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[1]*School of Life Sciences, Ecology and Evolutionary Biology, Graham Kerr Building, University of Glasgow, Scotland, UK;* [2]*Institute of Biomedical and Life Sciences, Division of Molecular Genetics, University of Glasgow, Scotland, UK;* [3]*Instituto de Investigación en Recursos Cinegéticos, CSIC-UCLM-JCCM, Ronda de Toledo, Ciudad Real, Spain;* [4]*Museo del Instituto de Zoología Agrícola, Facultad de Agronomía, Universidad Central de Venezuela, Maracay, Aragua, Venezuela*

Genetic differentiation in the Trinidad endemic *Mannophryne trinitatis* (Anura: Aromobatidae): Miocene vicariance, *in situ* diversification and lack of geographical structuring across the island

Michael J. Jowers[1,2], Iñigo Martínez-Solano[3], Bernard L. Cohen[2], Jesús Manzanilla[4] and Roger J. Downie[1]

Abstract

Trinidad offers a unique study system within the Caribbean to assess the processes and patterns of amphibian speciation. We used mitochondrial DNA sequences to investigate the phylogenetic relationships and patterns of intraspecific genetic variation of *Mannophryne trinitatis* from Trinidad. Molecular clock estimates point to a genetic split between *M. trinitatis* and its sister species, *M. venezuelensis*, dating to the Late Miocene (c. 7–8 Mya), suggesting vicariance as a means of speciation when Trinidad pulled apart from northern Venezuela. *M. trinitatis* phylogenetic population analyses from ten Northern Range and four Central Range localities recovered three well-resolved clades: a larger clade formed by haplotypes from Northern Range localities and two additional clades, one formed by haplotypes from the Central Range and another including haplotypes from Northern Range localities and one haplotype from the Central Range. Overall, our results show that the genetic diversity in *M. trinitatis* is not geographically structured but it is distributed among the various Northern and Central Range localities. In congruence with the vicariance speciation hypothesis, we attribute *M. trinitatis* present distribution and lack of genetic structure to multiple admixture events caused by climate changes that severely affected the topology of Trinidad throughout the Pliocene/Pleistocene periods.

Key words: Divergence – *Mannophryne trinitatis* – vicariance – Trinidad – mtDNA – speciation

Introduction

Trinidad and Tobago are the southernmost islands of the Lesser Antilles. Unlike the other Antillean islands, their geology, flora and fauna mark them as outliers of the South American continent (Murphy 1997). From an evolutionary perspective, Trinidad offers a unique study system within the Caribbean to assess the processes and patterns of amphibian speciation. Trinidad's geology, close proximity to northern Venezuela and the existence of Pliocene/Pleistocene land-bridge formations between the mainland and Trinidad, makes all three competing biogeographical hypotheses for the origin of species in the Caribbean (Rosen 1975, 1985; Pregill 1981; Hedges 1989, 1996a,b,c, 2006) equally plausible for Trinidad: overseas dispersal, land-bridge connections and vicariance (Boos 1984a,b; Rodríguez and López 2003; Camargo et al. 2009; Manzanilla et al. 2009). Furthermore, repeated Pleistocene sea-level falls and rises and flooding of Trinidad's basins by the Orinoco river discharges are key climatic and environmental changes likely to have shaped patterns of intraspecific genetic variation across different species in Trinidad (Jowers et al. 2008a).

The aromobatid genus *Mannophryne* La Marca 1992 currently comprises nineteen species that occur throughout the Western and Northern mountains of Venezuela, from the Andean Cordillera of Mérida to Paria and in the islands of Trinidad and Tobago (Fig. 1), where *M. trinitatis* and *M. olmonae*, respectively, occur. *Mannophryne* are characterized by the presence of dark collars and elaborate throat display and toe tip jumping behaviours, and recent evidence from mtDNA supports generic monophyly (Manzanilla et al. 2007, 2009). Until recently, isolated populations of *M. trinitatis* (Garman, 1887) were thought to inhabit some areas of the Paria peninsula, part of the adjacent mainland, but differences in chromosome banding (Kaiser et al. 2003), morphology, call and mitochondrial DNA strongly indicate that these mainland populations belong to a different species, *M. venezuelensis* (Manzanilla et al. 2007). The only other West Indian *Mannophryne* species, *M. olmonae* (Hardy 1983), inhabits Tobago's Main Ridge mountains and is similar to *M. trinitatis* in ecology and morphology (Hardy 1983; MJJ pers. observation). Like *M. venezuelensis*, *M. olmonae* was distinguished from *M. trinitatis* relatively recently (Hardy 1983). Thus, *M. trinitatis* and *M. olmonae* are endemic to Trinidad and Tobago, respectively, and are the only species of *Mannophryne* that do not occur on the South American mainland. A recently published phylogeny of the genus based on mitochondrial DNA shows that it is composed of two monophyletic sister clades, the Obliterata and the Collaris groups. Interestingly, within this latter group, *M. trinitatis* and *M. venezuelensis* are sister clades to *Mannophryne sp*, an undescribed species found in western Venezuela, and *M. olmonae* is sister clade to *M. riveroi*, with *olmonae* + *riveroi* being sister to the rest of the group (Manzanilla et al. 2009). Thus, from a biogeographical perspective, it is apparent that phylogenetic relationships within *Mannophryne*, at least in some cases, do not match today's species distribution.

