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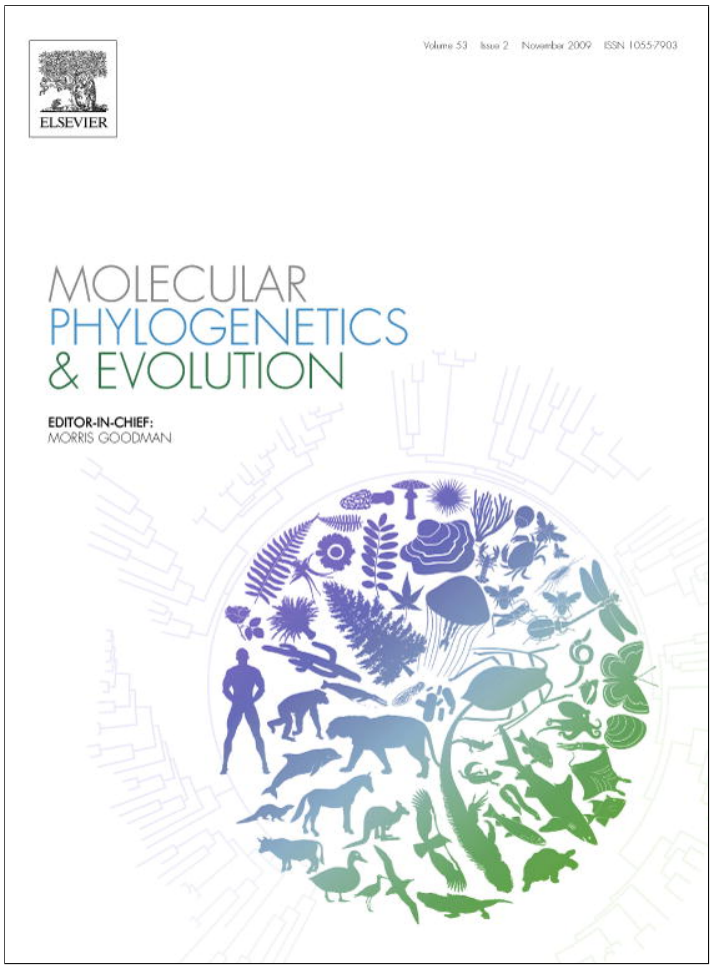

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Phylogeography of the frog *Leptodactylus validus* (Amphibia: Anura): Patterns and timing of colonization events in the Lesser Antilles

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ABSTRACT

The frog *Leptodactylus validus* occurs in northern South America, Trinidad and Tobago, and the southern Lesser Antilles (Grenada and St. Vincent). Mitochondrial DNA sequences were used to perform a nested clade phylogeographic analysis (NCPA), to date colonization events, and to analyze colonization patterns using on a relaxed molecular clock and coalescent simulations. *L. validus* originated on the mainland and first colonized Trinidad with subsequent independent colonizations of Tobago and the Lesser Antilles from Trinidad. The NCPA suggests a historical vicariant event between populations in Trinidad and Tobago from those in the Lesser Antilles. The colonization of Trinidad occurred ~1 million years ago (mya) and the colonization of the Lesser Antillean islands occurred ~0.4 mya. The coalescent approach supported the scenario where *L. validus* dispersed from Trinidad to St. Vincent and from there to Grenada, a dispersal event that could have been mediated by human introduction as recent as 1600 years ago.

1. Introduction

The West Indies is the group of islands comprising the Greater Antilles (Cuba, Jamaica, Hispaniola, Puerto Rico), the Lesser Antilles, Bahamas, and other small islands in the Caribbean Sea. The complex geological history of this archipelago with a unique balance between geographic isolation and size of the islands have provided an opportunity for colonization and adaptive radiation (Ricklefs and Bermingham, 2007). Most West Indian terrestrial vertebrates have their closest relatives in South America whereas fish and volant tetrapods (birds and bats) have closer ties with North and Central America (Hedges, 1996a). The accumulated evidence from biogeographic patterns, fossil records, phylogenetic relationships, molecular clock divergence estimates, and ocean currents support the idea of over-water dispersal for the vast majority of the terrestrial vertebrate taxa in the West Indies (Hedges, 1996a, 2006). The over-water dispersal hypothesis predicts that terrestrial vertebrates arrived in the West Indies predominantly from mainland South America (also from Central and North America) by active or passive (rafting) means (Hedges, 2006). However, there are two additional competing biogeographic hypotheses concerning the origin of the terrestrial vertebrates in the West Indies: (a) the proto-Antillean vicariance model and (b) the land-bridge model. The proto-Antillean vicariance model proposes that the West Indian fauna originated during the Cretaceous when the West Indies were an island arc between North America and South America that subsequently drifted eastward and fragmented until its current position (Hedges, 2006). Others have proposed that a land-bridge connection between the Greater Antilles and northern South America, called the Aves Ridge in the Caribbean Sea, occurred for a relatively short-time interval during the mid-Tertiary between 33 and 35 mya (Iturralde-Vinent and MacPhee, 1999). Whereas the vicariance hypothesis has been shown to represent a plausible explanation for a few ancient lineages (e.g., *Eleutherodactylus* frogs, *Solenodon* mammals, and *Cricosaura* lizards), the land-bridge explanation has not been confirmed with either paleogeographical or molecular divergence data (Hedges, 2006). The vicariant hypothesis predicts pre-Cenozoic divergences (>65 mya) and the land-bridge hypothesis predicts divergences that coincide with the putative emergence of a stable land bridge (33–35 mya). The available data for a number of lineages indicates divergences spread throughout the Cenozoic as predicted by the dispersal hypothesis (Hedges, 2006).

