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Molecular clocks and geological dates: cytochrome *b* of *Anolis extremus* substantially contradicts dating of Barbados emergence

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Abstract

Even though molecular clocks vary in rate to some extent, they are widely used and very important in a range of evolutionary studies, not least in interpreting cause and colonization in phylogeography. Evolutionists may use island age and emergence to give the earliest possible date for colonization by a species and hence give the lower limit in a molecular clock calibration. The geology of the Lesser Antilles is well studied and Barbados, although composed of some ancient rocks, is thought to have emerged only about 1million years ago (Ma). The cytochrome *b* mitochondrial gene is the most widely used gene in vertebrate phylogeography, and generally evolves at a rate of 1–2% per million years (Myr) for poikilothermic vertebrates. Divergence measured across almost all of this gene in the endemic anole (*Anolis extremus*) reveals a mean patristic distance of approximately 8.3% between this clade and its sister, together with distinct divergence and phylogeographical structure within Barbados. The divergence time, estimated by a range of procedures using four calibration points, is not in the least compatible with the proposed geological time of emergence of Barbados. Hence, either the molecular clock rate does not apply to the Barbadian anole population, or the geological dating of the emergence of Barbados is erroneous. The compatibility of geological times and molecular divergence of this complex on Martinique, together with relative rates tests comparing the rates on Barbados and Martinique, do not suggest atypical clock rates. The question of whether Barbados emerged much earlier than is currently thought, or whether the molecular clock assumptions are inappropriate, remains open.

Keywords: anole molecular clock, Barbados geology, cytochrome *b*

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Introduction

The application of molecular techniques to phylogenetic analysis (Page & Holmes 1998; Avise 2000) has shown that not only are there interisland differences (Creer *et al*. 2001; Stenson *et al*. 2004), but also that deep phylogenetic divisions exist even within species occupying small geographical areas such as islands (Malhotra & Thorpe 2000a; Thorpe 2002; Thorpe & Stenson 2003). Both inter- and intraisland divisions are usually commensurate with the age of an island and geological data is widely used to evaluate the direction and age of colonization events as well as to assist with the calibration of rates of gene evolution, and give the lower limit in molecular clock calibration (Zamudio & Greene 1997). The assessment of molecular clocks in evolutionary studies enables estimation of times of species divergence by the comparison of gene sequences because it is assumed that genes evolve at a constant rate (Page & Holmes 1998; Avise 2000). However, the rate of the molecular clock has been shown to vary within and between species as a result of many factors including differences in metabolic rate, variation in DNA repair efficiency, mutagen exposure differences, and variation in generation time and body size (Martin & Palumbi 1993; Bromham 2002). Cytochrome *b* is the most widely used mitochondrial gene in phylogenetic and phylogeographical studies of vertebrates with a generic 2% per million years (Myr) rate of evolution often referred to, albeit with an often slower rate for poikilotherms (Martin

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& Palumbi 1993; Johns & Avise 1998; Bromham 2002). As well as difficulties of finely calibrating a molecular clock there are different methods of estimation (e.g. uncorrected and corrected genetic distances and patristic distances), and different sections and combinations of mitochondrial genes. These make comparing detailed rates across the literature difficult, or inappropriate. Even so, broad comparisons are useful, and the divergence rate of cytochrome *b* (and similar genes such as *ND2*) for small lizards tends to be in the middle to slower section of the broad 1–2% band (Gübitz *et al*. 2000; Malhotra & Thorpe 2000a; Creer *et al*. 2001; Thorpe 2002; Thorpe & Stenson 2003). This tends to hold when cytochrome *b* is combined to other mitochondrial genes (Brown & Pestano 1998; Brown *et al*. 2001) and for multiple gene mtDNA studies of small lizards (Macey *et al*. 1998).