The presence of endemic *Mannophryne* species in Trinidad and Tobago led to the suggestion that colonization of the islands by these frogs may have occurred during the most recent periods of connection to the mainland, which resulted

Corresponding author: Michael J. Jowers (michaeljowers@hotmail.com)
Contributing author: Iñigo Martínez-Solano (inigomsolano@irec.csic.es), Bernard L. Cohen (b.l.cohen@bio.gla.ac.uk), Jesús Manzanilla (jmanzanilla@minamb.gob.ve), Roger J. Downie (J.downie@bio.gla.ac.uk)

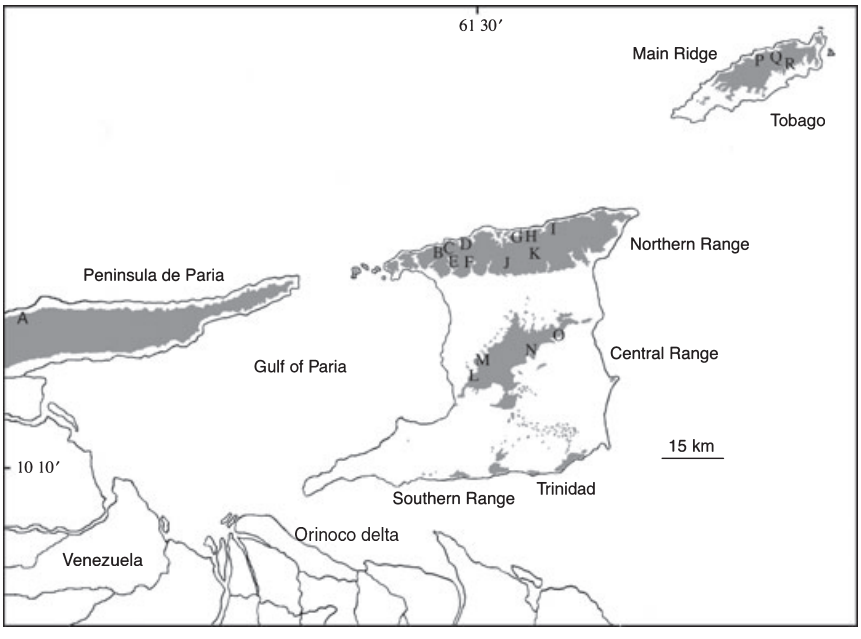

Fig. 1. Map of the localities mentioned in the text. Shading: elevations over 25 m above sea level. A, San Juan de las Galdonas; B, Blue Basin Waterfall; C, El Tucuche; D, Maracas Bay; E, Maracas Waterfall; F, Mount Saint Benedict; G, Paria Bay; H, Grand Tacaribe; I, Matelot; J, Lopinot; K, Simla; L, Gran Couva; M, Pepper Village; N, Tamana Cave; O, Mount Harris; P, Parlatuvier site 1; Q, Parlatuvier – Roxborough site 2; R, Parlatuvier – Roxborough site 3