Except for *Eleutherodactylus* frogs, most amphibian lineages support the over-water dispersal hypothesis (Hedges, 1996b). A few frog genera radiated in the West Indies; however, others have only occasionally dispersed into the West Indies whereas they are very diversified on mainland. The latter is the case of the neotrop-

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ical genus *Leptodactylus*, which is represented in the West Indies by a few species: the endemics *L. albilabris* and *L. fallax* (Hedges and Heinicke, 2007; Frost, 2009), and the recently demonstrated non-endemic *L. validus* (Yanek et al., 2006). Even though the divergence times of *L. albilabris* and *L. fallax* from their South American relatives (24–58 and 23–34 mya, respectively) overlap with the hypothesized emergence of the land bridge (33–35 mya), Hedges and Heinicke (2007) discarded this hypothesis due to the lack of geological and biological evidence, and concluded that these species dispersed over-water to their present locations during the Cenozoic. However, there are a number of reasons to suspect that time estimates from their study could be biased towards older divergences and consequently, their data may in fact support earlier divergence times (<33 mya) as predicted by the over-water dispersal hypothesis. First, Hedges and Heinicke (2007) sampled three of the four traditionally recognized "species groups" of *Leptodactylus* and calibrated the root of their tree at 65 mya because "many interspecific divergences in *Leptodactylus* date to the early Cenozoic". Actually, the 65 mya estimate corresponds to the minimum divergence of the four species groups of *Leptodactylus* based on an immunological study of the genus by Maxson and Heyer (1988). Because Hedges and Heinicke (2007) did not sample the four species groups, we cannot discard the possibility that the root in their tree actually represents a divergence event within *Leptodactylus* more recent than 65 mya. Second, particularly in the case of *L. albilabris*, Hedges and Heinicke (2007) acknowledged that the closest relative to the West Indian species may not have been included in their analyses given their limited sampling, which allowed them only to set an upper boundary in their estimates. Finally, *L. albilabris* occurs on the Puerto Rican bank and the Dominican Republic, and *L. fallax* occurs only in the northern Lesser Antilles, which makes it difficult to infer if these species dispersed over-water to their present locations either directly from the mainland or via sequential colonization of intermediate islands. In contrast, the wider distribution of *Leptodactylus validus* in northern South America, Trinidad, Tobago, and the southern Lesser Antilles offers an opportunity to reconstruct and to date a colonization history based on a sampling of all inhabited islands, the mainland as well as closely related species.

The aim of this study is to analyze the phylogeographic patterns of *Leptodactylus validus* to: (1) reconstruct the sequence in which islands were colonized and (2) infer colonization times of the Lesser Antilles, Trinidad, and Tobago islands. Previously, Yanek et al. (2006) demonstrated that populations on the islands form a monophyletic group relative to mainland populations. In addition to sampling the entire distribution of *L. validus* for performing a nested clade phylogeographic analysis (NCPA), multiple outgroup species were included to represent the taxonomic diversity within *Leptodactylus* and to reconstruct a key node for which a molecular calibration is possible: the *Leptodactylus* root. This study combines strategic taxon sampling with a relaxed-clock model in a Bayesian framework to obtain estimates of colonization times that incorporate uncertainty in rate variation across the tree, and in tree topology/branch lengths. We also employed a coalescent method in a statistical phylogeographic approach to distinguish between alternative colonization routes that could not be resolved with a classical phylogeographic analysis. This coalescent method has the unique feature of estimating the probability of mutations occurring in different subpopulations (Kuhner, 2008), but this approach has been rarely used in biogeographic studies despite its powerful ability to resolve alternative dispersal histories (see Milot et al., 2000, for an example). In addition to testing the dispersal, vicariant, and land-bridge biogeographic hypotheses, we also used the divergence-time estimates to evaluate the plausibility of human-mediated transportation of *L. validus* between the Lesser Antillean islands (Murphy, 1997).

2. Materials and methods

2.1. Geographic sampling

Mitochondrial DNA sequences of the 12S and 16S ribosomal genes (2286 bp) from 52 individuals from the mainland and several islands where *L. validus* occurs were taken from GenBank. These sequences were previously used in a phylogenetic study to elucidate the taxonomic status of *L. validus* and *L. pallidirostris* (Yanek et al., 2006; GenBank Accession Nos.: EF613120–EF613180, EF632000–EF632060). Excluding indel positions, there are 13 haplotypes in the combined 12S + 16S dataset of *L. validus* distributed among the islands of Grenada ($N = 20$), St. Vincent ($N = 13$), Trinidad ($N = 15$), Tobago ($N = 2$), and the mainland ($N = 2$) (Table 1 and Fig. 1). This sampling was based on all available tissues at hand, which is sufficient to determine the source of the populations that colonized the islands, although we acknowledge that we might not have sampled all haplotypes on the mainland. In addition, the directionality of dispersal events within *L. validus* relied on the inclusion of both closely and distantly related outgroups: two species from the *melanonotus* species group (*L. wagneri* and *L. podicipinus*) and three members of other species groups (*L. chaquensis* and *L. knudseni* of the *ocellatus* and *pentadactylus* groups, respectively). We also added the 12S and 16S sequences of *L. fuscus* to this dataset (GenBank Accession No.: DQ283404) to complete the sampling of the taxonomic diversity, i.e., the four traditionally recognized species groups within *Leptodactylus*, and to apply an appropriate calibration to the root of the tree (see below).

2.2. Relaxed-clock divergence estimates

The software program BEAST v1.4.8 (Drummond and Rambaut, 2007) was used to estimate time of divergence of clades under a Bayesian inference framework. The sampling design concentrated on *L. validus* but also included closely related species and representatives of the diversity within *Leptodactylus* to date divergences using a more reliable calibration of the tree root. We used BEAUti (provided in the BEAST package) to set the substitution model, priors, and MCMC conditions for estimating posterior distributions of the time to the most recent common ancestor (TMRCA) of four splitting events of interest. The analysis used the GTR+I+Γ model of nucleotide substitution, which was the best-fitting model to the data as found with Modeltest based on both likelihood-ratio tests and Akaike information criterion (Posada and Crandall, 1998). The input file was modified by hand to partition analysis between genes via duplication of model parameters and MCMC operators. Maximum likelihood analyses in PAUP* (Swofford, 2002) based on 10 independent searches with random addition of sequences and the GTR+I+ Γ model were run to compare the likelihood of an unconstrained topology against an enforced molecular clock using a likelihood-ratio test. This test rejected a strict molecular clock ($-\ln L$ unconstrained = 7646.0; $-\ln L$ molecular clock = 7680.6, $\chi^2 = 69.2$, df = 21, $P < 0.001$) and therefore, we selected a relaxed-clock model in BEAST with an uncorrelated, log-normally distributed rate across branches (Drummond et al., 2006). Default priors were used for all parameters except for the tree and root-height priors. We used a coalescent tree prior with constant population size for the *L. validus* clade (demographic reconstructions showed constant population size in Bayesian Skyline plots, see Supplementary data) and an unspecified prior for other branches of the *Leptodactylus* tree following a multi-demographic approach (Ho et al., 2008). The mean of the tree root height was set to 65 mya (standard deviation 20 my) because this calibration corresponds to the presence of the four traditionally recog-

Table 1
List of *L. validus* localities sampled in this study. Codes refer to the locality numbers in Fig. 1. Haplotype names are those used in the nesting clade design in Fig. 3 and *N* is the sample size for each haplotype on each locality.