The *Anolis* lizards are one of the most speciose vertebrate genera (Creer *et al*. 2001) with *c*. 150 species in the Caribbean. *Anolis extremus* is endemic to the island of Barbados, where it is ubiquitous. This species is nested deep within the *Anolis roquet* group of Martinique, being a sister taxon to the central Martinique clade of *A. roquet* (Thorpe & Stenson 2003). Based only on a single specimen of *A. extremus*, but exhaustive sampling of Martinique, this earlier study (Thorpe & Stenson 2003) suggested that the uncorrected pairwise genetic divergence of the cytochrome *b* gene between these sister lineages is about 6%. This divergence figure conflicts with the geological estimation of emergence of Barbados, which is around 1 million years ago (Ma) (Speed 1994). This conflict could be apparent rather than real if there were multiple colonization, or if the precursor to the Barbadian *A. extremus* existed on another island (e.g. the Caravelle Peninsula, now attached to Martinique) over the intervening period, but has become extinct after recently colonizing an emerged Barbados. This can be resolved by (i) establishing whether the Barbados populations are monophyletic (re multiple colonization), and (ii) establishing the molecular phylogenetic relationships and extent of divergence of *A. extremus* within Barbados. The 'intervening island' hypothesis predicts little or no divergence, or phylogeographical structure, of *A. extremus* within Barbados. However, if the Barbados populations are monophyletic, substantial divergence and phylogeographical structure within Barbados would indicate the species has been on Barbados for a substantial time, and the conflict between suggested geological and molecular times is real.

Materials and methods

The analyses are based on previously published sequences (Thorpe & Stenson 2003) of the cytochrome *b* gene from the *Anolis roquet/extremus* complex together with a set of new sequences from *A. extremus* in Barbados. The former comprise 74 full sequences of cytochrome *b* of *Anolis roquet* from Martinique together with a haplotype of *A. extremus* from St Lucia where it has been introduced (Gorman 1976; Schwartz & Henderson 1991), while the latter comprise 28 new, comparable Barbadian sequences. For Barbados, two specimens were sampled from 13 localities together with single specimens from a further two localities, with the localities spread evenly across the island (Fig. 1). Individuals