from Pleistocene or Holocene interglacial sea-level low-stands (Murphy 1997). On the basis of the shallow marine depth of the Gulf of Paria (38 m), Murphy (1997) argued that sea-level drops of up to 130 m (Gascoyne et al. 1979; Fairbanks 1989) during Pleistocene interglacials resulted in connection of Trinidad and the Paria peninsula (northern Venezuela), facilitating the herpetofauna to colonize Trinidad from the mainland. Other more likely connections may have formed between the shallow depths separating the south-western peninsula and the mainland (Kenny 1995) but the lack of hills in this area and the absence of *Mannophryne* species in the south of Trinidad (Jowers and Downie 2004) suggest that the ancestor of *M. trinitatis* did not arrive from the south. The maximum channel depth separating Trinidad and Tobago is 91 m, and thus, these two islands have only been connected twice during the Pleistocene (Murphy 1997), the last occasion as recently as 17 000 years ago (Fairbanks 1989). The similar number of different endemic herpetofauna species in both islands and the fact that Tobago is less than half the size of Trinidad suggest long isolation between the islands previous to Pleistocene connections. However, evidence of connections derives from the four endemic species found in both islands (two frogs and two snakes) (Murphy 1997). MtDNA data on *Leptodactylus fuscus* (Camargo et al. 2006, 2009) and *L. validus* (Yanek et al. 2006) from Trinidad and Tobago have shown very low levels of genetic divergence between the islands, suggesting that these species may have colonized the islands recently by oversea dispersal or rafting. Additionally, studies on the freshwater fish *Rivulus hartii* (Jowers et al. 2008a) and *Poecilia reticulata* (Alexander et al. 2006) have shown gene flow between Venezuela and western Trinidad attributed to Pleistocene glacial cycles when low sea stands facilitated the confluence of rivers. However, the estimated divergence between *M. venezuelensis* and *M. trinitatis* is considerably older (Manzanilla et al. 2007), indicating that *M. trinitatis* and possibly *M. olmonae* were already established on the islands before low sea-level stands made possible dispersal from the mainland. If so, this implies that the present-day distribution of genetic variation within *M. trinitatis* reflects the various environmental changes that affected the topology of Trinidad throughout the Pleistocene, such as flooding of the basins caused by the Orinoco river discharges and forest expansion and reduction during glacial and interglacial periods.

Mannophryne trinitatis (the 'stream frog') is the only endemic anuran found in the Northern and Central Ranges of Trinidad (Fig. 1). There is an additional Trinidad endemic, the golden tree frog *Phytotriades* (=*Phyllodytes*) *auratus*, found only on a few peaks in the Northern Range (Jowers et al. 2008b). In the Northern Range, *M. trinitatis* is widespread and common in and around small streams in steep forested valleys from sea level to high elevation. However, its distribution in the Central Range is much more restricted. Here, it is known only from four localities (Jowers and Downie 2004); a large (but variable-size) population is known from Tamana Cave (Kenny 1969) and populations of uncertain size at three other separated sites in open forest, where the elusive behaviour of adults makes them hard to find (Jowers and Downie 2004).

In this study, we investigated the phylogenetic relationships and patterns of intraspecific genetic variation of *M. trinitatis* populations using mtDNA data. For this purpose, we used two rapidly evolving gene fragments, the cytochrome-b and control region. The aims were to assess (1) *M. trinitatis* tempo and mode of speciation from its mainland sister species *M. venezuelensis*, (2) the level of haplotypic genetic differentiation within and between localities and (3) the genetic structure of *M. trinitatis* populations throughout Trinidad and more precisely between both mountain ranges.

Materials and methods

Sample collection

Recent work (Manzanilla et al. 2007, 2009) has shown that *Mannophryne venezuelensis* is sister taxon to *M. trinitatis*. Additionally, we added samples of the more distantly related *M. olmonae* from Tobago. A total of 44 specimens comprising *Mannophryne venezuelensis* (n = 4), *M. trinitatis* (n = 35) *and M. olmonae* (n = 5) were captured

from ravines and around mountain streams during the wet season of 2001–2004 (Table S1). After collection, frogs and tadpoles were killed in benzocaine (5%) and preserved in 95% alcohol.

Laboratory protocols

DNA extraction protocols were similar to those described by Sambrook et al. (1989). Total DNA was extracted from fingers (metamorphosed individuals) and tails (tadpoles) in 95% alcohol and purified using standard phenol/chloroform protocols. The oligonucleotide primers used for amplification and sequencing are shown in Table 1. The cytochrome-b forward primer L15179 (Ptacek et al. 1994) and the custom-designed reverse control region primer (CR)P-H (Table 1) were used to amplify a fragment of approximately 2500 base pairs (bp) corresponding to a large portion of the cytochrome-b 5′ end and a 3′ segment of the control region. This sequence was used to design specific primers (Table 1) that were used successfully to amplify cytochrome-b and control region fragments in all three *Mannophryne* species. Amplified fragments were purified by gel electrophoresis and recovered by silica/chaotrope spin column purification (Qiagen, Crawley, UK). Sequencing of both strands was performed by the in-house sequencing unit with amplification primers and fluorescent dideoxy chain terminators (BigDyes, PE Biosystems, Warrington, UK) and analysed in an ABI 377 (Applied Biosystems, Perkin Elmer). Templates were sequenced on both strands, and the complementary reads were used to resolve rare, ambiguous base-calls. After incomplete terminal sequences and PCR primers were removed, the sequences were used to prepare the alignments described in the next section.

