Biogeography of the túngara frog, *Physalaemus pustulosus*: a molecular perspective

LEE A. WEIGT,* ANDREW J. CRAWFORD,† A. STANLEY RAND† and MICHAEL J. RYAN†‡

*Smithsonian Institution, National Museum of Natural History, Washington, D.C. 20250, †Smithsonian Tropical Research Institute, Apartado 2072, Balboa, Ancón, Republic of Panama, ‡Section of Integrative Biology, 1 University Place C0930, University of Texas, Austin, TX 78712

Abstract

Physalaemus pustulosus, a small leptodactylid frog with South American affinities, ranges across northern South America through Middle America to southern Mexico. To investigate its geographic variation and evolutionary origins, we analysed the presumptive gene products of 14 allozyme loci and sequenced a portion of the mitochondrial COI gene from individuals sampled throughout the distribution. Generally, allozyme dissimilarities and sequence divergences are correlated with each other and with geographic proximity. The greatest discontinuity in genetic variation was found between populations in Middle America vs. South America + Panama. Based on two Bayesian MCMC (Markov chain Monte Carlo) divergence time estimates involving two independent temporal constraints, the timing of the separation of northern and southern túngara frog lineages is significantly older than the time since completion of the current Panama land bridge. *P. pustulosus* first invaded Middle America from South America about 6–10 million years ago giving rise to the northern lineage. The southern lineage then invaded Panama independently after land bridge completion. Despite millions of years of independent evolution, the multilocus allozyme data revealed that western Panama populations represent a contact zone containing individuals with alleles from both groups present.

Keywords: allozymes, biogeography, intergradation, mtDNA

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Introduction

The túngara frog, *Physalaemus pustulosus*, ranges from Veracruz and Yucatan in southern Mexico down the Pacific coast of Middle America and across the Caribbean coast of northern South America to Trinidad and Guyana. Many dryforest amphibians and reptiles show a similar distribution in Middle America and northern South America (Dunn 1940). The family Leptodactylidae, subfamily Leptodactylinae, and genus *Physalaemus* are largely South American (Savage 2002). The genus *Physalaemus* contains at least 45 species (Frost 2004). All but *P. pustulosus* are confined to South America and most species in this genus occur east of the Andes and south of Amazonia.

Early work on the species focused on mate recognition signals, female mating preferences, sexual selection, and life history (see Ryan 1985; Ryan & Rand 2003 for reviews).

Correspondence: Lee A. Weigt, Fax: 301 238 3059; E-mail: weigtl@si.edu

Recent investigations include genetic studies of relationships of several members of the genus (Cannatella *et al.* 1998), geographic variation in mating calls (Ryan *et al.* 1996), and microsatellite primer development (Pröhl *et al.* 2002). All of these studies assume *P. pustulosus* is a single taxonomic entity.

The last decade has seen a great increase in our understanding of the geological events culminating in the rise of the Isthmus of Panama and its effect on the biota. Reconstructed from diverse lines of evidence, the emergence took millions of years, with the seaway closing with the surfacing of the Panama land bridge in late Pliocene (Coates *et al.* 1992, 2004; Farrell *et al.* 1995; Coates & Obando 1996; Díaz de Gamero 1996 — see Table 5, p. 744 for chronology of events).

Movements of animals between North and South America in the past have been complex and their interpretation controversial (Stehli & Webb 1985). Vanzolini & Heyer (1985) suggest that the emergence of the Panama land bridge did not produce the sort of massive interchange between South

and North American herpetofaunas that is described for mammals (Webb 1985). They assert that most of the herpetofaunal interchange, including the leptodactylid frogs, took place prior to the emergence of the land bridge in Panama. Duellman (1966) and Savage (1966, 1982) express a contrasting view. They include *P. pustulosus* among the species that they think invaded Middle America from South America after the present Panama land bridge emerged. Modern-day western Panama populations of *P. pustulosus* are separated from the rest of the Central American populations by a gap in distribution in southwestern Costa Rica (Savage 2002).

In the context of the above geological history, we examined nuclear allozyme data and mitochondrial DNA (mtDNA) sequences for one species of frog that occurs in both South and Middle America. Molecular data provide additional information, along with data on present-day distributions, the fossil record, and morphology for reconstructing migration patterns and vicariant events (e.g. Bermingham & Avise 1986). Such reconstructions not only provide information about the history of the taxa under study, but also constrain interpretations of the geological history of the region.

Reconstructing the geographic origins of a given species requires knowledge of the phylogenetic relationship of that species with its nearest relatives. In their analysis of relationships among species within the *pustulosus* group, Cannatella *et al.* (1998) found that the inferred phylogenetic position of *P. pustulosus* differed among data sets. The COI data suggested that *P. pustulosus* is sister to the other six species of the *pustulosus* species group, while the morphology, 12S mitochondrial DNA sequences, allozyme data and the combined data all favoured *P. pustulosus* as the sister to the pair, *Physalaemus petersi* + *Physalaemus freibergi*. Among these data sets, only the morphology gave strong support to a particular sister relationship for *P. pustulosus*. In the present study, we revisit the relationships among species, and among populations of *P. pustulosus*.

Materials and methods

Collection sites and sampling

Figure 1 and Table 1 show the collecting localities sampled throughout the range of *Physalaemus pustulosus*. Ryan *et al.*
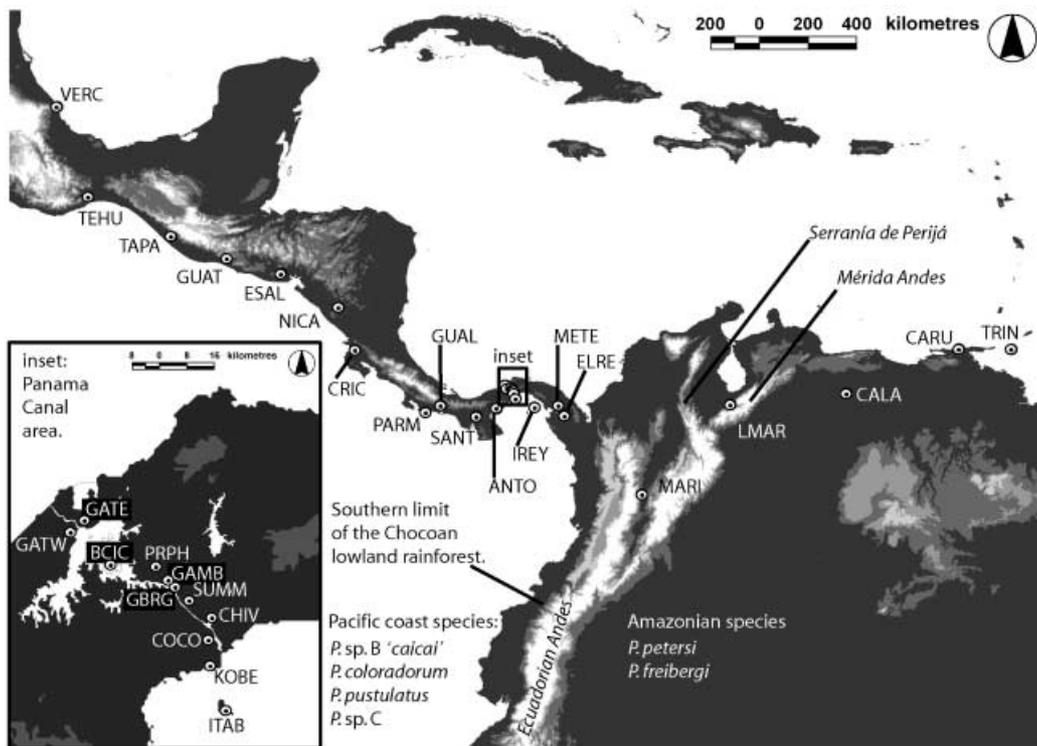
Fig. 1 Map showing collecting localities for *Physalaemus pustulosus* (circled dots) and the mountain ranges whose origins are used to constrain divergence time estimates. Each locality is labelled with the same four-letter code used in Table 1. A close-up map of the Panama Canal Zone localities (inset) is shown on the lower left. In one divergence time analysis, the Ecuadorian Andes are assumed to have split the four Pacific coast *Physalameus* from the two Amazonian species plus *P. pustulosus*. In a second, independent analysis the northern Andes, comprised of the Serran'a de Perijá and Mérida Andes, are assumed to have divided the CALA, LMAR, and MARI populations at least 2 million years ago. Note, *Physalaemus* is absent from the lowland rainforest of the Chocó. Elevation is indicated by five different shades, with the darkest shade representing 1–500 m and the lightest shade representing elevations above 2000 m.