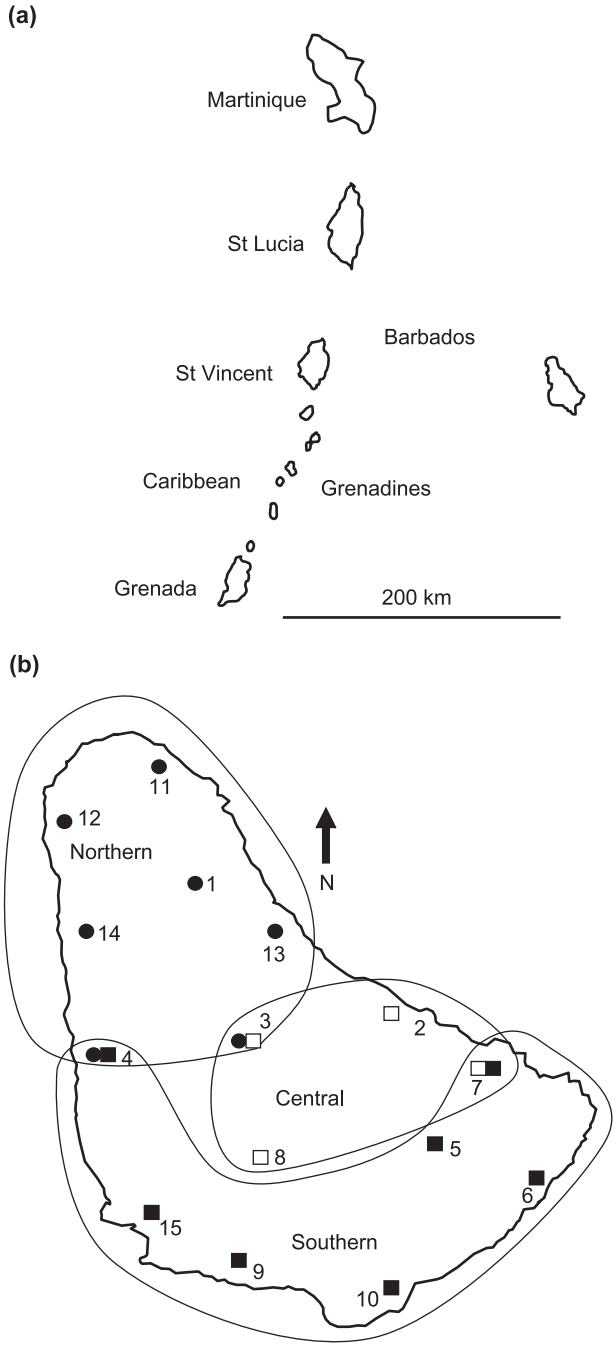

Fig. 1 (a) Map of the southern Lesser Antilles. (b) Map of Barbados showing the geographical distribution of the numbered samples and the lineages from Fig. 3.

were sampled by non-intrusive removal of tail tips (tissue was preserved in 80% ethanol). Whole genomic DNA was extracted using standard protocols. A 1139 bp segment of the cytochrome *b* gene, effectively the entire gene (Irwin *et al*. 1991), was then amplified with squamate primers (Thorpe & Stenson 2003). These were modified (Malhotra & Thorpe 2000b) Mt-A (Lenk & Wink 1997) and Mt-F (Wink 1995) primers. The primer sequences were Mt-A (5′-CTCCCAGCCCCATCCAACATCTCAGCATGATGAA-ACTTCG-3′) and Mt-F (5′-AGGGTGGAGTCTTCTG-TTTTTGGTTTACAAGACCAATG-3′).

Polymerase chain reaction (PCR) was carried out in 50 μL volumes containing 20 ng template DNA, 20 mM Tris-HCl, 50 mM KCl, 2 mM $MgCl_2$, 0.2 mM each dNTP, 0.4 mM each primer, and 2 units of *Taq* DNA polymerase (Gibco BRL). The PCR cycling parameters were 94 °C for 3 min, 30 cycles of 93 °C for 1 min, 48 °C for 2 min, 72 °C for 2 min, 1 cycle 72 °C for 3 min. Purification of PCR products involved isolation by electrophoresis through a 1% agarose gel, followed by one treatment with the Wizard® (Promega) purification system and one with the QIAquick® Gel Extraction system (QIAGEN). Sequence reactions were set up using BigDye™ Terminator (PE Biosystems) and cycle sequencing, for analysis on an ABI377 sequencer. Forward and reverse sequences were edited using CHROMAS 1.51, aligned by eye. Characteristics such as the absence of stop codons, insertions and/or deletions, and comparison with sequences from other taxa (Gleason *et al*. 1997) confirmed that the fragment was mitochondrial cytochrome *b* and not a pseudogene.

There are two sets of phylogenetic analyses each with its own optimized model determined by MODELTEST 3.0 (Posada & Crandall 1998). Analysis 1 was based on all sequences (75 published plus 28 new Barbadian) and was carried out to check for monophyly of the Barbadian haplotypes vs. multiple colonization of Barbados from Martinique, and to compare divergence and relative rates across the entire complex. The outgroup consisted of the sister taxon *Anolis aeneus* (Thorpe & Stenson 2003) together with *Anolis oculatus* from the *bimaculatus* series. The Bayesian method (MRBAYES version 3.0b4) was used to reconstruct the tree (Huelsenbeck & Ronquist 2001) based on an optimized model of sequence evolution, i.e. HKY85 + Gamma + I (Hasegawa *et al*. 1985), determined by the likelihood ratio test in MODELTEST 3.0 (Posada & Crandall 1998), for the specific data set. Four simultaneous Markov chains were run (three heated, one cold) for 2 million generations, sampling the chains every 100th generation. A plot of log-likelihood scores of sample points against generation showed that stationarity was achieved after the first 100 generations, thus the first 10 000 trees were ultimately discarded. A majority rule consensus tree ('Bayesian' tree) was then calculated from the posterior distribution of trees, and the posterior probabilities calculated as the percentage of samples recovering any particular clade (Huelsenbeck & Ronquist 2001), where probabilities ≥ 95% indicate significant support. Two further independent Bayesian analyses were run so that global likelihood scores, individual parameter values, topology and nodal support could be compared to check for local optima. Moreover, the topology was compared that of the equivalent (except fewer Barbadian samples) maximum-parsimony (MP) and maximum-likelihood (ML) trees in Thorpe & Stenson (2003), which gives bootstrap support for critical nodes.