Code	Locality	Latitude	Longitude	Haplotype(*N*): Voucher
1	Grenada: St. Andrew; Spring Gardens Estate	12 06 00N	61 41 00W	A(1): BWMC 06881
2	Grenada: St. Andrew; Birch Grove	12 06 00N	61 40 00W	A(2): BWMC 06882–06883
3	Grenada: St. George; Beausejour	12 06 00N	61 44 00W	A(1): BWMC 06939
4	Grenada: St. George; Grand Anse Bay	12 01 16N	61 46 00W	A(1): USNM 314794 B(4): USNM 314793, 314795, 314796, 314798
5	Grenada: St. George; inland from Grand Anse Bay	12 00 58N	61 46 00W	A(4): USNM 314813, 314819, 314820, 314831 B(7): USNM 314814–314818, 314821, 314822
6	St. Vincent: St. Andrew; near Vermont	13 12 00N	61 14 00W	A(1): USNM 314512
7	St. Vincent: St. George; Arnos Vale	13 08 37N	61 13 20W	A(5): USNM 314513–314515, 314719, 314722 B(5): USNM 314720, 314721, 314723, 314724, 314718
8	St. Vincent: St. George; Rose Cottage	13 08 00N	61 12 00W	A(2): USNM 314516, 314517
9	Tobago: St. Paul; Delaford, Louis d'Or River	11 16 00N	60 34 00W	C(2): USNM 523940, 523941
10	Trinidad: St. Patrick; near Chatham Beach	10 07 33N	61 44 40W	D(7): USNM 314627, 314628, 314631–314635 E(1): USNM 314629 F(1): USNM 314630 K(1): USNM 314636
11	Trinidad: St. George; west of Carapo	10 35 21N	61 17 27W	D(1): USNM 314672
12	Trinidad: St. George; north of Simla Research Station	10 42 00N	61 18 00W	G(1): USNM 286959
13	Trinidad: St. George; Carapo	10 35 27N	61 18 41W	H(1): USNM 314671
14	Trinidad: St. George; Simla Research Station	10 41 00N	61 17 00W	I(1): USNM 286948
15	Trinidad: St. George; near Brasso Seco Village	10 45 00N	61 16 00W	J(1): USNM 306105
Guyana	Guyana: Northwest District; Baramita	07 21 00N	60 29 00W	L(1): USNM 535774
Brazil	Brazil: Roraima, Igarapé Cocal	03 45 00N	61 44 00W	M(1): USNM 302408

Museum abbreviations: BWMC = Bobby Witcher Memorial Collection, Avila University; USNM = National Museum of Natural History, Smithsonian Institution.

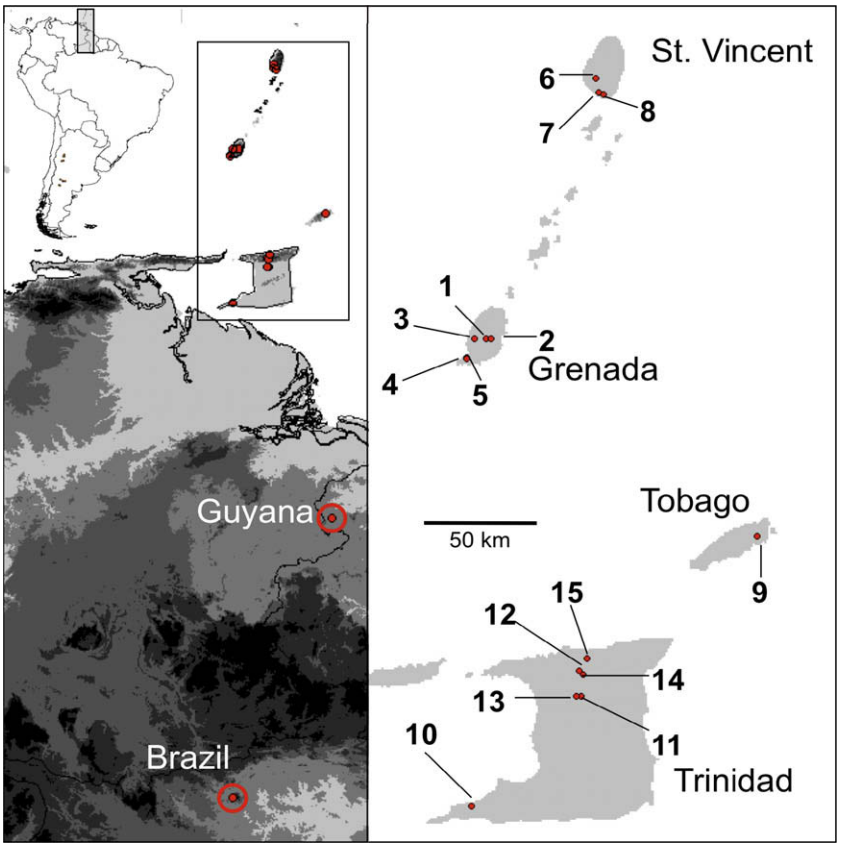

Fig. 1. Map of South America showing sampled *L. validus* localities. Locality numbers correspond to the code used in Table 1.

nized *Leptodactylus* species groups (Maxson and Heyer, 1988). Since the actual origin of *Leptodactylus* could in fact be older than 65 mya, we used a log–normal distribution for the tree root-height prior that is skewed towards older times to accommodate uncertainty in our calibration. No other calibrations are available for *Leptodactylus*; we preferred not to use geological events such as island ages because we would have to assume immediate colonization after island emergence. Dates of four splitting events were estimated in the analysis using TMRCA statistics: (1) insular clade vs. mainland sample, (2) Lesser Antilles vs. Trinidad, (3) Tobago vs. Trinidad, and (4) haplotype A vs. B. We used the auto-optimize option for the operators of the MCMC chain that was run for 20 mil-

lion states, sampled every 1000 generations, and a burn-in of 2 million states. The analysis was run 5 times to test for stability and convergence of MCMC chains in plots of posterior log likelihoods in Tracer v1.4 (Rambaut and Drummond, 2007). Posterior samples from all runs were combined and analyzed in Tracer v1.4 to obtain mean estimates and 95% highest posterior densities (HPD) of TMRCAs.