Table 1 Populations, locality information and sample sizes of 30 populations of *Physalaemus pustulosus* analysed in this study. Right-hand columns show biosys-1 output statistics on genetic variability within populations (standard errors in parentheses)

Population	Locality information (Latitude)	(Longitude)	Sample size	Mean sample size/locus	Mean no. of alleles/locus	Percentage of polymorphic loci	Mean heterozygosity (left, direct count; right, Hardy–Weinberg expected)
VERC	Laguna Verde, Veracruz, Mexico 19.73	−96.43	11	10.9 (0.1)	1.2 (0.10)	21.40 (0.06)	0.10 (0.06) 0.10
TEHU	Tehuantepec, Chiapas, Mexico 16.35	−95.28	11	11.0 (0.0)	1.4 (0.20)	35.70 (0.05)	0.12 (0.05) 0.13
TAPA	Tapachula, Chiapas, Mexico 14.86	−92.22	16	15.0 (0.1)	1.5 (0.20)	35.70 (0.05)	0.11 (0.05) 0.11
GUAT	Taxisco, Santa Rosa, Guatemala 14.03	−90.18	10	10.0 (0.0)	1.4 (0.20)	35.70 (0.06)	0.11 (0.04) 0.10
ESAL	San Miguel, El Salvador 13.49	−88.18	12	12.0 (0.0)	1.3 (0.10)	28.60 (0.05)	0.10 (0.04) 0.08
NICA	Tipitapa, Managua, Nicaragua 12.20	−86.07	12	12.0 (0.0)	1.4 (0.20)	28.60 (0.03)	0.07 (0.03) 0.06
CRIC	Liberia, Guanacaste, Costa Rica 10.61	−85.45	11	11.0 (0.0)	1.4 (0.10)	35.70 (0.06)	0.11 (0.05) 0.09
PARM	Puerto Armuelles, Chiriqui, Panama 8.27	−82.86	15	14.9 (0.1)	1.5 (0.10)	50.00 (0.07)	0.14 (0.04) 0.12
GUAL	Gualaca, Chiriqui, Panama 8.53	−82.29	14	14.0 (0.0)	2.0 (0.30)	64.30 (0.07)	0.23 (0.06) 0.20
SANT	Santiago, Veraguas, Panama 8.13	−80.98	16	15.4 (0.6)	1.8 (0.30)	42.90 (0.06)	0.19 (0.06) 0.17
ANTO	Anton, Cocle, Panama 8.40	−80.24	15	15.0 (0.0)	1.6 (0.20)	42.90 (0.05)	0.13 (0.05) 0.12
GATW	Gatun West Bank, Colón, Panama 9.25	−79.95	14	14.6 (0.2)	1.4 (0.10)	42.90 (0.07)	0.18 (0.06) 0.17
GATE	Gatun East Bank, Colón, Panama 9.28	−79.92	15	14.9 (0.1)	1.5 (0.20)	42.90 (0.06)	0.18 (0.06) 0.19
BCIC	Barro Colorado Island, Colón, Panama 9.17	−79.85	14	13.9 (0.1)	1.4 (0.20)	28.60 (0.06)	0.11 (0.05) 0.11
PRPH	Pipeline Road, Colón, Panama 9.16	−79.73	15	14.9 (0.1)	1.5 (0.10)	50.00 (0.05)	0.13 (0.05) 0.12
GAMB	Gamboa, Colón, Panama 9.12	−79.70	30	29.6 (0.3)	1.6 (0.10)	57.10 (0.06)	0.13 (0.05) 0.12

Table 1 *Continued*

Population	Locality information (Latitude)	(Longitude)	Sample size	Mean sample size/locus	Mean no. of alleles/locus	Percentage of polymorphic loci	Mean heterozygosity (left, direct count; right, Hardy–Weinberg expected)
GBRG	Gamboa Bridge, Colón, Panama 9.11	−79.69	16	16.0 (0.0)	1.5 (0.20)	35.70 (0.05)	0.12 (0.06) 0.14
SUMM	Summit Gardens, Panama, Panama 9.07	−79.65	13	12.9 (0.1)	1.5 (0.10)	50.00 (0.06)	0.16 (0.06) 0.15
CHIV	Chiva Chiva Road, Panama, Panama 9.02	−79.59	15	14.8 (0.2)	1.8 (0.20)	64.30 (0.06)	0.17 (0.05) 0.17
COCO	Coco Solo, Panama, Panama 8.97	−79.59	14	15.0 (0.0)	1.6 (0.20)	50.00 (0.06)	0.15 (0.05) 0.15
KOBE	Ft. Kobbe, Panama, Panama 8.90	−79.59	14	13.8 (0.2)	1.4 (0.20)	35.70 (0.06)	0.13 (0.05) 0.12
ITAB	Isla Taboga, Panama, Panama 8.80	−79.55	14	14.9 (0.1)	1.3 (0.10)	28.60 (0.04)	0.07 (0.04) 0.07
IREY	Isla del Rey, Las Perlas, Panama 8.45	−78.85	15	15.0 (0.0)	1.4 (0.10)	35.70 (0.03)	0.05 (0.04) 0.07
METE	Metet', Darien, Panama 8.50	−77.97	15	14.9 (0.1)	1.5 (0.20)	42.90 (0.08)	0.17 (0.06) 0.13
ELRE	El Real, Darien, Panama 8.13	−77.73	15	14.4 (0.6)	1.5 (0.10)	50.00 (0.08)	0.18 (0.06) 0.15
MARI	Mariquita, Tolima, Colombia 5.18	−74.90	15	14.1 (0.9)	1.6 (0.30)	35.70 (0.09)	0.22 (0.07) 0.18
LMAR	L. Maricaibo, Venezuela 8.56	−71.63	2	2.0 (0.0)	1.2 (0.10)	21.40 (0.06)	0.11 (0.06) 0.11
CALA	Calobozo, Guarico, Venezuela 8.98	−67.35	12	11.9 (0.0)	1.5 (0.20)	42.90 (0.07)	0.13 (0.04) 0.10
CARU	Carúpano, Sucre, Venezuela 10.64	−63.22	14	13.6 (0.2)	1.4 (0.20)	28.60 (0.06)	0.12 (0.06) 0.14
TRIN	Trinidad 10.63	−61.28	5	3.7 (0.5)	1.5 (0.20)	35.70 (0.09)	0.23 (0.07) 0.18

(1996) reported and compared geographic call dissimilarity and Nei unbiased genetic distances (D_N) for these 30 populations. The four-letter population abbreviations used here follow Ryan *et al.* (1996). Within a locality, male frogs, and occasionally females, were sampled from sites within an area radius of less than 300 m. Material from Trinidad came from Smithsonian Museum of Natural History collections. For this study one individual, of the 15 animals per site used for allozymes, was dissected in the field and flash frozen in liquid nitrogen, or prepared in the laboratory, and stored frozen at −80 °C for mtDNA analyses.

Molecular methods

Allozyme methods and results presented in Ryan *et al.* (1996) are not repeated here — only new analytical results and their methods are presented. Buffers and products resulting from standard starch gel electrophoresis (Murphy *et al.* 1990) are reported in Table 2. We consider the allozyme data to reflect genome-wide (nuclear) gene differences (Garcia-Paris *et al.* 2003). Allozyme frequency data were analysed with the BIOSYS-1 (Swofford & Selander 1981), and multidimensional scaling was performed using NTSYS-PC version 1.8 (Rohlf 1993). Maximum-parsimony phylogenetic analysis of allozyme frequencies was calculated with FREQPARS (Swofford & Berlocher 1987) and PAUP* 4.1b (Swofford 1998). Mantel tests were used to determine correlations between all matrices (MANTEL version 2.0, Mantel nonparametric calculator, Copyright 1999, Adam Liedoff). The probability of rejecting the null hypothesis was based on 1000 randomization simulations. The new comparisons not presented in Ryan *et al.* (1996) were those involving the mtDNA divergence matrix.

For mtDNA analysis, thigh muscle was minced in DNA storage buffer (Seutin *et al.* 1991). Genomic DNA was extracted from a 100 mg wet-weight portion of tissue with the Gnome DNA extraction kit (BIO 101)) with all kit volumes reduced to one-third volume. The final resuspension was in 100 μL of sddH$_2$O. Polymerase chain reaction (PCR) thermal cycler profiles, methods, and primers are listed in Appendix II. Double-strand sequences were unambiguously aligned with SEQUENCHER (Gene Codes).

In addition to the 30 *P. pustulosus* samples obtained here, the final data set also included nine (one sample from each) of the following members of the *P. pustulosus* species group: *Physalaemus colouradorum*, *Physalaemus pustulatus* (Ecuador) *P.* sp. B ('caicai'), and *P.* sp. C, *Physalaemus petersi* and *Physalaemus freibergi* (Cannatella *et al.* 1998). These latter two species comprise the sister clade to *P. pustulosus* in all data sets of Cannatella *et al.* (1998) except COI. We also included as outgroups one COI sequence each from *Physalaemus enesefae*, *Physalaemus ephippifer*, and *P.* sp. A (Roriama, Brazil). Choice of outgroup was based on Cannatella & Duellman (1984) and Cannatella *et al.* (1998).

Name usage follows Ryan *et al.* (1996) and Cannatella *et al.* (1998), but names of *P. pustulatus* from west of the Andes are in the process of being changed by Ron *et al.* (2004; 2005).