Divergence between key lineages at eight major nodes of the *A. roquet/extremus* complex is estimated using several methods. All except the corrected genetic distance allow a measure of spread or confidence. This is not readily available when the appropriate correction model is HKY85 + I + G (as in this case), but this distance is included for comparative purposes. Simple uncorrected mean pairwise genetic distances (with standard errors) were computed using MEGA2 (Kumar *et al*. 2001). Genetic distances corrected by the evolutionary model selected for the phylogenetic Analysis 2, and their corresponding patristic distances were computed in PAUP (Swofford 2001). For patristic distances, standard deviations in divergence time (node height) were calculated by parametric bootstrapping using the following procedure. First, 100 pseudoreplicate nucleotide matrices were generated in SEQ-GEN (Rambaut & Grassly 1997) from the original, unconstrained, ML phylogeny and the original data matrix, under the optimal evolutionary model and parameter values. One hundred ML trees were then generated in PAUP using a heuristic search of 1000 replicates with nearest-neighbour interchange branch swapping, under the optimal evolutionary model with a clock assumption. Because this procedure relies on a close match between model and data (Felsenstein 2004) the adequacy of the HKY85 + Gamma + I model was first tested using the absolute goodness of fit procedure expounded by Whelan *et al*. (2001). Both corrected and uncorrected distances are given as percentages for ease of comparison and ease of translation into divergence per Myr.

In addition, divergence times with confidence limits were estimated using the nonparametric rate smoothing (NPRS; Sanderson 1997) and penalized likelihood (PL; Sanderson 2002) methods with the Powell algorithm implemented in R8S (Sanderson 2003). The former method relaxes the assumption of a molecular clock by using a least squares smoothing of local estimates of substitution rates, whilst PL is a semiparametric approach combining a parametric model with different substitution rates on every branch with a nonparametric roughness penalty, costing the model more if rates change too rapidly from branch to branch (Sanderson 2003). Calculation of the confidence limits is based on the procedure outlined by Cutler (2000) and adopted by Sanderson (2003) in the R8S program.

The divergence time between lineages was calibrated by four geological dates of volcanic precursor island origin in the Martinique complex, separately for each method. These volcanic events relate to four nodes in the *Anolis roquet/extremus* phylogeny. These nodes are distributed throughout this phylogeny and are at both higher and lower divergence levels to the critical nodes to be dated, i.e. the divergence between the Barbadian lineage and the sister lineage in Martinique, and divergence between the primary lineages within Barbados. The primary phylogenetic division within the *A. roquet/extremus* complex is the split between the western populations associated with the younger (late Miocene) arc and those further east, which include populations associated the older arc. The younger arc could have been colonized at the origin of the southwest (Trois Ilet) Martinique peninsular at *c*. 8 Ma (Maury *et al*. 1990). Divergence between the southwest and southeast lineages may be dated to the origin of northwest precursor island at *c*. 6 Ma (Bouysse *et al*. 1983). Divergence within the southeast lineage and within the southwest lineage may be dated from volcanic activity at Le Diamant (2.0 Ma) and at Case Pilote (2.0 Ma), respectively (Andreieff *et al*. 1976). For the genetic and patristic distances the time of divergence between the lineages of interest was computed from the average rate across these four calibration points. For the NRPS and PL approaches the maximum ages of these nodes were constrained at the geological dates.