2.3. Nested clade analysis

To assess evidence for colonization or vicariance, a statistical parsimony network of *L. validus* haplotypes (Templeton et al., 1992) was constructed using the program TCS v1.21 (Clement et al., 2000) considering indels as a fifth state and enforcing a 95%-confidence limit for connecting haplotypes. The loops in the haplotype network were resolved based on expectations of coalescence theory and haplotypes were nested in groups to perform a nested clade phylogeographic analysis (NCPA) (Templeton et al., 1995; Templeton, 2004). The NCPA was used to test for non-random associations of haplotype groups and geography and therefore to test for geographic breaks in haplotype distributions. The nested design and geographic coordinates of sampled localities were input in GeoDis v2.5 (Posada et al., 2000) to infer historical and demographic population histories based on the latest inference key (updated on December 15, 2008).

2.4. Coalescent-based analysis

Following a statistical phylogeographic approach (Knowles and Maddison, 2002), we also applied a coalescent-based method that obtains the probabilities of specific mutations originating in separate geographic populations (=islands) as implemented in the program Genetree 9.0 (Griffiths, 1994; Bahlo and Griffiths, 2000). The method uses coalescent simulations for obtaining joint-likelihood estimates of the temporal and spatial distribution of mutations and most recent common ancestors (MRCAs) assuming a neutral coalescent process and an infinite-sites model (ISM) (Griffiths and Tavaré, 1994, 1997). We evaluated the fit of our sequence data from Grenada, St. Vincent, and Trinidad to the assumptions of the ISM with the program *seq2tr*, which is included in the Genetree package. First, we removed all sites incompatible with ISM (base positions 12, 464, 631, 891, and 950), but we had to exclude four rare haplotypes from Trinidad to retain eight polymorphic sites (base positions 191, 903, 582, 1336, 1546, 1581, 1968, and 2089) that distinguish between the haplotypes occurring in Grenada, St. Vincent, and four representative haplotypes from Trinidad (F, G, H, and I). The program *seq2tr* was also used to estimate a gene tree from the empirical sequence data that serves as an input file for Genetree. All individuals from each of the three islands were used to estimate genetic diversity ($\theta_w = N_e\mu$) and the migration rates between islands ($M = N_e m$) using Migrate-n 3.0.3 (Beerli and Felsenstein, 2001) based on two replicate MCMC-maximum likelihood analyses consisting of 10 short and 6 long runs, each of them with four incrementally heated chains. We input genetic diversity values and migration rates together with the pruned dataset to estimate distributions of mutations among subpopulations, using 10 million replications and assuming constant population sizes since demographic reconstructions demonstrate stable population sizes through time in all three islands (see Supplementary data). Analyses were run 10 times with different random seeds to estimate mean and standard deviations of probabilities and as a procedure for validation of the analyses (see Kuhner, 2008). A gene tree with the ages of mutations and scaled with the TMRCA of all haplotypes was produced with *treepic* (provided with the Genetree package). The TMRCA of all haplotypes and the mutation ages estimated in coalescent units (T) were transformed to time in years (t) using the equation $t = 2N_eT$ described in the Genetree manual, where N_e (inbreeding effective population size) was calculated using θ_w and μ was estimated with a relaxed molecular clock (see below). A mean generation length of 3 years was assumed based on the available estimates for *Leptodactylus* species (*L. bufonius*, Reading and Jofré, 2003; *L. pentadactylus*, Galatti, 1992) to transform the time units of the mutation rate from generations to years, and this rate was multiplied by the number of base pairs (2286) to transform the rate from substitutions per nucleotide to substitutions per gene.

3. Results

3.1. Colonization patterns

The Bayesian phylogeny of haplotypes shows that mainland haplotypes (L and M) are basal within *L. validus* (Fig. 2). The mainland lineage gave rise to haplotype F in Trinidad, which gave rise to the Tobago haplotype (C) and to haplotype D also found in Trinidad as shown in the haplotype network (Fig. 3). Haplotype D is ancestral to haplotype B, currently found in Grenada and St. Vincent, and haplotype A, which is present on both islands, is derived from haplotype B (Fig. 3). Thus, based on the data and the underlying assumption of parsimony, both Grenada and St. Vincent were colonized from a single lineage, i.e., a single wave of colonization from Trinidad, but which island was colonized first is unresolved based on the geographic distribution of haplotypes A and B. In addition, it is unclear if haplotypes A and B originated on the same island and dispersed in the same direction to the other island or if each haplotype originated on different islands and coexist today in both islands due to dispersal in opposite directions. The NCPA detected significant association of groups with geographic distances in three nesting groups: group 2-2, group 3-1, and the total cladogram (see nested design in Fig. 3). The inference for group 2-2 in Trinidad was inadequate geographic sampling. For group 3-1 that compares group 2-1 in Grenada and St. Vincent with group 2-2 in Trinidad, allopatric fragmentation was inferred. At the level of the total cladogram, the sampling design is inadequate to discriminate between isolation by distance vs. long distance dispersal (Table 2). Although results show significant geographic association of mainland vs. insular groups at the highest nesting level, additional mainland sampling is needed to clarify historical or demographic processes among mainland and insular populations.

3.2. Timing of colonization events

The Bayesian analysis in BEAST showed convergence of posterior likelihoods between runs. For all parameters of interest, the effective sample sizes were higher than 200, suggesting stabilization and good mixing of the MCMC chains. Therefore, samples from the five independent runs were combined for obtaining summary statistics. The estimated mean substitution rate was 1.79×10^{-3} substitutions/site/million years with a 95% HPD between 1.27 and 2.35×10^{-3}. The colonization of Trinidad that corresponds to the TMRCA of all insular samples and the mainland sample from Guyana (haplotype L) occurred 1.025 mya (95% HPD: 0.333–1.894) (Fig. 4). The colonization of Grenada and St. Vincent islands, estimated as the TMRCA of Lesser Antillean haplotypes A and B, and the Trinidad's haplotypes D, E, K, J, and I, occurred 399 kya (95% HPD: 117–761). The colonization of Tobago, represented by the splitting event between haplotype C from Tobago and haplotype F from Trinidad, was dated to 219 kya (95% HPD: 15–761). Finally, the split between haplotypes A and B present in Grenada and St. Vincent occurred 85,250 years ago (95% HPD: 1677–220,000) (Fig. 4).