Analytical methods

The 39 aligned COI sequences were subjected to the following phylogenetic analyses. Fitch (1971) parsimony (FP) trees were inferred from heuristic searches using PAUP* 4.0b10 for Unix (Swofford 1998). Starting trees were obtained by stepwise addition of taxa in random order in 50 000 replicate searches, with a maximum of 5000 trees used for tree-bisection–reconnection (TBR) branch swapping in each replicate. Clade support in parsimony trees was evaluated by two methods. First, we employed the bootstrap procedure (Felsenstein 1985) using 2000 replicates with five random addition sequences and TBR branch swapping in each replicate, with a 1000-tree limit placed on each replicate. Second, we calculated decay indices (Bremer 1988) for each node using TREEROT version 2c (Sorenson 1999) and PAUP*.

Maximum-likelihood (ML) inference began with a test of significant heterogeneity in base frequencies in the COI data across n taxa using a $\chi^2[3(n-1)]$ test. Fifty-six models of sequence evolution were then evaluated using three criteria: by hierarchical likelihood-ratio test (hLRT; Felsenstein 1981) and Akaike information criteria (AIC; Akaike 1974), both implemented in MODELTEST version 3.5 (Posada & Crandall 1998), and by the Bayesian information criterion as implemented in the program DT-MODSEL (version 13-Aug-02) by Minin *et al.* (2003). This latter technique tends to suggest simpler models than the former two criteria. Because branch lengths are an integral part of phylogeographic analyses, we would prefer that model suggested by the DT method over the hLRT or AIC model should they differ. In all cases, ML models were evaluated and parameters calculated from neighbour-joining (NJ) trees (Saitou & Nei 1987). In MODELTEST, this was based on JC69 distances (Jukes & Cantor 1969), whereas DT-MODSEL used an NJ tree based on LogDet distances (e.g. Lockhart *et al.* 1994). We also re-ran MODELTEST starting from a consensus topology of the most parsimonious trees.

ML inference also used heuristic searches and TBR swapping, but started from NJ trees. ML analyses used the model/s suggested by the above criteria and assumed the given parameter values. These analyses were repeated using full searches in which both the topology and the parameter values for the given model/s were estimated. We tested for evolutionary rate heterogeneity across the phylogeny by a LRT test comparing the difference in support between trees with and without a molecular clock enforced. Rather than attempt a bootstrap analysis of clade support on ML trees, we conducted Bayesian MCMC

Table 2 Allele frequencies of polymorphic loci in 30 populations of *Physalaemus pustulosus*. Three gene products (ME, SOD1, SOD2) were invariant for all populations. Horizontal shading indicates (non-rare) alleles showing intergradation and vertical double lines show the western Panama populations where the intergradation occurs

Gene product	Allele	VERC	TEHU	TAPA	GUAT	ESAL	NICA	CRIC	PARM	GUAL	SANT	ANTO	GATW	GATE	BCIC	PRPH	GAMB	GBRG	SUMM	CHIV	COCO	KOBE	ITAB	IREY	METE	ELRE	MARI	LMAR	CALA	CARV	TRIN
Sample size	(*N*)	11	11	16	10	12	12	11	15	14	16	15	14	15	14	15	30	16	13	15	15	14	14	15	15	15	15	2	12	14	5
Phosphogluconate dehyrogenase																															
6PGDH	A																											0.03				
	B																									0.14		0.07				
	C										0.81	0.63	0.67	0.46	0.73	0.80	0.83	0.62	0.58	0.46	0.63	0.58	0.03	1.00	0.54	0.50	0.60				
	D					0.04			0.04																		0.30				
	E		0.18			0.04					0.06	0.33	0.33	0.54	0.27	0.20	0.17	0.38	0.42	0.54	0.37	0.42	0.97		0.32	0.50		0.75	0.04		0.50
	F								0.07																			0.25	0.96	1.00	0.50
	G	1.00	0.82	0.97	0.90	0.79	0.75	0.36	0.93	0.96	0.13	0.03																			
	H		0.03	0.10	0.21	0.17	0.64																								
Aspartate transaminase																															
ATA	A	1.00	1.00	1.00	1.00	0.88	1.00	1.00	0.82	0.82	0.34	0.37	0.30	0.23		0.03	0.12	0.16	0.08	0.23	0.17	0.18			0.03	0.03	0.10		0.05		
	B																	0.03				0.07									0.20
	C					0.12			0.18	0.18	0.59	0.63	0.70	0.77	1.00	0.97	0.88	0.81	0.92	0.77	0.83	0.75	1.00	1.00	0.97	0.97	0.90	0.75	0.86	0.46	
	D										0.06																	0.25	0.09	0.54	0.80
Triose phosphate isomerase																															
TPI	A	0.50		0.03																											
	B																								0.27	0.50			0.17		
	C										0.22	0.03																			
	D	0.50	0.86	0.84	0.95	1.00	1.00	1.00	0.17	0.39											0.07	0.07									
	E																														0.20
	F												0.03	0.30	0.77	0.32	0.25	0.30	0.56	0.54	0.53	0.57	0.36	0.17	0.20						
	G				0.05																										
	H		0.14	0.13					0.83	0.61	0.72	0.93	0.70	0.23	0.68	0.75	0.70	0.44	0.46	0.40	0.37	0.64	0.83	0.80	0.73	0.50	1.00	1.00	0.83	1.00	0.80
	I										0.06																				
Lactate dehydrogenase																															
LDH1	A											0.07																			
	B		0.23	0.12	0.55		0.04		0.90	0.86	0.70	0.90	1.00	1.00	1.00	0.97	0.95	1.00	1.00	1.00	0.97	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
	C	0.41	0.73	0.88	0.45	1.00	0.96	1.00	0.10	0.14	0.30	0.03					0.03	0.05			0.03										
	D	0.59	0.04																												
LDH2	A		0.14	0.03																											
	B						0.04						0.03								0.03										
	C	1.00	0.86	0.66	1.00	1.00	1.00	0.96	1.00	1.00	1.00	1.00	0.97	1.00	1.00	1.00	1.00	1.00	1.00	0.97	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
	D			0.31																											

Table 2 *Continued*

Gene product	Allele	VERC	TEHU	TAPA	GUAT	ESAL	NICA	CRIC	PARM	GUAL	SANT	ANTO	GATW	GATE	BCIC	PRPH	GAMB	GBRG	SUMM	CHIV	COCO	KOBE	ITAB	IREY	METE	ELRE	MARI	LMAR	CALA	CARV	TRIN
Sample size	(N)	11	11	16	10	12	12	11	15	14	16	15	14	15	14	15	30	16	13	15	15	14	14	15	15	15	15	2	12	14	5
Phosphoglucomutase																															
PGM	A					0.08																									
	B				0.05		0.08												0.04	0.03											
	C									0.04																					
	D	1.00	1.00	1.00	0.90	0.92	0.92	0.96	0.90	0.96	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.96	0.97	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.25	0.04	0.36	
	E				0.05			0.04	0.10																			0.75	0.96	0.64	1.00
Glucose phosphate isomerase																															
GPI	A																								0.03						0.10
	B																								0.57				0.12		0.20
	C	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.07	0.50	1.00	1.00	1.00	1.00	1.00	1.00	0.98	1.00	1.00	0.90	1.00	1.00	1.00	0.97	1.00	0.80	0.40	1.00	0.88	1.00	0.60
	D									0.04										0.10											
	E									0.04							0.02							0.03		0.20					0.10
	F								0.93	0.39																					
	G									0.04																					
Malate dehyrogenase																															
MDH1	A																													0.04	
	B																			0.03										0.25	
	C	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.54	0.53	0.93	0.90	0.98	0.97	0.85	0.87	0.97	0.93	0.96	0.97	0.97	0.93	1.00	1.00	1.00	0.71	1.00
	D												0.46	0.47	0.07	0.10	0.02	0.03	0.15	0.10	0.03	0.07		0.03	0.03	0.07					
MDH2	A											0.23		0.06																	
	B									0.36				0.17						0.17				0.03		0.03	0.40				
	C	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.64	0.88	0.77	1.00	0.77	1.00	0.93	0.97	1.00	0.92	0.97	0.83	1.00	1.00	0.97	0.93	0.97		1.00	1.00	1.00	1.00
	D										0.03																				
	E										0.06					0.07	0.03			0.08	0.03				0.07		0.60				
	F										0.03																				
Isocitrate dehydrogenase																															
ICD1	A																			0.03											
	B	0.25	0.50	0.25	0.20	0.29	0.13	0.36	0.47	0.14	0.78	0.97	0.29	0.60	0.68	0.57	0.55	0.53	0.62	0.87	0.83	0.96	0.37	0.27	0.50	0.57	0.50		0.46	0.36	0.50
	C								0.07						0.03																
	D	0.75	0.50	0.75	0.80	0.71	0.87	0.64	0.53	0.71		0.03	0.71	0.40	0.29	0.43	0.45	0.44	0.38	0.13	0.17	0.04	0.63	0.73	0.50	0.43	0.50	1.00	0.54	0.64	0.50
	E									0.07	0.22																				
ICD2	A						0.04			0.04																					
	B	1.00	1.00	1.00	1.00	1.00	1.00	0.96	1.00	0.96	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00

(Markov chain Monte Carlo) analyses of phylogenetic relationships (Rannala & Yang 1996; Yang & Rannala 1997) using MRBAYES version 3.0b4 for Unix (Huelsenbeck & Ronquist 2001). Each MCMC analysis involved six chains with default heating. After a conservative burn-in period of 1001 generations, 9000 trees were sampled at a frequency of one per 200 generations.

