples. *Pristimantis* sp. nov. A from Pacific Darien is also very similar to *P. ridens*. As the molecular data confirm, we were unable to find *P. ridens* at either site in the Darien. The inferred absence of some reptiles and amphibians from Darien may be related to the complex biogeography of the region and not simply an artifact of insufficient collecting (Myers et al., 2007).

4.2. Origin of P. ridens

We refrain from making biogeographic inferences concerning the early evolution of the *P. cruentus* group because the group as a whole is not monophyletic with respect to South American species (Heinicke et al., 2007). Focusing on results for intraspecific samples, however, allows us to make several useful biogeographic inferences. Clearly the taxon *P. ridens* is very old, and while the intraspecific basal divergence time of 12–22 Ma seems extreme, it matches the 27–15 Ma divergence estimated for a widespread *Pristimantis* species from Ecuador (Elmer et al., 2007). Thus, our data show that *P. ridens* already occupied the isthmian landscape prior to the completion of the isthmian land bridge 2.8–3.1 Ma (Coates and Obando, 1996). Other poikliothermic taxa of South American origin believed to have entered Central America prior to 3.1 Ma include primary freshwater fishes (Bermingham and Martin, 1998), túngara frogs (Weigt et al., 2005), and vipers (Zamudio and Greene, 1997).

While the diversification of *P. ridens* apparently predated even the time of interchange between continents for raccoons and giant ground sloths, as estimated from the fossil records (Marshall et al., 1979; Webb and Rancy, 1996), it happens to coincide with the presence of a community of large mammals in central Panama very similar to that found contemporaneously in North America. This fossilized mammal community suggests that central Panama was the terminus of a long-peninsula stretching south and eastward (Whitmore and Stewart, 1965; Kirby and MacFadden, 2005). Clearly, this landmass supported northern elements of the modern-day frog fauna during the Miocene (Savage, 1982; Crawford and Smith, 2005; Crawford et al., 2007), and it may have received anuran colonists from South America, such as *P. ridens* and the túngara frog (Weigt et al., 2005), by the Late Miocene. *Pristimantis ridens* could have arrived in Central America before the completion of a land bridge by rafting (e.g., Vences et al., 2004), or during a period of low sea levels during the end of the Miocene when the sea level was roughly 60 m below today's level (Bermingham and Martin, 1998; Perdices et al. 2002).

4.3. Early expansion of P. ridens

Once in Central America, *P. ridens* underwent a first expansion during the Miocene 12+ Ma, with three separate lineages covering eastern Panama, western Panama, and some portion of Costa Rica. Which region harbored the more ancestral lineage we cannot determine, but we can make the following parsimonious inference. Since Panama (sampling regions B, O, C, and E in Fig. 2) contains two of the three old lineages of *P. ridens* while Costa Rica + Honduras (sampling regions A, G, S, R, and T and H in Fig. 2) contain only one, we infer that Panama, specifically Central Panama as suggested by the ML topology (Fig. 3), contained the ancestral lineage. This inference also conforms to our prior expectation of a colonizing lineage from South America (e.g., Duellman, 2001; Vanzolini and Heyer, 1985).

4.4. Origin of Golfo Dulce P. ridens

Given the above results, we infer that *P. ridens* occupied at least some portion of Costa Rica by 12 Ma. The monophyly of all Costa Rican + Honduran samples relative to Panamanian samples clearly indicates that that the two coasts of Costa Rica were not colonized independently from Panama (Fig. 5). Because the SOWH test, network, and R_2 test results together suggest that, within Costa Rica, *P. ridens* moved from the Pacific to the Caribbean (Fig. 5), the Golfo Dulce population on the Pacific coast was probably the source of the entire Costa Rica + Honduras clade. Other lineages within the Golfo Dulce have also been shown to be old: in another eleutherodactyline frog, *C. crassidigitus*, with a similar distribution to *P. ridens*, populations of the Golfo Dulce region were estimated to have originated 4–8 Ma (Crawford et al., 2007).

4.5. Recent expansion of P. ridens

Pristimantis ridens shows an interesting pattern of low intra-lineage variation in the Costa Rica + Honduras clade, coupled with very high inter-lineage divergence between this clade and the two Panama clades (Fig. 3). We interpret the appearances of three particular polytomies in our phylogenetic tree as evidence of hard polytomies at these junctures. We encountered a trichotomy at the root of *P. ridens*, a trichotomy at the root of the Costa Rica + Honduras clade, and a massive polytomy at the base of the TSRH clade. Our contention is that the lack of resolution is not due to homoplasy, but rather to a minimal number of substitutions falling on these internodes, or essentially, that the time between splitting events was very brief relative to the mutation rate. Thus, rather than an equilibrium panmictic population in Caribbean Costa Rica and Honduras, our evolutionary genetic analyses are consistent with the low divergence within this clade resulting instead from a recent and rapid demographic expansion through this region. This recent expansion may serve as a model for how the basal expansion may have taken place across Panama and Costa Rica 12+ Ma.