Sequence choice and alignment

Mitochondrial DNA sequences were chosen for use because the well-known properties of this maternally inherited, non-recombining genome have proved useful in innumerable studies of amphibian phylogeny. We compiled a concatenated (1217 base pairs) alignment of 588 bp from the cytochrome-b (*cytb*) protein-coding region and 629 bp from the control region (CR) for 44 individuals from all three *Mannophryne* species (Table S1). The *cytb* sequences were aligned manually, without gaps, and when translated into amino acids with the vertebrate mitochondrial code, an open reading frame was found. For subsequent analyses, all *cytb* and CR sequences (which included a few indels) were concatenated.

Phylogenetic analyses

Analyses were performed with PAUP*4.b.10 (Swofford 2002) and MrBayes v.3.1.2 (Ronquist and Huelsenbeck 2003). In PAUP*, heuristic search of 1000 pseudoreplicates was made to test for incongruence of the data partitions (partition homogeneity test, Farris et al. 1995). TCS 1.18 (Clement et al. 2000) was used to reduce sequences to haplotypes, and because of low variation between sequences, all phylogenetic analyses were performed on haplotypes.

We used MrBayes to infer gene trees based on mtDNA sequence data. Several alternative data partitioning schemes were compared: (1) the data were analysed as an unpartitioned data set, (2) two partitions were defined, corresponding to the two mitochondrial DNA regions sequenced (control region and cytochrome-b), (3) three partitions were defined, corresponding to the control region, 1st + 2nd positions in the cytochrome-b alignment and 3rd positions in cytochrome-b sequences and (4) four partitions, corresponding to control region and 1st, 2nd and 3rd positions in the cytochrome-b alignment. In all cases, the DNA substitution model for each partition was selected by FindModel (available at http://www.hiv.lanl.gov). Each run consisted of four chains of 20 000 000 generations, sampled each 10 000 generations for a total of 2000 trees. The best partitioning scheme was selected by comparing the harmonic mean of the log-likelihoods in each analysis and calculating Bayes factors (Kass and Raftery 1995). Twenty-five per cent of the trees resulting from the analyses were discarded as 'burn in'.

We used the program BEAST 1.5.4 (Drummond and Rambaut 2007) to estimate coalescence times between haplotypes (TMRCAs). We defined groups corresponding to the three species (*M. olmonae, M. trinitatis* and *M. venezuelensis*) and to *M. trinitatis* + *M. venezuelensis* and run the analyses under a birth-death prior using both a strict and a relaxed (uncorrelated-lognormal) molecular clock model. To assess whether the molecular clock dates between *M. venezuelensis* and *M. trinitatis* were underestimated because of the fact that *M. olmonae* was not the most recent common ancestor of these two species, all analyses were also performed excluding *M. olmonae*.

Inspection of trace files indicated that a strict-clock model could not be rejected (the parameter 'Coefficient of Variance' in relaxed clock analyses abuts zero), and thus, we report results based on this model only. Analyses using the optimal partitioning scheme according to MrBayes analyses suffered from overparameterization, according to low effective sample sizes (ESSs) of parameter estimates in the model. Therefore, to incorporate differences between partitions and information on optimal substitution models while avoiding the use of models with too many parameters, we specified two different partitions corresponding to the two mitochondrial regions sequenced. For each partition, the HKY + G model of substitution was selected, with model parameters unlinked across partitions. In the absence of a reliable prior on the substitution rate or of a well-dated calibration point, we specified a normal prior on the clock rate with a mean value of 0.0075 substitutions per lineage per million years and a standard deviation of 0.0025 substitutions/lineage/million years to encompass a

Table 1. Primers used to amplify and sequence the cytochrome-b and control region fragments

Primers	Gene	Sequence	References
L15179	Cytb	5′-TGAGGACAAATATCATTCTGAGG-3′	Ptacek et al. (1994)
(CR)P-H	*CR*	5′-GTCCATAGATTCRSWTCCGTCAG-3′	This study
Cyt B-F	Cytb	5′-GTTCAATGAATTTGAGGCGG-3′	This study
Cyt B-R	Cytb	5′-CCAAGTGTGGGAGAGAAGATG-3′	This study
CR-F	*CR*	5′-CCCTACTATCCTTATATTCTAGGAC-3′	This study
CR-R	*CR*	5′-GCTTTTATGACTACCAGGTCC-3′	This study

Table 2. Median time since most recent common ancestor (TMRCA, in million years) and 95% highest posterior density (HPD) intervals of haplotypes in all three *Mannophryne* species studied. In the second and fourth columns, *M. olmonae* haplotypes were excluded from the analyses