To estimate divergence times, we employed the highly parametric Bayesian MCMC method of Thorne *et al.* (1998; Kishino *et al.* 2001) as implemented in the software package MULTIDISTRIBUTE version 05-Aug-03 (Thorne & Kishino 2002). In applying this method to the *pustulosus* group, we compared and contrasted two different sets of assumptions based on two different geological events that may reasonably be considered independent. First, following the preferred phylogeny of Cannatella *et al.* (1998), we assumed that the rise of Ecuadorian Andes separated the ancestor of the Pacific coast species (*P. coloradorum*, *P. pustulatus*, *P.* sp. B '*caicai*', and *P.* sp. C) from the ancestor of the Amazonian species (*P. freibergi*, *P. petersi*) + *P. pustulosus*. We further assumed that this vicariant event happened at some point in the mid-Miocene, 16.4–11.2 million years ago (Ma) (Hoorn 1993; Hoorn *et al.* 1995; Gregory-Wodzicki 2000). We repeated the analysis removing the 16.4 Ma constraint on maximal age of divergence.

Second, we ran an independent analysis assuming that the northernmost Andes split ancestral *P. pustulosus* populations prior to 2.0 Ma. The eastern chain of the northern Andes terminate in a Y-shaped pattern, with the Serranía de Perijá along the Colombian border forming the western fork of the Y, and the Mérida Andes of Venezuela forming the eastern fork. Among our samples, Mariquita (MARI) lies to the west of the Andes, Lago Maricaibo (LMAR) in the centre of the 'Y', and Calabozo (CALA), Carupano (CARU), and Trinidad (TRIN) lie to the east. The northern Andes are thought to have reached their current elevation by around 2.7 Ma (Gregory-Wodzicki 2000), but the Sierra de Perijá and Mérida Andes are probably much older than the Eastern Cordillera (Hoorn *et al.* 1995). Therefore, our constraint of ≥ 2.0 Ma is conservative.

The COI sequence data in Cannatella *et al.* (1998) and the COI results in this study (see below) supported *P. pustulosus* as sister to the other six species of the *P. pustulosus* group. However, most data sets as well as the combined data set presented in Cannatella *et al.* (1998) suggested that *P. pustulosus* is the sister to the *P. freibergi* + *P. petersi* clade. Given these two alternative topologies, we chose to assume the latter hypothesis for our divergence time estimation because this topology would again bias our results towards obtaining a younger age for túngara frogs. We also assumed that our two island samples, Trinidad (TRIN) and Isla del Rey (IREY), did not share a common ancestor with any mainland sample more recently than 10 000 years ago. Priors were chosen following the guidelines

in the multidistribute documentation. Mean of the prior distribution of evolutionary rates was based on a rate calibration of 0.69% uncorrected sequence divergence per lineage per million years (Myr) from a study of Mongolian toads by Macey *et al.* (1998). To compare our non-clock divergence time estimation with a traditional molecular clock calibration approach, we also applied this rate of 0.69% to our data by averaging all pairwise uncorrected genetic distances across a particular node. Thus we report three wholly independent divergence time estimates: (i) Ecuadorian Andes splitting Pacific and Amazonian species in the mid-Miocene, (ii) northern Andes splitting Colombian and Venezuelan populations 2.0 Ma or more, and (iii) standard application of a 'frog clock'.

Results

Allozymes

Variation in the presumptive gene products of 14 loci is shown in Table 1. There are no significant differences among populations in any of the statistics. Levels of heterozygosity are close to those reported by Nevo & Beiles (1991) for seven tropical anurans (mean = 0.069 ± 0.055). Subsequent years sampling in Gamboa (GAMB) resulted in nearly identical allozyme frequencies (pooled), which suggests that the differences we observe among populations do not simply reflect year-to-year variation. The 11 polymorphic loci are presented in Table 2 as allele frequencies and in Appendix I as genotypes.

Intergradations at four putative gene loci (6PGD, LDH, AAT, and TPI) is highlighted in Table 2 and is illustrated in Fig. 2 as an ordination of the allozyme frequency data
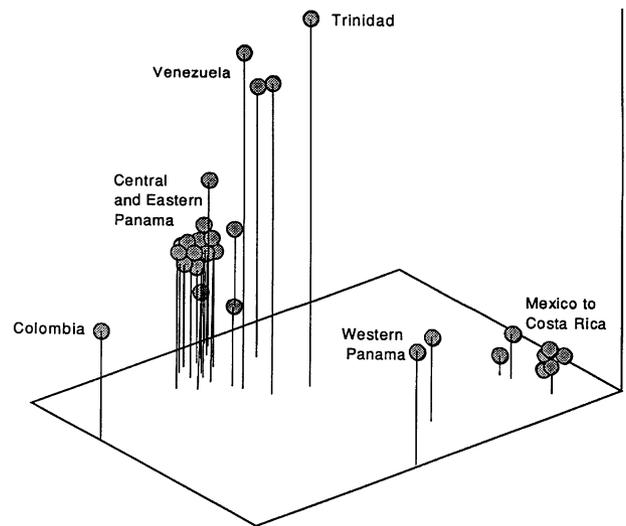

Fig. 2 Multidimensional scaling (MDS) plot of three principal ordinations for allozyme data on *Physalaemus pustulosus* populations.

Table 3 The upper portion is a group averaging of Nei's (1978) unbiased genetic distances (D_N) from allozyme data. The lower portion is a group averaging of the HKY-IG sequence divergences (*100). ***** indicate no within-area comparisons, as only one population represents the area

	Mexico to Costa Rica	Western Panama	Central Panama	Eastern Panama	Colombia	Western Venezuela	Eastern Venezuela and Trinidad
Mexico to Costa Rica	0.03 / 3.9	0.15	0.27	0.28	0.41	0.33	0.37
Western Panama	10.8	0.04 / 1.1	0.19	0.19	0.21	0.22	0.25
Central Panama	13.4	4.3	0.03 / 0.6	0.03	0.12	0.13	0.18
Eastern Panama	11.1	6.2	7.8	0.02 / 0.1	0.10	0.10	0.17
Colombia	10.7	5.5	6.4	5.7	*****	0.20	0.25
Western Venezuela	10.7	5.9	6.6	5.8	3.5	*****	0.05
Eastern Venezuela and Trinidad	13.4	6.4	6.7	6.6	5.3	5.8	0.05 / 0.9

by multidimensional scaling (MDS) (Lessa 1990). Multidimensional scaling allows populations to be shown as intermediate rather than with one group or another group (Lessa 1990). This is an advantage over a phenetic clustering, which has other shortcomings as well (de Queiroz & Good 1997). There is a mean Nei's unbiased genetic distance (D_N) of 0.294 (range 0.171–0.434) between Middle and South American + Panama populations (Table 3). This distance is 3- to 10-fold greater than the mean D_N of 0.026 and 0.083 within these two groups of populations.

Previously, a matrix of geographic distances was calculated as the shortest distance between pairs of populations (appendix A in Ryan *et al.* 1996). Here we present a Mantel test comparing the matrices of pairwise geographic and both pairwise genetic distances (only mtDNA data are new; other data from Appendix A, B, and C in Ryan *et al.* 1996), along with call dissimilarities matrix between all populations showed a strong correlation for the two measure of genetic distances, allozymes and mtDNA, with each other and with geography. Table 4 shows correlations among all matrix comparisons.

Mitochondrial DNA

The 564 bp of COI data presented here (GenBank Accession nos DQ120012–DQ120050) correspond to bases 8114–8677 of the *Xenopus laevis* mitochondrial genome (GenBank Accession no. NC_001573; Roe *et al.* 1985). All phylogenetic analyses included additional members of the *P. pustulosus* species group plus three outgroup taxa. In this alignment, 369 characters were constant, 19 sites contained singletons, leaving 176 parsimony-informative characters.

Base frequencies across taxa were not heterogeneous

Table 4 Matrix correlation table, Mantel tests using 1000 iterations. The first three rows are taken from Ryan *et al.* (1996). G, standard normal variate; Z, Mantel coefficient; R, correlation coefficient between matrices; P, probability

Comparison matrices	G	Z	R	P
Advertisement call vs. geography	4.87	571 540.52	0.502	0.001
Allozymes vs. advertisement call	5.32	211.75	0.430	0.001
Allozymes vs. geography	7.37	247 214.34	0.732	0.001
mtDNA vs. advertisement call	4.29	93.83	0.353	0.001
mtDNA vs. allozymes	9.78	14.40	0.781	0.001
mtDNA vs. geography	6.34	104 084.00	0.643	0.001

($\chi^2_{[114]} = 24.82$, $P = 1.0$). On both the NJ and the MP starting trees, the hLRT suggested the most complex, 10-parameter model, GTR + Γ + I, which assumes unequal base frequencies, an independent reversible rate for each of the six possible types of nucleotide substitutions, heterogeneity among nucleotide sites described by a discrete Γ distribution with shape parameter, α (Yang 1994), plus a proportion of invariable sites, I (Hasegawa *et al.* 1987). The AIC suggested a simpler nine-parameter model (TVM + Γ + I) that assumes a single rate for both types of transitions. Values of ML model parameters were similar when estimated from NJ versus parsimony starting trees. The decision theory (DT) technique of model selection, which seeks to minimize error branch length estimation, also recommended the Γ and I parameters, but found that only two substitution rates were sufficient in the model, one each for transitions and transversions. This corresponds to a HKY + Γ + I model (Hasegawa *et al.* 1985).