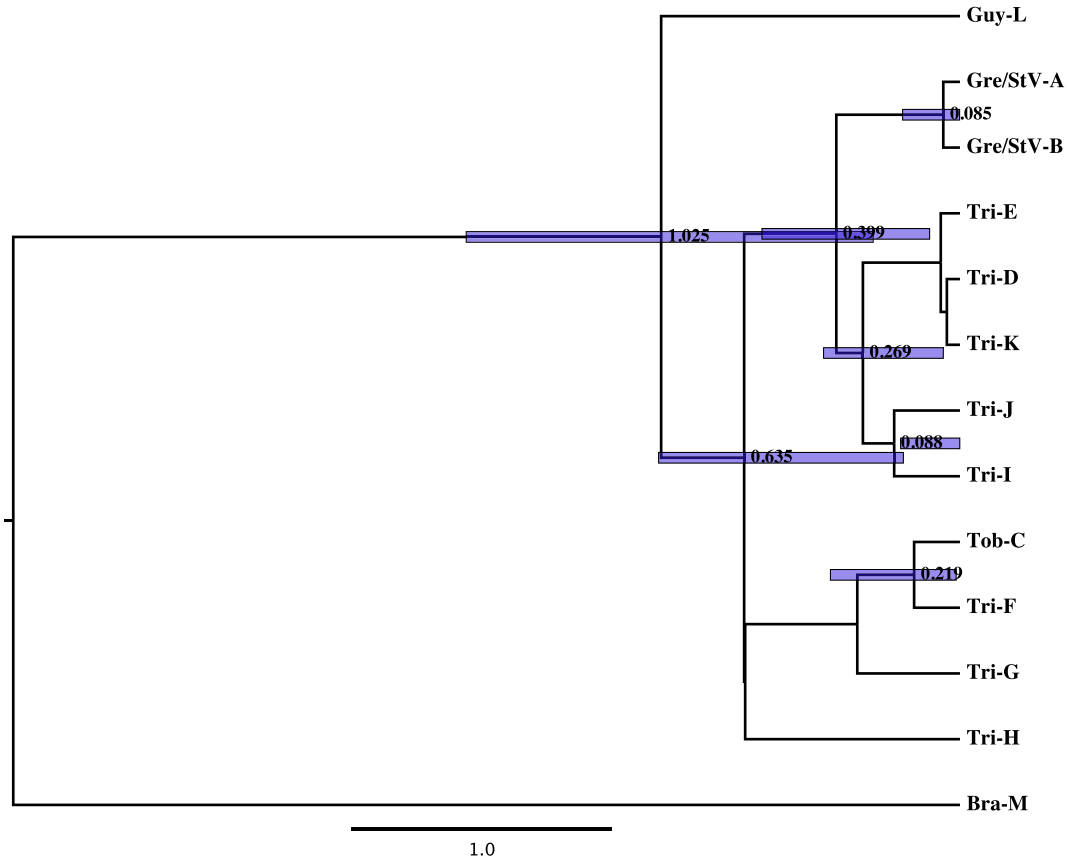

Fig. 2. Maximum credibility tree from Bayesian analysis with Beast based on 36,000 sampled trees from the posterior distribution. Shaded bars on nodes represent 95% highest posterior density and numbers next to the nodes are mean age estimates in million of years. Outgroup taxa used in the analysis were excluded from the tree to show divergence times within *L. validus*. Names of terminal taxa indicate the population(s)-haplotype: Bra, Brazil; Guy, Guyana; Tri, Trinidad; Tob, Tobago; Gre, Grenada; and StV, St. Vincent. Haplotype names match those used in Table 1. The scale bar represents 1 million years.

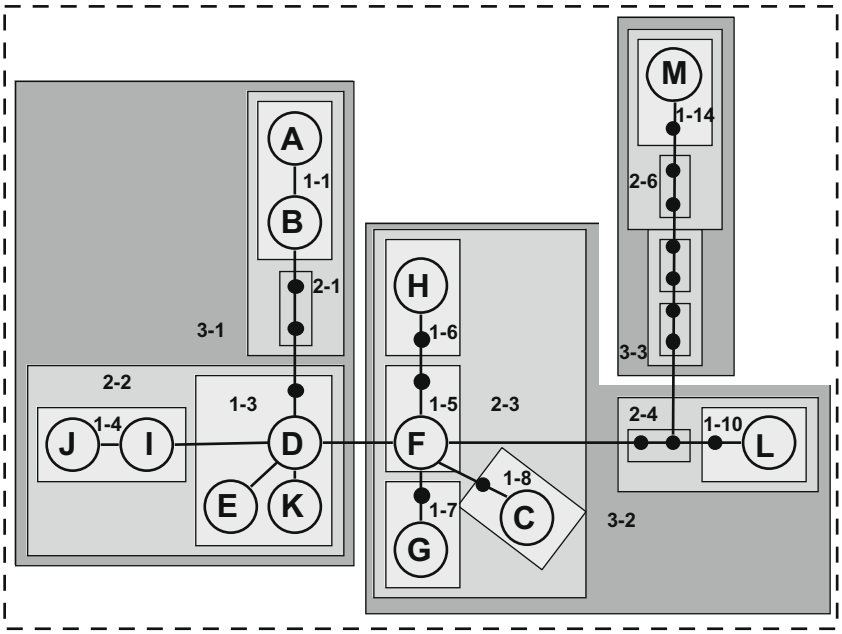

Fig. 3. Nested group design of *L. validus* haplotype network. Haplotype names match those used in Table 1. Dots represent missing haplotypes. Nested group are shown in boxes with darker shades corresponding to higher nesting levels. Numbers identify nested groups: the number before the dash indicates the nesting level and the number after the dash is the group number.

3.3. Coalescent-based analysis

The average genetic diversity among Trinidad, Grenada, and St. Vincent was $\theta_w = 5.05$, and for each island separately these were: $\theta_w = 8.94$ (Trinidad), $\theta_w = 4.92$ (Grenada), and $\theta_w = 1.30$ (St. Vincent). Average migration rates based on two replicate runs in Migrate-n, and measured as the number of migrants per generation going one way, was zero between all pair-wise comparisons except

Table 2
NCPA results and inferences based on latest inference key.

Nesting clade	Test of I vs. T		Nested clades	Geographic distance		Inference chain
	Dc	Dn		Dc	Dn	
2-2	31.8(L)*	12.5	1-3(I)	35.6(L)*	34.9(L)*	1. yes, 19. yes, 20. no: IGS
			1-4(T)	3.8	22.5	
3-1	−33.2	69.8(L)**	2-1(T)	61.8(S)**	76.7(S)**	1. yes, 19. no: AF
			2-2(I)	28.6(S)**	146.5(L)**	
Total cladogram	54.9	−5.6	3-1(T)	99.1(S)**	132.9(S)*	1. no, 2. yes, 3. yes, 5. no, 6. no, 7. no, 8. no: IBD/LDD
			3-2(I)	151.8	141.8	
			3-3(T)	0	799.1(L)*	

Abbreviations: Dc, within group distance; Dn, nested group distance; (I), interior group; (T), tip group; (S), significantly smaller distance than expected; (L), significantly larger distance than expected; IGS, inadequate geographic sampling; AF, allopatric fragmentation; IBD/LDD, isolation by distance or long distance dispersal.
* $P < 0.05$.
** $P < 0.01$.