	TMRCA (million years)		95% HPD interval	
Species	*M. olmonae* as outgroup	*M. olmonae* excluded	*M. olmonae* as outgroup	*M. olmonae* excluded
M. olmonae	0.87	–	0.35–1.80	–
M. trinitatis	1.63	1.72	0.76–3.31	0.79–3.38
M. venezuelensis	0.31	0.32	0.09–0.71	0.09–0.73
M. venezuelensis–M. trinitatis	7.28	7.91	3.67–14.23	3.69–15.23

wide range of possible substitution rates in amphibian mtDNA (1–2% pairwise differences per million years). Three different analyses were run for 40,000,000 generations, and posterior distributions of parameter estimates were visually inspected in Tracer v1.5 (Rambaut and Drummond 2007) to check for convergence before combining them into a single log file to estimate median values of the parameters of interest as well as their 95% highest posterior density intervals. Ten per cent of the resulting topologies and parameter values were discarded as 'burn in'.

Haplotype diversity and population analyses

The extent of geographical structuring of genetic variation between *M. trinitatis* populations was evaluated by Φ-statistics using the analysis of molecular variance (AMOVA) in ARLEQUIN 3.0 (Excoffier et al. 1992; Schneider et al. 2000). The significance of variance components and Φ-statistics were assessed by permutations (10 000) of the data sets. The haplotype frequency and a parsimony minimum spanning network (MSN) of the sequences were analysed in TCS 1.18 (Clement et al. 2000) following the method of Templeton et al. (1992) in which >95% confidence was required for haplotypes to be connected. We assessed the extent of geographical structuring in *M. trinitatis* between different groups. The genetic structure was assessed for (1) all localities within the same group, (2) Northern versus Central Range localities, (3) haplotypes from localities unique to clade one (Maracas Bay, Paria Bay, Grand Tacaribe, Matelot and Simla versus all other localities), (4) three groups, haplotypes from localities recovered only in clade one versus Central Range haplotypes (clade III) versus the rest of populations (clade II) (see Results section).

Results

Base composition was not heterogeneous (χ^2 tests p = 1.0). There was no significant partition heterogeneity between *cytb* and CR partitions (p = 0.80).

The harmonic means of log-likelihood values for partitioning schemes 1–4 in Bayesian analyses are as follows: (1) −3610.86, (2) −3586.51, (3) −3489.65 and (4) −3473.50. Thus, there is considerable statistical support for the most complex partitioning scheme (4: four partitions). Figure 2 shows a consensus phylogram resulting from this analysis. There is strong support for the monophyly of each species as well as for