Based on the above results, we conducted six likelihood-based phylogenetic analyses. An ML search with fixed parameter values was conducted under the TVM + Γ + I model
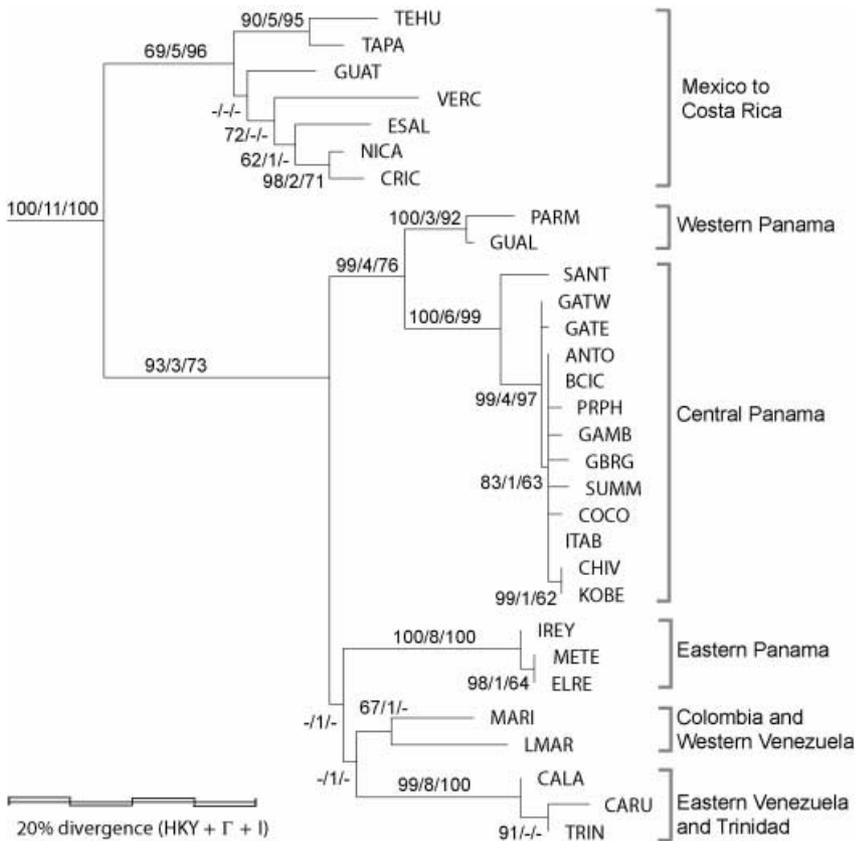
Fig. 3 Phylogeography of *Physalaemus pustulosus* population samples (indicated by four-letter abbreviations) inferred from ML analysis of COI gene sequences under the HKY + Γ + I model. Additional species of the *P. pustulosus* group and outgroups used in the analyses are not shown. Statistical support for branches is indicated by three numbers, separated by slashes. The first number shows the Bayesian marginal posterior probabilities (MPP) multiplied by 100, and assuming an HKY + Γ + I model. The middle number reflects the decay index, a.k.a. Bremer support value. The third number shows the percent parsimony bootstrap support (BSS). A dash indicates in the first position an MPP of less than one-half, in the middle position a node not found in the most-parsimonious tree, and in the second position a level of BSS less than 50%. This tree corresponds to node (2) in Fig. 4.

suggested by the AIC. Full ML and Bayesian analyses were conducted using the GTR + Γ + I model. The fixed parameter, full ML, and Bayesian analyses were repeated under the simpler HKY + Γ + I model suggested by the DT criterion. The full ML heuristic searches yielded a tree with support –Ln = 3417.31974 under the GTR + Γ + I model, and –Ln = 3422.30150 under the HKY + Γ + I model.

Figure 3 presents the ML tree obtained under the HKY + Γ + I model, with levels of statistical support obtained by Bayesian analysis under the HKY + Γ + I model, parsimony bootstrap analysis, and decay indices. Parsimony analyses recover six most-parsimonious trees of 621 steps. Only one conflicting difference is observed among ML and consensus parsimony topologies, and this involved the root node of the northwestern clade [Mexico–Costa Rica; (ML = GUAT or TEHU + TAPA; parsimony = VERC; Bayesian = trichotomy)].

Our phylogenetic results corroborate the break between northern and southern lineages of *P. pustulosus* (Ryan *et al.* 1996) and reveal significant differences among populations in western, central, and eastern Panama. The southern samples of túngara frogs can be divided into at least five or six clades. Western and central Panama form a well-supported group, whereas phylogenetic relationships among eastern Panama, Colombia, western Venezuela, and eastern Venezuela + Trinidad are unresolved (Fig. 4).

The molecular clock hypothesis was not rejected under either model of sequence evolution tested. Under HKY + Γ + I with fixed parameter values, ML tree support, –Ln = 3422.3187, clock enforced tree –Ln = 3443.334, for a difference of $21.015 \times 2 = 42.030$. Under GTR + Γ + I with fixed parameter values, ML tree –Ln = 3418.028, clock tree –Ln = 3439.333, for a difference of $21.305 \times 2 = 42.610$. With $(n-2) = 37$ degrees of freedom, $\chi^2_{(P=0.1)} = 48.363$.

All optimal trees placed the túngara frogs, *Physalaemus pustulosus*, as sister to the rest of the *pustulosus* species group (not shown), in agreement with the COI gene tree in Cannatella *et al.* (1998), although their other data sets placed the túngara frogs as sister to the *Physalaemus freibergi* + *Physalaemus petersi* sister pair. To test whether our COI topology was significantly better than one constraining the monophyly of (*P. freibergi*, *P. petersi*, all *P. pustulosus* samples), we performed a paired-sites test of Shimodaira & Hasegawa (1999) as implemented in PAUP*. The SH test used RELL optimization on ML trees obtained with fixed parameter values under both HKY + Γ + I and GTR + Γ + I models. Despite the '100% certainty' of the Bayesian analysis, the difference in –ln L support between optimal and constrained trees was nonsignificant under either model, and this also held true when the 12S data of Cannatella *et al.* (1998) was also included and re-analysed using DT-MODSEL or MRBAYES.
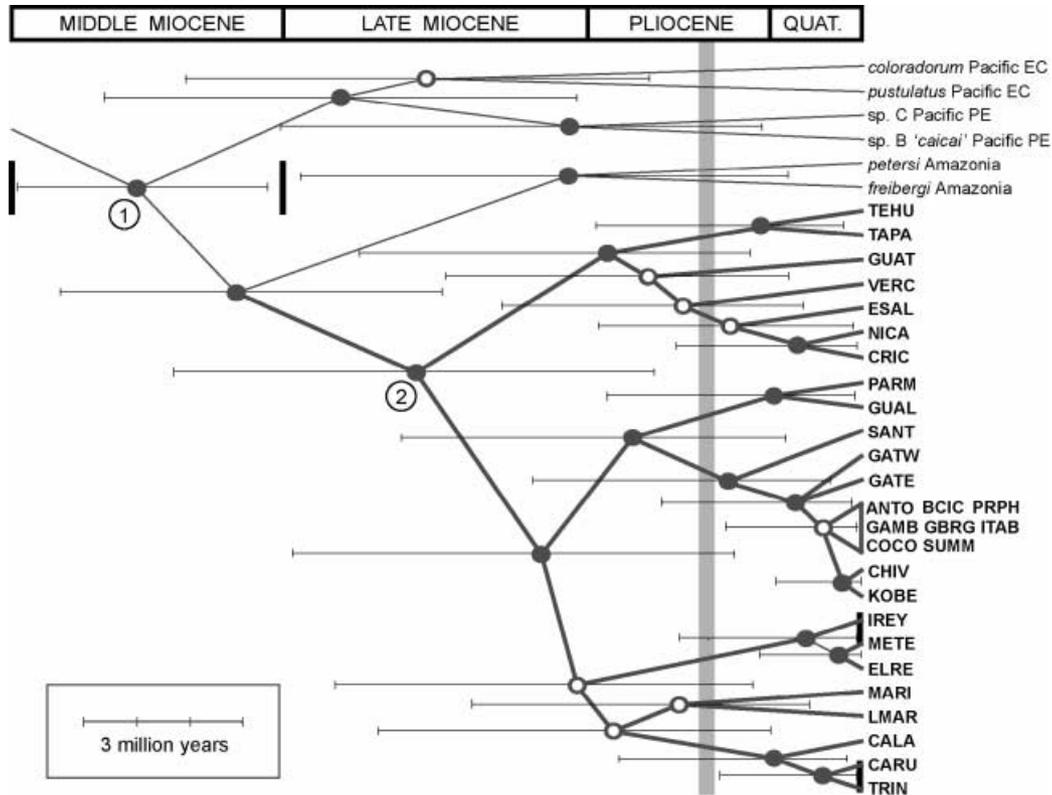
Table 5 Pairwise HKY-IG sequence divergences (× 100)