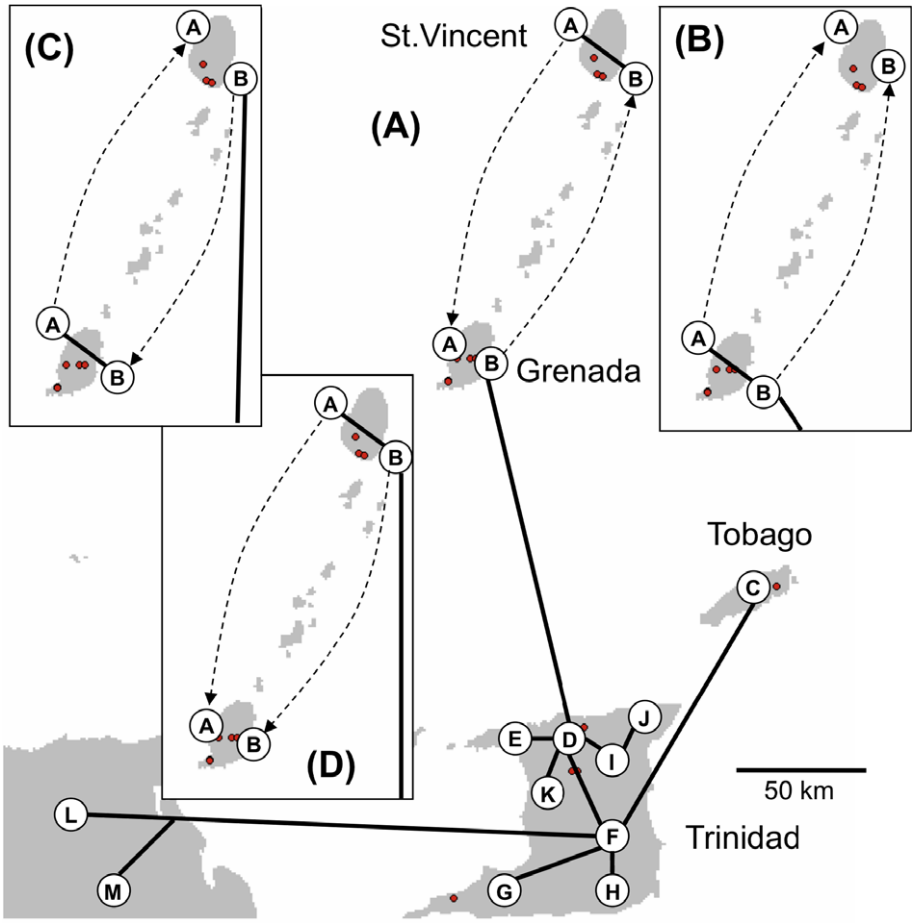

Fig. 4. Geographic distribution of the *L. validus* haplotype network. Mutational steps between haplotypes are shown in Fig. 3. Dotted lines represent dispersal events carrying haplotypes between islands. Haplotype names match those used in Table 1. Insets B, C, and D show alternative colonization scenarios for Grenada and St. Vincent (see text).

for the migration from St. Vincent to Grenada which was $M = 32.2$. After pruning the dataset to meet the assumption of the ISM, eight base positions (191, 582, 903, 1336, 1546, 1581, 1968, and 2089) representing six haplotypes (A, B, D, E, J, and K) were analyzed to estimate the geographic origin of mutations. Three mutations (1546, 1581, and 2089) in the gene tree support a sister-lineage relationship between haplotypes A and B, which are present in Grenada and St. Vincent, whereas the relationships with other haplotypes from Trinidad were unresolved (Fig. 5). Across the 10 independent runs in Genetree with different seeds, the mutation in position 2089, which produced haplotype B (present today in both Grenada and St. Vincent), and the mutation in position 191, which defines haplotype A (also present in both islands), had a probability >0.95 of having occurred in St. Vincent (Table 3).

The mutation rate was 1.23×10^{-5} substitutions per gene per generation after transforming the BEAST estimate expressed in substitutions per site per million years and assuming a generation length of 3 years. Based on this mutation rate, the genetic diversity estimated in DnaSP ($\theta_w = 5.05$) corresponds to an inbreeding effective population size (Ne) of 410,569. The average TMRCA of all samples from Grenada, St. Vincent, and Trinidad, which dates back to the colonization of Trinidad, was $T = 1.735$ (±0.167), which is equivalent to $t = 1.425$ (±0.137) million years. The age of mutation 2089, which dates back to the MRCA of haplotypes A and B, was $T = 0.941$ (±0.184) coalescent units, which equals $t = 772{,}691$ (±151,089) years. Based on the same parameters, the age of mutation 191 exclusive of haplotype A was $T = 0.458$ (±0.052), which is equal to $t = 371{,}959$ (±42,700) years (Table 3 and Fig. 5).