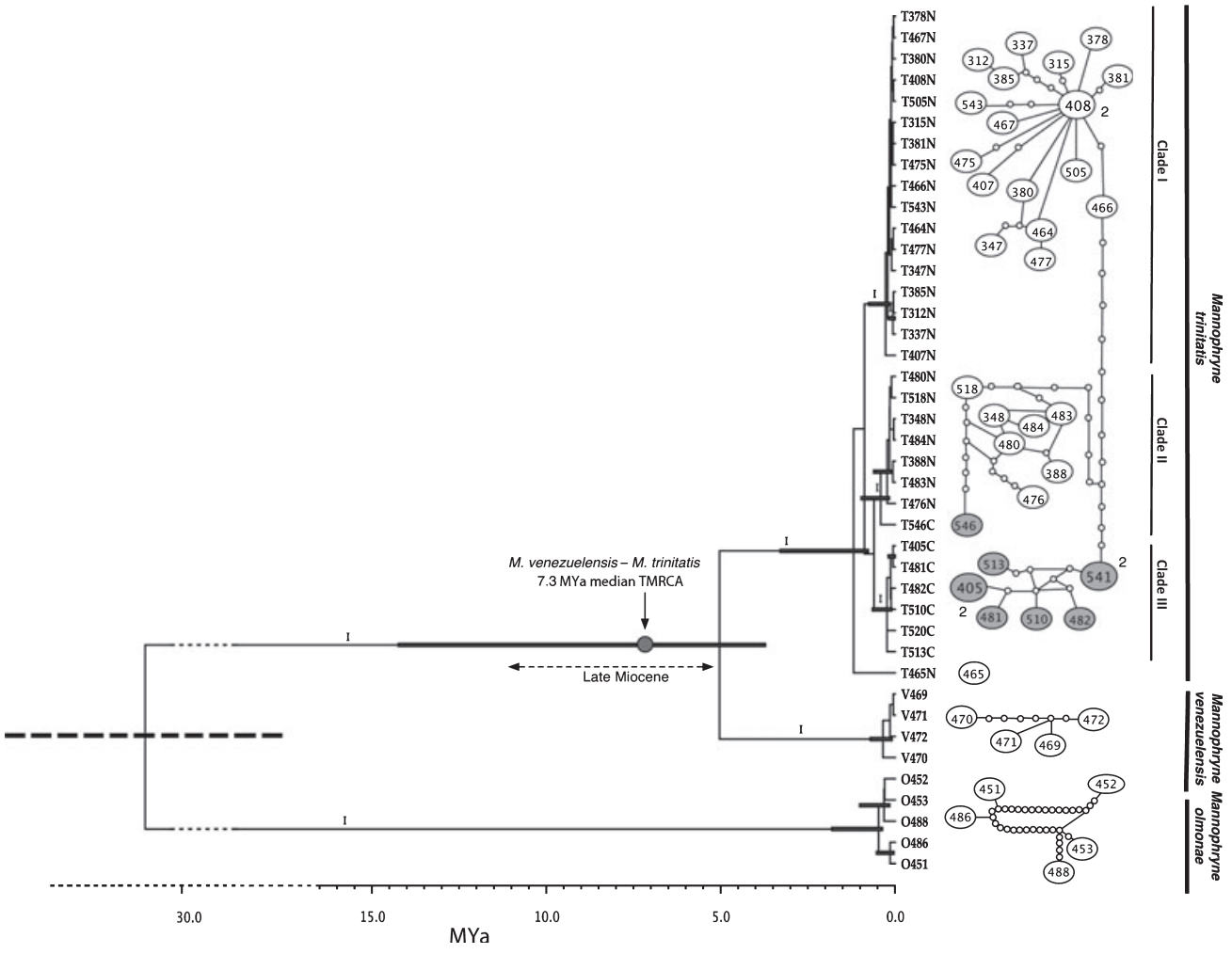

Fig. 2. Bayesian consensus phylogram and minimum spanning networks. The letters 'C' and 'N' by haplotypes indicate Central and Northern Range, respectively. In the minimum spanning networks, the haplotypes are displayed as circles. The size of the circles corresponds to the haplotype frequency. The number by circles indicates the haplotype frequency when higher than one. Small circles indicate hypothetical intermediate haplotypes. Due to the number of mutational steps separating *M. venezuelensis*, *M. trinitatis* and *M. olmonae*, they could not be joined to the network with greater than 95% confidence. Three haplotypes were recovered from more than one individual: T408N was also present in T479N, T405C also occurred in T404C, and T541C and T520C also shared haplotype. Long tree branches and highest posterior density intervals are replaced by dashed lines for visualization purposes

a sister-group relationship between *M. trinitatis* and *M. venezuelensis* (Bayesian posterior probabilities = 1.0). Within *M. trinitatis*, there is a strongly supported clade formed exclusively by haplotypes found in populations from the Central Range (Bayesian posterior probability, BPP = 1.0, clade III), and there is another well-supported clade including most haplotypes found in the Northern Range plus haplotype T546C from the Central Range (BPP = 1.0) (Fig. 2, clade II). Haplotypes resulting from the concatenated *cytb* and control region sequences from Paria Bay, Gran Tacaribe, Matelot, Maracas Bay and Simla formed another clade (Fig. 2, clade I). Conversely, haplotypes from Maracas Waterfall, Lopinot, Blue Basin, Mount Saint Benedict and Pepper village were not grouped in the analyses. Individuals T408 and T479 from Lopinot and Paria Bay, respectively, carried the same haplotype. Identical *cytb* haplotypes from different localities were frequent (Table S1). Haplotype T465N from Blue Basin did not group with any of the three *M. trinitatis* clades and showed the highest genetic divergence with all other haplotypes (mean Kimura 2 parameter distances: 2.6 ± 0.4% standard deviation).

The topologies resulting from BEAST analyses are fully concordant with those resulting from MrBayes analyses, including support for the same clades indicated earlier (Fig. 2). Table 2 summarizes estimates for TMRCAs of the different groups studied, including median values and 95% highest posterior density (HPD) intervals (in million years). The results suggest an old divergence between *M. trinitatis* and *M. venezuelensis* (median value for the TMRCA: 7.28 million years, the 95% HPD interval ranging between 3.67 and 14.23 Mya) and Pleistocene divergence for populations within *M. trinitatis* (median TMRCA for all *M. trinitatis* haplotypes = 1.63 million years) These results were very similar with the exclusion of *M. olmonae* from the analyses (Table 2). However, it is important to keep in mind that these estimates represent divergence times between haplotypes and thus necessarily predate population divergence times (although the magnitude of this offset between haplotype and population divergence is unknown).

Table 3 shows mean genetic distances within and between species. The AMOVA results showed no significant degree of genetic subdivision in any of the analyses (not shown, see Materials and methods for the levels of genetic structure assessed).