	VERC	TEHU	TAPA	GUAT	ESAL	NICA	CRIC	PARM	GUAL	SANT	ANTO	GATW	GATE	BCIC	PRPH	GAMB	GBRG	SUMM	CHIV	COCO	KOBE	ITAB	IREY	METE	ELRE	MARI	LMAR	CALA	CARU	TRIN
VERC	VERC																													
TEHU	6.7	TEHU																												
TAPA	5.9	2.2	TAPA																											
GUAT	4.4	4.5	3.8	GUAT																										
ESAL	4.3	5.4	4.4	3.1	ESAL																									
NICA	3.5	4.9	4.0	3.1	2.2	NICA																								
CRIC	4.1	5.6	4.7	2.9	2.4	0.9	CRIC																							
PARM	11.5	12.4	12.4	9.8	11.6	10.2	10.1	PARM																						
GUAL	10.9	11.7	11.1	9.1	11.0	9.7	9.5	1.1	GUAL																					
SANT	14.1	14.2	13.7	10.9	14.1	12.7	13.2	4.3	3.9	SANT																				
ANTO	13.9	14.1	13.5	10.8	14.7	12.5	13.1	4.4	3.9	1.8	ANTO																			
GATW	13.6	13.8	13.2	10.4	14.3	12.2	12.7	4.2	3.7	1.6	0.2	GATW																		
GATE	13.9	14.1	12.8	10.8	14.7	12.5	13.1	4.4	3.9	1.8	0.4	0.2	GATE																	
BCIC	13.9	14.1	13.5	10.8	14.7	12.5	13.1	4.4	3.9	1.8	0.0	0.2	0.4	BCIC																
PRPH	14.3	14.5	13.8	11.1	15.0	12.8	13.4	4.6	4.2	2.0	0.2	0.4	0.5	0.2	PRPH															
GAMB	13.9	14.1	13.5	10.8	14.7	12.5	13.1	4.6	4.2	2.0	0.2	0.4	0.5	0.2	0.4	GAMB														
GBRG	14.6	14.8	14.2	11.4	15.3	13.2	13.7	4.8	4.4	2.2	0.3	0.5	0.7	0.3	0.5	0.5	GBRG													
SUMM	13.9	14.8	14.2	10.7	14.6	12.5	13.0	4.8	4.4	2.2	0.3	0.5	0.7	0.3	0.5	0.5	0.7	SUMM												
CHIV	14.5	14.4	13.7	11.3	15.3	13.1	13.6	4.7	4.2	2.1	0.2	0.4	0.5	0.2	0.4	0.4	0.5	0.5	CHIV											
COCO	14.3	14.5	13.8	11.1	15.0	12.8	13.4	4.6	4.2	2.0	0.2	0.4	0.5	0.2	0.3	0.4	0.5	0.5	0.4	COCO										
KOBE	14.5	14.4	13.7	11.3	15.3	13.1	13.6	4.7	4.2	2.1	0.2	0.4	0.5	0.2	0.4	0.4	0.5	0.5	0.0	0.4	KOBE									
ITAB	13.9	14.1	13.5	10.8	14.7	12.5	13.1	4.4	3.9	1.8	0.0	0.2	0.4	0.0	0.2	0.2	0.3	0.3	0.2	0.2	0.2	ITAB								
IREY	12.2	11.1	10.5	10.4	11.1	9.7	10.2	6.3	5.8	6.7	7.6	7.3	7.1	7.6	7.9	7.9	8.1	7.6	7.7	7.9	7.7	7.6	IREY							
METE	12.7	11.6	11.0	10.9	11.5	10.2	10.6	6.6	6.1	7.1	8.0	7.7	7.4	8.0	8.2	8.3	8.5	8.0	8.1	8.2	8.1	8.0	0.2	METE						
ELRE	12.7	11.6	11.0	10.9	11.5	10.2	10.6	6.6	6.1	7.1	8.0	7.7	7.4	8.0	8.2	8.3	8.5	8.0	8.1	8.2	8.1	8.0	0.2	0.0	ELRE					
MARI	11.9	12.1	10.1	9.2	12.0	10.0	9.9	6.0	5.1	6.5	6.3	6.0	5.8	6.3	6.5	6.6	6.8	6.8	6.6	6.0	6.6	6.3	5.5	5.8	5.8	MARI				
LMAR	12.0	11.6	11.0	9.0	11.5	10.1	10.0	6.1	5.6	6.4	6.2	6.4	6.4	6.7	6.7	6.9	6.9	6.8	6.7	6.8	6.4	5.6	5.9	5.9	3.5	LMAR			
CALA	15.2	14.7	12.7	11.4	14.0	13.8	13.7	6.0	6.0	6.0	6.0	5.8	5.5	6.0	6.3	6.3	6.0	6.5	6.3	6.3	6.3	6.0	6.5	6.8	6.8	4.8	5.4	CALA		
CARU	14.2	15.1	13.7	11.0	13.6	13.1	12.6	6.5	7.0	7.0	7.1	6.8	6.5	7.1	7.3	7.3	7.6	7.0	7.4	7.3	7.4	7.1	6.5	6.8	6.8	5.7	6.3	1.4	CARU	
TRIN	14.9	14.4	13.0	11.0	13.6	13.4	13.3	6.2	6.7	6.7	6.8	6.5	6.3	6.8	7.0	7.0	6.8	7.3	7.1	7.0	7.1	6.8	6.2	6.5	6.5	5.5	5.6	0.5	0.9	TRIN

Fig. 4 Estimation of absolute divergence time for each node using the Bayesian MCMC method of Kishino *et al*. (2001) given the ML HKY + Γ + I topology and assuming the rise of the Ecuadorian Andes (Fig. 1) between 16.4 and 11.2 million years ago precipitated the split at the ingroup root node (1). Heavy black bars on either side of node (1) illustrate this temporal constraint. Nodes are placed according to their mean divergence time relative to the geological timescale indicated above (Quat., Quaternary). Black nodes received higher statistical support than open nodes (Fig. 4). Error bars on each node denote the central 95% of the estimated posterior probability distribution of divergence time. The long thick vertical grey bar indicates the rise of the Isthmus of Panama between 3.1 and 2.8 million years ago. Node (2) represents the split between northeast and southwest lineages of *Physalaemus pustulosus*. Note: the estimated divergence time of node (2) is roughly 1 million years older (Table 6) when the Ecuadorian Andes constraint is removed and instead the northern Andes are constrained to be at least 2 million years old (corresponding to the two nodes connecting MARI, LMAR, and CALA). The two heavy vertical black lines next to IREY and TRIN indicate the constraint that these two continental island populations diverged from their mainland relatives at least 10 000 years ago.

To avoid biasing our results towards obtaining a very old túngara lineage, we conservatively ran the Bayesian MCMC divergence time analyses on a topology corresponding to the COI ML tree (HKY + Γ + I model), but with *P. freibergi* + *P. petersi* placed as the sister lineage to *P. pustulosus*. Among all three Bayesian analyses, the youngest mean divergence times were obtained with the Ecuadorian Andes scenario with both upper and lower temporal constraints applied. These results are shown in Fig. 4. Yet even this analysis placed the 95% confidence interval (CI) of the divergence time of northern vs. southern clades of *P. pustulosus* nearly 1 Ma prior to the rise of the isthmus (3.1–2.8 Ma, Coates & Obando 1996). Under the northern Andes time constraint, the 95% CI around the basal split within the túngara clade preceded the rise of the isthmus by almost 2 Ma (Table 6). Applying a traditional molecular clock also resulted in a *P. pustulosus* split happening almost 2 Ma prior to the rise of the isthmus.

Repeating the analysis using the optimal COI topology with *P. pustulosus* as sister group to all other *pustulosus* group species, túngara populations would be older still.