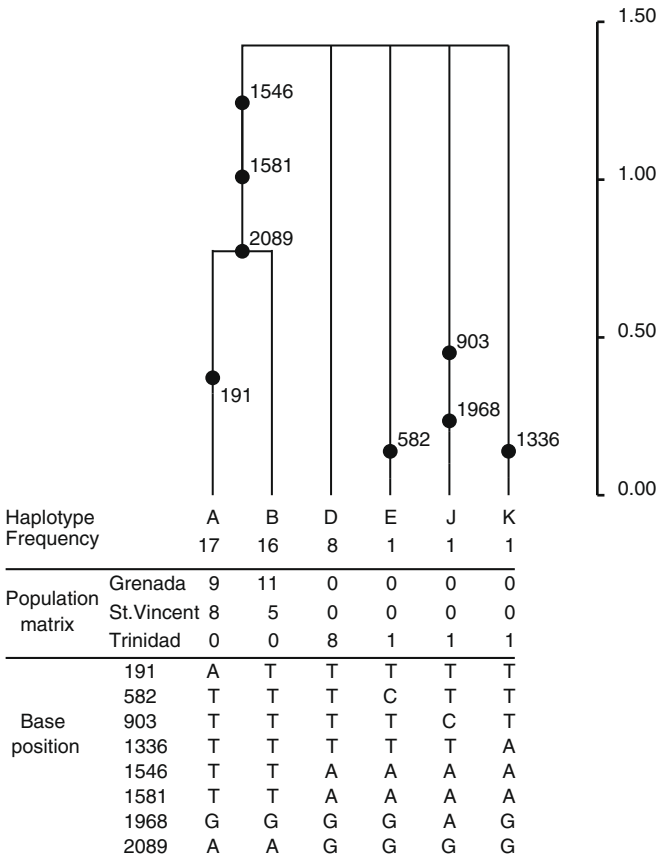

Fig. 5. Distribution of mutations across the gene tree as estimated in Genetree. Dots represent point mutations that are identified with the base position in the dataset. Total haplotype frequencies, haplotype frequencies within populations, and nucleotide bases in each of eight mutation sites are shown for all six haplotypes. The scale represents time in millions of years. Haplotype names match those used in Table 1.

4. Discussion

4.1. Colonization patterns

Because all divergences within *Leptodactylus validus* occurred in the Pleistocene, ruling out the vicariance and land-bridge hypotheses, we spend the rest of the discussion in deciphering the sequence of colonization events and the timing of these events that took place via over-water dispersal. The pattern of exclusive haplotypes in Trinidad, Tobago, and the southern Lesser Antilles allowed the reconstruction of a partially resolved history of island colonizations using a statistical parsimony network. Except for showing that haplotypes from Grenada and St. Vincent form a clade, previous phylogenetic analyses were unable to resolve relationships among *L. validus* haplotypes (Yanek et al., 2006). The phylogenetic tree rooted with several *Leptodactylus* species shows that the Trinidad populations of *L. validus* are derived from the mainland (Fig. 2) and the statistical parsimony network shows that the Lesser Antillean islands and Tobago were colonized in separate events from different lineages in Trinidad (Fig. 3).

The NCPA inferences suggest a significant allopatric fragmentation event between the Lesser Antilles and Trinidad. Available data on advertisement call, a common indicator of species boundaries in frogs, show no differences among islands, but there is slight differentiation in adult morphology between Trinidad and the Lesser Antillean islands (Heyer, 1994). The exclusive haplotypes in Grenada and St. Vincent and the morphological differentiation suggest that Lesser Antillean populations could represent a different evolutionary lineage from the one found in Trinidad. Species limits involving the Lesser Antillean populations should be tested with additional data; ideally nuclear DNA markers with high rates of variation could confirm or refute the distinctness of the Lesser Antillean populations. The presumed occurrence of a distinct species restricted to Grenada and St. Vincent [and probably in the Grenadines where *L. validus* also occurs (Daudin and de Silva, 2007)] would fit typical biogeographic patterns in the region (Heyer, 1994) and support the interpretation that the Lesser Antilles have functioned as a selective barrier ('biogeographic filter') for some mainland species that have dispersed to the West Indies (Hedges, 1996a, 2006). In the case of *L. validus*, a lineage was able to colonize Grenada, St. Vincent and the Grenadines, and differentiate from ancestral lineages in Trinidad. The Grenada/St. Vincent/Grenadines lineage has not been able to disperse further north along the chain of islands in the Lesser Antilles.

The colonization(s) of the Lesser Antilles from Trinidad is consistent with the expected dispersal patterns through rafting based on the direction of the Guiana current (Hedges, 2006). Rafts formed of natural vegetation originating from the mouth of major South American rivers have been proposed as a mechanism to explain the scattered pattern of divergences of West Indian taxa from their South American ancestors throughout the Tertiary. Similar colonization patterns, probably also mediated by rafting, have been found in other vertebrates, e.g., the snakes *Corallus hortulanus* (Henderson, 1997) and *Liophis melanotus* (Dixon and Michaud,

Table 3
Probability of mutations in each of three subpopulations and ages of these mutations and mRCA derived from coalescent simulations in Genetree. Results from 10 runs each with different starting random seeds are shown. Mean and standard deviation (SD) of probabilities across the 10 runs for each subpopulation (Grenada = Gre, St. Vincent = StV, and Trinidad = Tri) are shown. Ages of mutations and mRCA for each run and mean and SD values averaged across runs are shown.

Mutations	Probability						Time		
	191			2089			mRCA	191	2089
Runs	Gre	StV	Tri	Gre	StV	Tri			
1	0.000	1.000	0.000	0.000	0.947	0.053	1.745	0.464	0.919
2	0.000	1.000	0.000	0.000	0.943	0.057	1.917	0.571	1.190
3	0.000	1.000	0.000	0.000	0.999	0.001	1.462	0.430	0.523
4	0.000	1.000	0.000	0.000	0.988	0.012	1.522	0.425	0.969
5	0.000	1.000	0.000	0.000	0.982	0.018	1.778	0.438	0.853
6	0.000	1.000	0.000	0.000	0.985	0.015	1.826	0.405	0.918
7	0.000	1.000	0.000	0.000	0.995	0.005	2.007	0.454	1.170
8	0.000	1.000	0.000	0.000	0.991	0.009	1.701	0.455	0.914
9	0.000	1.000	0.000	0.000	0.985	0.015	1.622	0.524	1.020
10	0.000	1.000	0.000	0.000	0.825	0.175	1.766	0.414	0.938
Mean	0.000	1.000	0.000	0.000	0.964	0.036	1.735	0.458	0.941
SD	0.000	0.000	0.000	0.000	0.052	0.052	0.167	0.052	0.184

1992), and the parthenogenetic lizards *Gymnophthalmus underwoodi* (Kizirian and Cole, 1999) and *Kentropyx borckiana* (Cole et al., 1995).

4.2. Timing of colonization events

The Pleistocene colonization of Trinidad, Tobago, and the Lesser Antilles are consistent with over-water dispersal and do not support the hypotheses of proto-Antillean vicariance or land-bridge dispersal that predict much older divergences than those found in this study. Therefore, this study corroborates earlier results that *L. validus* originated in northern South America and invaded Trinidad ~1 mya and then Tobago ~220 kya, suggesting these populations could have maintained genetic contact with the mainland during any of their intermittent connections during the Pleistocene glacial cycles. Trinidad and Tobago are continental islands with a maximum depth of 91 m between them and 38 m between Trinidad and the mainland (Murphy, 1997). Therefore, repeated episodes of connection and isolation between Trinidad and Tobago and the mainland occurred during the glacial cycles up to the Last Glacial Maximum (LGM, ~21 kya), when the sea level was 120–135 below current sea levels (Clark and Mix, 2002) and the islands were contiguous with the mainland of South America for the last time (Murphy, 1997).