Discussion

Phylogenetic and divergence time estimates

The findings of this study concur with others that have proposed independent species status for the three *Mannophryne* forms here investigated (Hardy 1983; Kaiser et al. 2003; Manzanilla et al. 2007, 2009). Our molecular clock calibration resulted in a median value for the most recent common ancestor (TMRCA) of haplotypes in *M. venezuelensis* and *M. trinitatis* of 7.28 million years and thus rejects the possibility that *M. trinitatis* colonized Trinidad through the much more recent Pleistocene land-bridge formations that joined the Paria Peninsula to Trinidad's Northern Range on several occasions, as suggested by some authors (Murphy 1997). Our results concur mostly with those of Manzanilla et al. (2007), in which by using mtDNA *COI* sequence divergence they proposed that vicariance was the likely cause for the observed genetic divergence between *M. venezuelensis* and *M. trinitatis*, dating between 4 and 6 million years ago, before Trinidad pulled apart from the South American continent around the Late Miocene (Liddle 1946; Salvador and Stainforth 1968; Vierbuchen 1984; Tyson et al. 1991; Audemard and Audemard 2002; Moretti et al. 2007; Persad 2009). Although the divergence times between both species here reported and those of Manzanilla et al.'s (2007) are mostly in agreement, our TMRCA median and 95% highest posterior density interval point to (Table 2) a much older genetic split (Late-Middle Miocene) than previously estimated (Late Miocene/Pliocene period) and thus strengthen the vicariance hypothesis as a means of speciation. To the best of our knowledge, this is the only example of genetic isolation and thus speciation by vicariance in Trinidad.

Although the vicariance scenario seems to fit the observed genetic divergence between *M. venezuelensis* and *M. trinitatis*, there is some evidence that herpetofauna have colonized Trinidad through rafting. Boos (1984a,b) argues that reptile species found in certain coastal areas of Trinidad and its north-western islands (Patos, Monos, Huevos, Chacachacare and Gaspar Grande) and Tobago are recent South American immigrants carried by the mid-Atlantic current from the Amazon and Orinoco Delta to north-western Trinidad and to south-eastern Tobago. A good example is the widespread mainland lizard *Cnemidophorus lemniscatus lemniscatus,* found only in small coastal areas where these currents touch or pass. South American reptiles present only in the north-western islands of Trinidad indicate that these are most likely recent colonists and not relicts that otherwise would have been established in Trinidad (Boos 1984a,b). Despite this evidence, we reject the rafting hypothesis for several reasons. First, the ancestor of *M. trinitatis* was unlikely to colonize Trinidad by rafting, as rafts originate from large alluvial systems where lack of hill formations would make the presence of *Mannophryne* species unlikely (Manzanilla et al. 2007). Secondly, the lack of other *Mannophryne* species from the Paria peninsula (e.g. *M. riveroi*, *M. venezuelensis*) in Trinidad indicates that these frogs are unlikely to disperse by rafting. Thirdly, the evidence that herpetofauna use rafts

Table 3. Mean-corrected genetic distances (Kimura 2 parameter) and standard deviation (std) within and between species, including values between populations from the Northern and Central ranges in Trinidad

	Species				
	Mannophryne trinitatis	*M. venezuelensis*	*M. olmonae*	*M. trinitatis* Northern Range	*M. trinitatis* Central Range
M. trinitatis	1.2 ± 0.2	8.4 ± 0.8	21 ± 1.4	–	–
M. venezuelensis	8.4 ± 0.8	0.4 ± 0.1	22.2 ± 1.6	–	–
M. olmonae	21 ± 1.4	22.2 ± 1.6	1.0 ± 0.2	–	–
M. trinitatis Northern Range	–	8.4 ± 0.8	–	1.0 ± 0.2	1.4 ± 0.2

derives from recent colonization events favored by currents that were unlikely to be present or to flow in a similar motion several million years ago, because of significant changes in the coastal topology in the area since that time (Iturralde-Vinent and McPhee 1999; Garciacaro et al. 2011). Thus, the proposed age estimate calibration indicates that the ancestor of *M. trinitatis* did not colonize Trinidad during the proposed land-bridge connections of the Pliocene/Pleistocene because it was already established in Trinidad long before this period. Furthermore, as previously discussed, ecological and geological data strongly reject the colonization of Trinidad through rafting around the Late-Middle Miocene.