In contrast to *P. ridens*, *C. crassidigitus*, a member of a Central American genus and species group, shows no sign of ancient or recent demographic expansion, even though these two species are both eleutherodactyline frogs and show similar geographic distributions (Crawford et al., 2007). *Craugastor crassidigitus* showed very high levels of genetic divergence between the Caribbean lowlands (region R, Fig. 2) and Tilarán (T), as well as between versants within Costa Rica, whereas *P. ridens* showed relatively greater genetic divergence within central Panama. Thus, while a common environment may be what shapes these two species' geographic ranges they filled this common space by different routes at very different times.

4.6. Demographic history

This leads us to consider why *P. ridens* was able to move between Panama and the Golfo Dulce region 12 Ma, but only much more recently spread into Caribbean Costa Rica and Honduras. For an organism with the habitat specificity of *P. ridens*, we would have expected a dispersal route favoring more continuous and wet habitat of the Caribbean coast. The patches of drier forest along the Pacific coast (Fig. 1) made the Pacific dispersal route the least expected among the *a priori* hypotheses (Fig. 2). However, our analysis actually revealed very deep genetic structuring along the Caribbean coast, roughly corresponding to the present-day international border between Panama and Costa Rica. This deep phylogeographic break has been noticed earlier in frogs and other taxa, and recently dubbed the "Bocas Break" (Crawford et al., 2007). There is no obvious geographic barrier in this region, although the mountains do come rather close to the sea at this point (Fig. 1).

The deep genetic break along the continuous Caribbean habitat could be due to a cryptic barrier or the relict of a historical barrier that is now absent but existed previously (e.g., Cheviron et al., 2005; Patton and Da Silva, 2005). The latter possibility would indicate that current variation in Central American frogs stems at least partly from historical geography. Once established, the persistence of genetic differentiation across the "Bocas Break" could be explained by a priority effect stemming from the establishment of large populations in these regions. By this effect, established populations will either occupy niche space and exclude immigrants deterministically, or stochastically overwhelm immigrant haplotypes with the numerical superiority of the resident haplotypes (Reeves and Bermingham, 2006). Either mechanism makes the fixation or increased frequency of a migrant haplotype unlikely as long as two conditions are met: (1) that the resident population inhabited the region prior to immigration from other populations, and (2) that the resident population is large relative to the number of immigrants. Such a model may also explain the very large genetic break between the proximal populations at Cerro Campana (region C in Fig. 2) and Omar Torrijos NP (region O). The intervening valley where considerably fewer individuals are found would keep the migration rates low relative to the local population sizes at either site. The "priority effect" hypothesis would predict the existence of a zone of secondary contact along the Caribbean coast between Costa Rican and Panamanian *P. ridens* lineages, which could be tested with finer-scale geographic sampling.

4.7. Environmental history

Our study has provided us with an opportunity to infer the history of the environmental landscape in lower Central America from phylogeographic data. The inferred connection between western

Panama and the Golfo Dulce region supports the previous existence of a dispersal corridor of wet forest habitat in western Pacific Panama, a region that is currently dry. According to our phylogeographic data from wet forest frogs, this corridor disappeared 12+ Ma (this study) or 4–8 Ma (Crawford et al., 2007). This region had become dry forest >4 Ma according to plant macrofossils from central Panama (Graham and Dilcher, 1995). Subsequently, vegetation probably cycled between savannah and dry forest (Piperno and Pearsall, 1998), but wet forest never returned. As with studies of lowland frogs of South America, our hypothesis for the local environmental history allows for dynamic ecosystem variation during the Pleistocene (e.g., Noonan and Gaucher, 2006; Carnaval and Bates, 2007), but we infer that the spatial and temporal variation in Central America was much more bounded. In other words, we posit that during the past >4 Ma, the Golfo Dulce has remained wet while the Pacific coast of western Panama has remained dry (Piperno and Pearsall, 1998; Weigt et al., 2005). The finding that populations of *P. ridens* in Panama are quite old, substantially predating the completion of the Central American Isthmus, supports more broadly the idea that Central America was not only a corridor for interchange between North America and South America, but a staging ground for developing biodiversity as well.

4.8. Taxonomic implications

Our molecular phylogeny establishes the monophyly of a group comprised of *P. altae*, *P. pardalis*, and *P. pirrensis*. We designate this clade of morphologically and behaviorally distinctive frogs (Ibáñez and Crawford, 2004) as the *P. pardalis* species group (Fig. 3). The molecular phylogenetic results for *P. ridens* show that this taxon is comprised of three lineages separated by substantial mtDNA sequence divergence, suggesting the potential existence of two cryptic species (q.v., Fouquet et al., 2007; Stuart et al., 2006). Based on the type locality of *P. ridens* (Cope, 1866), this nominal taxon could be restricted to Costa Rica, Nicaragua, and Honduras. For the central Panama clade, the name *P. molinoi* Barbour is available (Barbour, 1928; Lynch, 1980; Savage, 1981). The type locality of *P. molinoi* is Barro Colorado Island, northeast of the midpoint between regions C and E in Fig. 2. We are unaware of any names previously associated with the western Panama lineage (samples from regions B and O in Fig. 2).

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