The Lesser Antillean islands were colonized ~400 kya (not earlier than 117 kya), which is also consistent with previous estimates that *L. validus* colonized the southern Lesser Antilles in the Pleistocene (Heyer, 1994; Hedges, 1996b; Murphy, 1997). The 117 kya lower bound for this event does preclude the possibility of human transportation between Trinidad and the Lesser Antilles since humans reached Trinidad only ~8 kya from the Orinoco river delta in Venezuela (Wilson, 2007). In addition, the dispersal event that carried haplotype A between the Lesser Antillean islands occurred ~85 kya, and as recently as ~1.6 kya, suggesting that *L. validus* could have been transported by humans between these islands as suggested in previous studies (Murphy, 1997). Archaeological evidence indicates that the first human populations arrived in Grenada by canoeing from Trinidad ~5 kya (Wilson, 2007), meaning that humans could have occupied the Lesser Antilles when haplotype B appeared in St. Vincent and migrated to Grenada (as suggested by Genetree analyses). On the other hand, the intervening Grenadines islet chain between Grenada and St. Vincent could also have facilitated natural dispersal when lower sea levels formed larger stepping-stones between Grenada and St. Vincent in the late Pleistocene (Pregill and Olson, 1981). Bathymetric maps show that maximum sea depths in the Grenadines archipelago are 200 m (Maury et al., 1990), and therefore these islands could have been larger, closer to each other and even forming contiguous landmasses at the LGM when sea levels were 120–135 m lower than today (Clark and Mix, 2002). The almost contemporaneous occurrence of this event and the wide confidence intervals associated with relaxed-clock estimates make human transportation conceivable.

4.3. Resolution of alternative colonization hypotheses

The unresolved colonization history of Grenada and St. Vincent based on the haplotype network yield four possible dispersal scenarios that account for the current haplotype distributions in the Lesser Antilles (Fig. 4). First, haplotype D could have colonized Grenada and given rise in situ to haplotype B, followed by dispersal of haplotype B to St. Vincent. Haplotype B could have given rise on St. Vincent to haplotype A, which subsequently dispersed to Grenada (Fig. 4A). Alternatively, haplotype A could have originated in Grenada and both haplotypes A and B dispersed to St. Vincent (Fig. 4B). Two additional dispersal scenarios are possible if haplotype B differentiated first in St. Vincent instead of Grenada (Fig. 4C and D). Taking into account geographic distances and a more parsimonious explanation of the data, we can conclude that dispersal scenarios A and B are more likely because Grenada is geographically closer to Trinidad than to St. Vincent. In this study, we used a coalescent-based simulation approach to discriminate between these alternative dispersal scenarios that cannot be resolved with a haplotype network alone. The results from Genetree unambiguously indicate that haplotypes A and B, derived from haplotype D in Trinidad, have a significantly higher probability of origin in St. Vincent and subsequently they dispersed to Grenada, supporting the colonization history shown in Fig. 4D. Although dispersal from St. Vincent to Grenada is not consistent with the more general patterns of natural over-water dispersal in other taxa driven by ocean currents (Hedges, 2006), because it was a very recent event (as recent as 1600 years ago), human introduction could have mediated this dispersal event as discussed above. Another possibility is that haplotype A might have evolved twice, once in Grenada and once in St. Vincent, since this haplotype is only one mutational step from haplotype B.

4.4. Integration of phylogeographic approaches

The pattern and timing of historical events are of primary interest in phylogeographic studies. A single approach cannot provide inferences for all these questions simultaneously, and every approach has its assumptions and limitations (Nielsen and Beaumont, 2009). It is of fundamental importance for phylogeographic inference first to identify a plausible set of alternative hypotheses about historical events and secondly, to use methods based on different assumptions that ideally can discriminate among the alternative hypotheses (Knowles and Maddison, 2002; Knowles, 2004). Moreover, the same historical events should ideally also be cross-validated using different methods that enable robust inferences not sensitive to the assumptions of any particular inferential procedure. In this study, we utilized two independent approaches, haplotype networks/NCPA and a coalescent-based method for discriminating between alternative colonization patterns in the Lesser Antillean islands. Despite recent criticisms about the usefulness of NCPA because of its high false positive rate (Petit, 2008; Knowles, 2008; but see Garrick et al., 2008; Templeton, 2008, 2009a,b), NCPA is still a useful method for formulating hypotheses about historical events and demographic processes to be tested with coalescent-simulation approaches. Because Grenada and St. Vincent share the same two haplotypes, phylogenetic analyses and haplotype networks are unable to resolve colonization events, but the Genetree results suggest that St. Vincent was colonized first. In addition, a Bayesian relaxed-clock method and Genetree cross-validated the timing of these colonization events, even though the latter method uses only a subset of the data and both methods are based on different assumptions: Beast is a Bayesian method based on correlated sampling of genealogies but Genetree is a likelihood approach that draws independent samples of parameter values. As Kuhner (2008) recently noted, coalescent genealogy samplers like Genetree are statistically powerful and robust but, due to their assumptions about the population model, their agreement with unrelated methods greatly strengthens the inferences made.

Estimated divergence times in Genetree are somewhat higher than those obtained with Beast but ranges overlap for the two older events: the dispersal from the mainland to Trinidad (BEAST ~1.025 mya, Genetree 1.425 mya) and the colonization of the Lesser Antilles (BEAST ~400 kya, Genetree ~772 kya). On the other hand, the divergence of haplotype A from B is definitively lower for the BEAST estimate (~85 kya) than for Genetree (~372 kya). It should be noted that these time estimates from Genetree are only approximate since they are based on Ne calculations that as-

sumed panmixis among the three island populations but population substructure could have biased upwards the coalescence times (Jesus et al., 2006). In addition, we also assumed that our point estimate of the mutation rate based on the Beast analysis of *Leptodactylus* is conserved and applies to the divergences within *L. validus*, and finally, the assumption about generation length is based on limited data for other species of *Leptodactylus*. The roughly similar time estimates from two separate methods lends further confidence to the probabilities of mutations found in Genetree that were used to distinguish between alternative colonization histories. This study highlights that descriptive and statistical phylogeographic methods can complement and/or cross-validate each other, whereas using them separately only provides a partial picture of the spatial and temporal patterns of intraspecific genetic structure.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2009.07.004.

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