Discussion

Our analyses of COI divergence in *Physalaemus pustulosus* provide the newest and perhaps strongest molecular evidence to date that organisms seemingly intolerant of salt water were able to move between Middle and South America in the late Tertiary well prior to the date of the final completion of the land bridge 3.1 Ma. Previous studies arguing for intercontinental migration prior to the completion of the Panamanian isthmus have relied on external calibrations and fixed-rate molecular clocks (e.g. Zamudio & Greene 1997; Bermingham & Martin 1998; Zeh *et al*. 2003). In contrast, our study uses not one but two independent temporal

Table 6 Comparing three independent estimates of rates and dates. The first four columns compare estimates of dates of divergence for four lineage-splitting events. The Sierra de Perijá separates sample localities Mariquita (MARI) and Lago Maracaibo (LMAR), whereas the Mérida Andes separate these two localities from Calabozo (CALA), Carupano (CARU), and Trinidad (TRIN) to the east. The Ecuadorian Andes separate the Pacific coast species from those in the Amazon. Each row corresponds to one analysis, and within each row assumptions and results are shown in bold and plain text, respectively. Rows 1 and 2 show results (with 95% confidence intervals) from 'multidivtime' analyses. Row 1 shows the same results as Fig. 4, plus estimates of the rates of evolution. Row 2 shows the resulting date and rate estimates assuming only that the northern Andes separated Colombian and Venezuelan lineages not less than 2 Myr. Row 3 shows the divergence date estimates (± two standard deviations from the mean) assuming a molecular clock of 0.69%/lineage/Myr

| | Dates in millions of years (Myr) | | | | Rates as percentage divergence per lineage per Myr |
| | Northern Andes | | | North vs. South *P. pustulosus* | |
Calibration	Sierra de Perijá	Mérida Andes	Ecuadorian Andes		
Ecuadorian Andes (F84 + Γ)	3.53 (1.03–7.53)	4.83 (1.74–9.31)	**11.2–16.4**	8.61 (4.07–13.3)	0.49% (0.10–1.3%)
Northern Andes (F84 + Γ)	**≥ 2.00**	**≥ 2.00**	15.5 (10.4–21.8)	9.73 (4.95–15.9)	0.43% (0.085–1.1%)
Toad clock (uncorrected percentage)	2.31 (2.31–2.31)	3.47 (2.96–3.98)	12.5 (10.6–14.5)	6.35 (5.04–7.66)	**0.69%**

constraints internal to the data at hand, and does not rely on the assumption of a strict molecular clock (Table 6).

Within *P. pustulosus*, both nuclear and mitochondrial genetic results define two distinct groups of populations: the northern group that includes populations from Mexico to northern Costa Rica, and the southern group that includes those from western Panama to Trinidad. With the exception of the western Panama populations at this junction, localities that are close geographically are generally similar in allozyme frequencies and have a small D_N. Exceptions include Mariquita (MARI), the Colombian population and Lago Maricaibo (LMAR), the western Venezuela population, which are more distinct. Related frogs in Argentina (*Pleurodema* spp.) showed much less allozyme or call differentiation than observed here (McLister *et al*. 1991). The D_N between *P. pustulosus* populations in the Middle American group and the southern group (mean = 0.294) are comparable to the value obtained by Cannatella *et al*. (1998) for *Physalaemus petersi* vs. *Physalaemus freibergi* (D_N = 0.323).

The level of allozyme differentiation between the northern and southern lineages (mean D_N = 0.294) is nearly equivalent to the level of differentiation observed between most closely related of the recognized species in the *P. pustulosus* species group (lowest D_N = 0.323; Cannatella *et al*. 1998) although these results do not share the same suite of loci, so interpretations should be limited. The mtDNA divergences are more striking. The average sequence divergence between the northern and southern groups is 12.6% and within each group is only 3.9% and 4.5%, respectively.

Nuclear allozyme results indicate populations in western Panama are intermediate with bidirectional intergradation (Table 2). The wide geographic distribution of this representative of an otherwise South American genus without any conspicuous morphological differentiation within Middle America (Freeman 1967) and only partial clinal variation in some advertisement call parameters, would

suggest that either *P. pustulosus* is a recent invader from South America or that there has been substantial gene flow among its populations. The genetic data do not support either of these inferences. How we interpret the information for these lineages depends on the species concept employed (see de Queiroz 1998).

Timing of events

Estimates of dates of divergence and rates of evolution can be highly variable, yet we found a remarkable correspondence in dates and rates among our three analyses (Table 6). Our two independent rate calibrations offer reciprocal support for one another, and are further corroborated by the molecular clock from Mongolian toads (Macey *et al*. 1998). In fact, the toads from Tibet appear to have *faster* rates of mtDNA sequence evolution than three lineages of Neotropical anurans: *Physalaemus* (Table 6), *Eleutherodactylus* (Crawford 2003), and *Bufo* (Mulcahy & Mendelson 2000).

Of primary interest is whether *P. pustulosus* crossed from South to Middle America prior or subsequent to the emergence of the isthmus 2.8–3.1 Ma (Coates & Obando 1996). Our data show convincingly that the northern and southern groups had diverged prior to the oldest hypothesized isthmus formation (Fig. 4, Table 6). Both the geological and palaeontological data suggest that the final closure of the isthmus took place in eastern Panama about 3.1–2.8 Ma (Whitmore & Stewart 1965; Coates & Obando 1996; Coates *et al*. 2004). The contact zone between northern and southern túngara frogs lies in western Panama. Therefore, we posit that *P. pustulosus* entered lower Middle America in two or more distinct events. If we average our two COI divergence time estimates (Table 6), the first event involved the basal split of *P. pustulosus* around 9 Ma (4.5–14.6 Ma) and the arrival of the northern lineage of túngara frogs in Middle America. The Panama microplate completed its

collision with South America by 7.1 Ma (Coates *et al.* 2004) and the mammalian interchange began at this time with raccoons migrating south and ground sloths moving north 9.3–8.0 Ma (Marshall *et al.* 1979; Marshall 1985; Webb 1985). Although amphibians are intolerant of salt water, they can on rare occasion disperse across oceans (Hedges 1996; Vences *et al.* 2003, 2004). One dispersal mechanism for túngara frogs could be on a raft launched from the Río Magdalena in Colombia yet situated north of Panama. The ocean currents along the Caribbean coast of South America then, as now, flow westwards so discharge from the Magdalena flows towards Panama (Díaz de Gamero 1996).

Our results support the earlier findings of Bermingham & Martin (1998) who were forced to rely on an externally calibrated molecular clock. In their analysis of three genera of freshwater fishes, they conclude that a wave of colonization from South America into Panama took place 7–4 Ma, with one or two additional invasions following the rise of the isthmus at ~3 Ma. We used two independent geological events to obtain rate and date estimates without assuming a molecular clock and obtained similar results but with even an older dispersal date. The mean mtDNA sequence divergence rate among branches we estimated at 0.46% per lineage per Myr, a rate 33% slower than an often-cited toad rate (0.69% — Table 6). Because of their smaller size, faster generation time, and perhaps their tropical environment, one might have expected túngara frogs to show the faster rate (Adachi *et al.* 1993; Martin & Palumbi 1993; Rand 1994). This observation suggests that frogs may have similar rates of mtDNA evolution, independent of size. The cane toad, *Bufo marinus*, and the túngara frog are geographically codistributed across the túngara frog's range. The largest genetic break among the cane toad populations corresponds to the northern Andes. Slade & Moritz (1998) proposed that the rise of the northern Andes 2.7 Ma (around the area of Bogotá, Colombia) divided east and west populations. Under this model two conclusions are reached. First, the overall rate of DNA sequence evolution at the ND3 gene and tRNA genes is 1% per lineage per Myr in cane toads, more than double the mean rate of COI evolution in *P. pustulosus*. Second, unlike túngara frogs, there is no evidence to suggest that cane toads entered Middle America prior to the emergence of the land bridge.

A discontinuity between Middle and South American animals in western Panama is not unique to *P. pustulosus*. Birds (Wetmore 1965; McDonald *et al.* 2001) and arthropods (Wilcox *et al.* 1997; Zeh *et al.* 2003) show similar patterns. A pseudoscorpion has a range similar to *P. pustulosus*, and exhibits similar COI genetic distances (Wilcox *et al.* 1997). The pseudoscorpion populations from western and central Panama, separated by only 400 km, are referred to as undergoing 'incipient speciation' owing to reproductive incompatibility and 8.2% sequence divergence and the authors date the separation at 6 Ma.

Limited sampling of the bushmaster, genus *Lachesis*, shows similar patterns (Zamudio & Greene 1997). They recognize the smallest of their Central American divergences (5.3%) as distinct evolutionary species. They use a size-specific reptilian clock calculation range of 0.47–1.32% per Myr to indicate that this predates the land bridge emergence with the two groups divergence time of 6.4–17.9 Ma and the Costa Rican 'species' at 4–11 Ma.

What do these divergences and intergradations mean for the frog populations in Panama? Wake & Jockusch (2000) describe problematic salamander taxonomy in the face of multiple data sets. They promote Ghiselin's (1997; p. 99) species concept: 'Biological species are populations within which there is, but between which there is not, sufficient cohesive capacity to preclude indefinite divergence', and state the critical nature of knowledge of population interaction and its role in inferring relationships. Under these criteria, the pseudoscorpion data on pre- and postzygotic isolating mechanisms would support their specific designation, whereas the bushmasters' elevation to specific status, lacking such study, would be premature. For *P. pustulosus*, the intergradation in western Panama populations in the nuclear allozyme data is pivotal. The fact that túngara frogs show this cohesion along with little to no ecological, morphological or call difference leads us to conclude that these interactions will contribute to the process of 'de-differentiation' (Wake & Jockusch 2000).

Many geological scenarios (Van Andel *et al.* 1971; Iturralde & MacPhee 1999) indicate connections of various blocks involved in the formation of the rising isthmus around 10 Ma. By 16 Ma, palaeontological data show that North American mammals were well established in central Panama (Whitmore & Stewart 1965). Savage (2002) includes *Physalaemus* with a South American element and characterizes the genus as a 'recent contributor to the faunal diversity'. We feel, however, that an earlier movement into Middle America is more likely, with the southern group moving again more recently into Middle America to re-establish contact with the earlier group. These two groups do not presently occupy portions of Costa Rica where suitable habitat seems to exist (Savage 2002), but frogs must have crossed this gap to produce the admixture seen in western Panama.