M. trinitatis population structure

The finding that most *M. trinitatis* haplotypes were not associated with specific localities (i.e. individuals from the same locality showed different haplotypes, while two individuals from different localities showed the same haplotype) agrees with results of studies on the savanna frog, *Leptodactylus validus,* in Trinidad, where different haplotypes showed little biogeographical structure (Camargo et al. 2009). The observed genetic diversity between and within localities does not match the present distribution of *M. trinitatis*, which therefore suggests either past events in the area that favored the mixing of genotypes, the retention of ancestral polymorphisms or a combination of both. However, climate changes that severely affected the topology of Trinidad during the Pleistocene are likely events to have contributed to the observed population structure. When colder and drier weather during glacial maxima reduced forests and expanded savannas, *M. trinitatis* may have been constrained to retreat from forest edge localities to more humid central forests. The reverse may have occurred during short interglacial periods, with expansion of forests into the savannas, and thus re-colonization of lower forest edge localities (Murphy 1997). Sea-level rises and flooding by the Orinoco River wet season discharges are known to have flooded most of the basins during long interglacial periods forcing species to re-colonize higher-altitude localities.

This may be corroborated by the fact that although the Southern Range shows suitable habitat conditions for the presence of *M. trinitatis*, extensive surveys have never found them there (Jowers and Downie 2004). Floods caused during the Orinoco River discharges would have covered most of this range, leaving very few and small isolated land peaks available. Thus, *M. trinitatis* present distribution and current lack of an apparent population structure is likely the result of higher-altitude habitat availability throughout prolonged Pleistocene high sea-level stands and subsequent recolonization events of newly available habitat during low sea-level stands. Further evidence of the effect on genotypic diversity through prolonged periods of flooding in Trinidad comes from recent work on the population structure of the freshwater fish *Rivulus hartii* based on mtDNA data (Jowers et al. 2008a). In this study, *R. hartii* from Northern Range high-altitude localities recovered distinct haplotypes and high genetic divergence compared with all other homogeneous haplotypes from the Southern slope of the Northern Range, Central and Southern Ranges, and these observations were attributed to mixing of genotypes owing to increased connectivity between lower-altitude river drainages following flooding of the basins during Pleistocene Orinoco River discharges.

Conclusions

We attribute the observed lack of geographical structure of *M. trinitatis* populations to continuous adaptation to fluctuating habitat conditions because it speciated from its most recent common ancestor in northern Venezuela by vicariance during the Miocene. As with *M. trinitatis*, most other herpetofauna populations in Trinidad are found in mountain ranges which repeatedly acted as refugia through glacial, interglacial periods and flooding of the basins. Therefore, species distribution appears to reflect past historical events that have drastically changed the topography of the island. These climatic changes throughout the Pliocene and Pleistocene are likely to have affected Trinidad's vegetation, changing macrohabitats and therefore species distributions. Although so far there have been few molecular studies in Trinidad (Camargo et al. 2006, 2009; Yanek et al. 2006; Jowers et al. 2008a) addressing the consequences of habitat change in the island and its effect on species population structure, it is plausible that other forest anurans with specific environmental and ecological specializations which inhabited Trinidad throughout the Pliocene and Pleistocene will show a similar pattern of population and geographical structure as *M. trinitatis*.

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Resumen

Diferenciación genética en el endemismo de Trinidad Mannophryne trinitatis *(Anura: Aromobatidae): vicarianza durante el Mioceno, diversificación in situ y ausencia de estructura geográfica en la isla*

Trinidad es un sistema único en el Caribe para estudiar patrones y procesos de especiación en anfibios. Empleamos secuencias de ADN mitocondrial para investigar las relaciones filogenéticas y patrones de variación genética en *Mannophryne trinitatis* de Trinidad. Estimaciones basadas en la aplicación de un reloj molecular apuntan a una divergencia genética entre *M. trinitatis* y su especie hermana *M. venezuelensis* que data del Mioceno superior (c. 7–8 Ma), lo que sugiere un proceso de especiación por vicarianza cuando Trinidad se separó del norte de Venezuela. El análisis filogenético de diez poblaciones de *M. trinitatis* de la Cordillera Norte y de cuatro de la Cordillera Central mostró la existencia de tres clados bien resueltos: un clado mayor, formado por haplotipos de localidades de la Cordillera Norte y dos clados más: uno formado por haplotipos de la Cordillera Central y otro que incluye haplotipos de la Cordillera Norte y un haplotipo de la Cordillera Central. En general, nuestros resultados muestran que la diversidad genética en *M. trinitatis* no está estructurada geográficamente. De acuerdo con la hipótesis de especiación por vicarianza, atribuimos la distribución actual de *M. trinitatis* y la falta de estructura genética a múltiples eventos de contacto secundario a consecuencia de cambios

climáticos que afectaron severamente la topología de Trinidad durante el Plioceno y Pleistoceno.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. List of specimens examined, genes sequenced and GenBank and museum accession numbers.

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