Conclusions

The pronounced genetic differences between populations of *Physalaemus pustulosus* in Middle and South America suggest that they have been separated from one another for 4–13 Myr. A comparison with differences among species in the *pustulosus* species group separated by the Andes suggests that the isolation between Middle and South American populations of *P. pustulosus* predates the completion of the Panama land bridge 2.8–3.1 Ma. In spite of the large molecular differences, the allozyme data alone

support the existence of some reproductive compatibility demonstrated by admixture in western Panama. Combined with the absence of documented pre- or post-mating barriers to gene flow, or mate choice preferences, recognition of two species is premature at this time.

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Lee A. Weigt pursues molecular systematics, cryptic species and biodiversity while running molecular facilities. Andrew J. Crawford studies evolutionary genetics and biogeography of Neotropical frogs, while A. Stanley Rand has long studied tropical biology, herpetology and behavioural ecology. Michael J. Ryan's research interests are the evolution of behaviour, animal communication and sexual selection.

Appendix I

Genotypes by population with electrophoretic conditions noted for each gene product (Buffers: RW, Ridgway *et al.* 1970; CT, Clayton & Tretiak 1972; TC , Selander *et al.* 1971; Enzyme numbers from IUCBN 1992)

Gene product	Genotype	VERC	TEHU	TAPA	GUAT	ESAL	NICA	CRIC	PARM	GUAL	SANT	ANTO	GATW	GATE	BCIC	PRPH	GAMB	GBRG	SUMM	CHIV	COCO	KOBE	ITAB	IREY	METE	ELRE	MARI	LMAR	CALA	CARV	TRIN
Phosphogluconate dehydrogenase																															
6PGDH	AB																										1				
1.1.1.44	BC																									4		1				
buffer = CT	CC										5	7	5	4	7	9	18	8	3	2	5	4		15	3	1	5				
	CD																													7	
	CE										1	4	6	5	5	6	7	4	9	7	9	7	1		5	5					
	CG										2	1																			
	DD																										1				
	DG									1																					
	DH						1																								
	EE											3	1	5	1		1	4	1	3	1	2	14		2	1		1			1
	EF																										1	1			3
	EG		4				1																								
	FF																												11	14	1
	FG								2																						
	GG	11	7	15	8	7	7	1	13	13																					
	GH			1	2	5	3	6																							
	HH							4																							
Aspartate transaminase																															
ATA	AA	11	11	16	10	9	12	11	10	8	1	1	2								1										
2.6.1.1	AB																1														
buffer = RW	AC				3				3	5	8	9	5	7		1	7	4	2	7	3	5			1	1	3		1		
	AD										1																				
	BC																				2										
	BD																														2
	CC								1		5	5	8	8	14	14	23	11	11	8	11	7	15	15	14	14	12	1	8	5	
	CD										1																	1	2	1	
	DD																													6	3
Triose phosphate isomerase																															
TPI	AA	4																													
5.3.1.1	AD	3		1																											
buffer = CT	BB																								4				1		
	BH																								8	7			2		
	CH										7	1																			
	DD	4	9	11	9	12	12	11		2																					
	DF																				1										
	DG				1																										
	DH		1	4					5	7										1	2										
	EH																														2
	FF										2	9					3	5	4	4	6	1	1	1							
	FH											1	5	5	9	7	12	8	6	7	5	8	3	4							
	HH		1						10	5	7	13	8	1	5	7	15	3	3	2	2	5	11	10	7	4	15	2	9	13	3
	HI										2																				
Lactate dehydrogenase																															
LDH1	AB											2																			
1.1.1.27	BB				2				12	10	8	12	15	15	14	14	27	16	13	15	14	14	15	15	15	15	15	2	12	14	5
buffer = TC	BC		5	4	7		1		3	4	5	1				1	3					1									
	CC		5	12	1	12	11	11			2																				
	CD	7	1																												
	DD	3																													
LDH2	AC		3	1																											
	BC							1				1									1										
	CC	11	8	7	10	12	12	10	15	14	16	15	14	15	14	15	30	16	13	14	15	14	15	15	15	15	15	2	12	14	5
	CD			6																											
	DD			2																											

Appendix I *Continued*

Gene product	Genotype	VERC	TEHU	TAPA	GUAT	ESAL	NICA	CRIC	PARM	GUAL	SANT	ANTO	GATW	GATE	BCIC	PRPH	GAMB	GBRG	SUMM	CHIV	COCO	KOBE	ITAB	IREY	METE	ELRE	MARI	LMAR	CALA	CARV	TRIN
																				Populations											
Phosphoglucomutase																															
PGM	AD					2																									
2.7.5.1	BD				1		2												1	1											
buffer = TC	CD									1																					
	DD	11	11	16	8	10	10	10	12	13	16	15	15	15	14	15	30	16	12	14	15	14	15	15	15	15	15			2	
	DE				1			1	3																			1	1	6	
	EE																											1	11	6	5
Glucose phosphate isomerase																															
GPI	AB																										1				
5.3.1.9	AC																														1
buffer = CT	BB																										3				
	BC																										10		3		2
	CC	11	11	16	10	12	12	11	1	3	16	15	15	15	14	15	29	16	13	12	15	14	15	14	15	10	1	2	10	14	1
	CD																			3											
	CE																1							1		4					1
	CF									8																					
	DE									1																					
	EE																									1					
	FF								14	1																					
	FG									1																					
Malate dehyrogenase																															
MDH1	AB																													1	
1.1.1.42	BC																							1						6	
buffer = RW	BD																1														
	CC	11	11	16	10	12	12	11	15	14	16	15	2	5	12	12	29	15	9	12	14	12	13	14	14	13	15	2	12	7	5
	CD												11	6	2	3	1	1	4	2	1	2	1		1	2					
	DD												1	4																	
MDH2	AC											7					2														
	BB																										2				
	BC									10				5							5			1							
	BE																										8				
	CC	11	11	16	10	12	12	11	15	4	12	8	15	8	14	13	28	16	11	14	10	14	14	14	13	15		2	12	13	5
	CD										1																				
	CE										2			2		2			2	1					2						
	CF										1																				
	EE																										5				
Isocitrate dehydrogenase																															
ICD1	AB																1														
1.1.1.42	BB		3								9	14	1	5	7	5	6	4	5	12	10	11	1	2		2					
buffer = TC	BC														1																
	BD	5	5	1	4	7	3	8	14	4		1	6	8	4	7	20	8	5	2	5	1	9	4	15	13			11	10	
	BE										7																				
	CE									2																					
	DD	5	3	1	6	5	9	3	1	8			7	2	2	3	3	3	2	1			5	9				2	1	4	
ICD2	AB							1		1																					
	BB	11	11	16	10	12	12	10	15	13	16	15	15	15	14	15	29	16	13	15	15	14	15	15	15	15	15	2	12	14	2
ME, SOD1, SOD2	AA	11	11	16	10	12	12	11	15	14	16	15	15	15	14	15	30	16	13	15	15	15	15	15	15	15	15	2	12	13	

ME, *Malate dehydrogenase* (+ NADP), 1.1.1.40, buffer = RW.
SOD, *Superoxide dismutase*, 1.15.1.1, buffer = TC.

Appendix II

PCR thermal cycler profiles and COI primers used in this study

PCR amplifications were 50 μL total volume with conserved or 'universal' primers for cytochrome oxidase I (COI) COIf (5′-CCTGCAGGAGGAGGAGAYCC-3′) and COIa (5′-AGTATAAGCGTCTGGGTAGTC-3′) (Kessing *et al.* 1989) or COIa2 (5′-CCTGCYARYCCTARRAARTGTTGAGG-3′), and *Taq* or *Tfl* at 1.25 units / 50 μL volume. The thermal cycler profile was: 1.5 min at 94 °C; 5 cycles: 30 s at 94 °C, 30 s at 45 °C, 45 s at 72 °C; 25 cycles: 30 s at 94 °C, 30 s at 52 °C, 45 s at 72 °C. After thermal cycling, 5 μL was run on a 1.5% agarose gel and bands were excised and resuspended in 400 μL, heated to 65 °C and 1 μL was used as template for a second round of PCR: 1.5 min at 94 °C; 25 cycles: 30 s at 94 °C, 30 s at 52 °C, 45 s at 72 °C. PCR products were purified via GeneClean (Bio 101) and cycle sequenced (50 °C annealing temperature) with ABI dye terminator chemistry following the manufacturer's instructions. Additional internal sequencing primers included: PP6 (5′-TCTGCAACAATAATYATYCGCAATTCCAAC-3′), PP7 (5′-GTTGGA ATTGCRATGATTATTGTTGCAGA-3′), PP8 (5′-TCTCTAGAYATTGTATTACATGA-3′), and PP9 (5′-TCATGTAATACAATGTCTAGAGA-3′)