Relative rates tests (Takezaki *et al*. 1995) were performed, using PHYLTEST (Kumar 1996), on the same major nodes of the *A. roquet/extremus* complex (Fig. 3) to test if the rate of evolution in Barbados is compatible with that in the rest of the complex. While relative rate tests are weak as clock tests they are effective at comparing rates between sister taxa (Bromham 2002). Here, the closest possible lineage is used as the outgroup.

Analysis 2 was based on the 28 Barbadian haplotypes with a central Martinique locality haplotype of *Anolis roquet* (*roquet* 30 in Thorpe & Stenson 2003), representing the sister lineage to the Barbadian lineage, as the outgroup. The Bayesian tree reconstruction procedure followed that of analysis 1 with the model (HKY85 + Gamma + I) and its parameters determined by for the specific data set in analysis 2. MP and ML trees were also reconstructed using PAUP* 4.0b8a (Swofford 2001), with the ML tree based on the same model of sequence evolution as the equivalent Bayesian tree. Maximum parsimony reconstructed the haplotype tree using the heuristic search algorithm, random addition of sequences and tree-bisection–reconnection with 100 replications. Bootstrap support is given for both the MP and ML trees in analysis 1. Pairwise matrix correspondence (Mantel) tests (Thorpe 2002) were run to evaluate the geographical structuring of the haplotypes on Barbados. A geographical proximity matrix based on haplotype locality was compared to (i) corrected genetic distance between haplotypes, (ii) patristic (Thorpe 2002) or tree distances (Page & Holmes 1998) between haplotypes based on the ML tree, and (iii) clade membership contrasts based on the three main clades (north, central, south).

Metropolis–Hastings simulations (Kuhner *et al*. 1998) were run on the Barbadian sequences to estimate theta (Θ) and growth rate (g) to indicate whether the sequence shows signs of a bottleneck. The program FLUCTUATE 1.4 also estimates the relationship between mutation rate (μ) and effective population size (N) such that:

$$N = (\Theta/\mu)/2.$$

Results

The new Barbadian sequences yielded 974 base pairs of comparable sequence (GenBank Accession nos DQ004586–DQ004613). For the analysis of the entire *Anolis roquet/extremus* complex (analysis 1) MODELTEST selected HKY85 + Gamma + I model, with the following parameter values: A, C, G, T base frequencies were 0.317, 0.228, 0.138 and 0.317, the gamma distribution shape parameter was 0.874, the transitions/transversion (ti/tv) ratio was 2.509 and the proportion of invariable sites, 0.408. The absolute goodness-of-fit procedure expounded in Whelan *et al*. (2001) showed the HKY85 + Gamma + I model to be a very good fit, with the chi-squared value of the real data falling within the 95% confidence limits. The additional Bayesian trees do not indicate a problem with local optima and the topology is congruence with both the MP and ML trees in Thorpe & Stenson (2003). The Bayesian tree (Fig. 2) has the critical nodes fully supported and shows the Barbadian and central Martinique haplotypes to be reciprocally monophyletic sister lineages. The Barbados population does not appear to be the product of multiple colonization from Martinique. The St Lucian haplotype is a sister haplotype to 10a from the southern lineage of Barbados suggesting that specimens were introduced into St Lucia from southern Barbados. Divergences among and within the primary lineages of the *A. roquet/extremus* complex are high (Table 1). For example, the divergence between the primary clades (node 1, Fig. 2) is 12.4% mean corrected pairwise genetic distance, and the divergence of the Barbados lineage to its sister central Martinique lineage (node 3) and divergence within Barbados (node 4) are commensurately high at 7.1% and 6.6% mean corrected genetic distance, respectively. All methods (Table 1) estimate the timing of the divergence between the Barbados lineage and the Martinique sister lineage (node 3) to be between 5.6 and 6.1 Ma, with the divergence within Barbados (node 4) to be between 5.2 and 5.7 Ma. The relative rates tests show that, at every tested level, there are no significant differences in rate of cytochrome *b* evolution (Table 1); notably there is no difference in rate between the Barbados lineage and its sister lineage in Martinique.

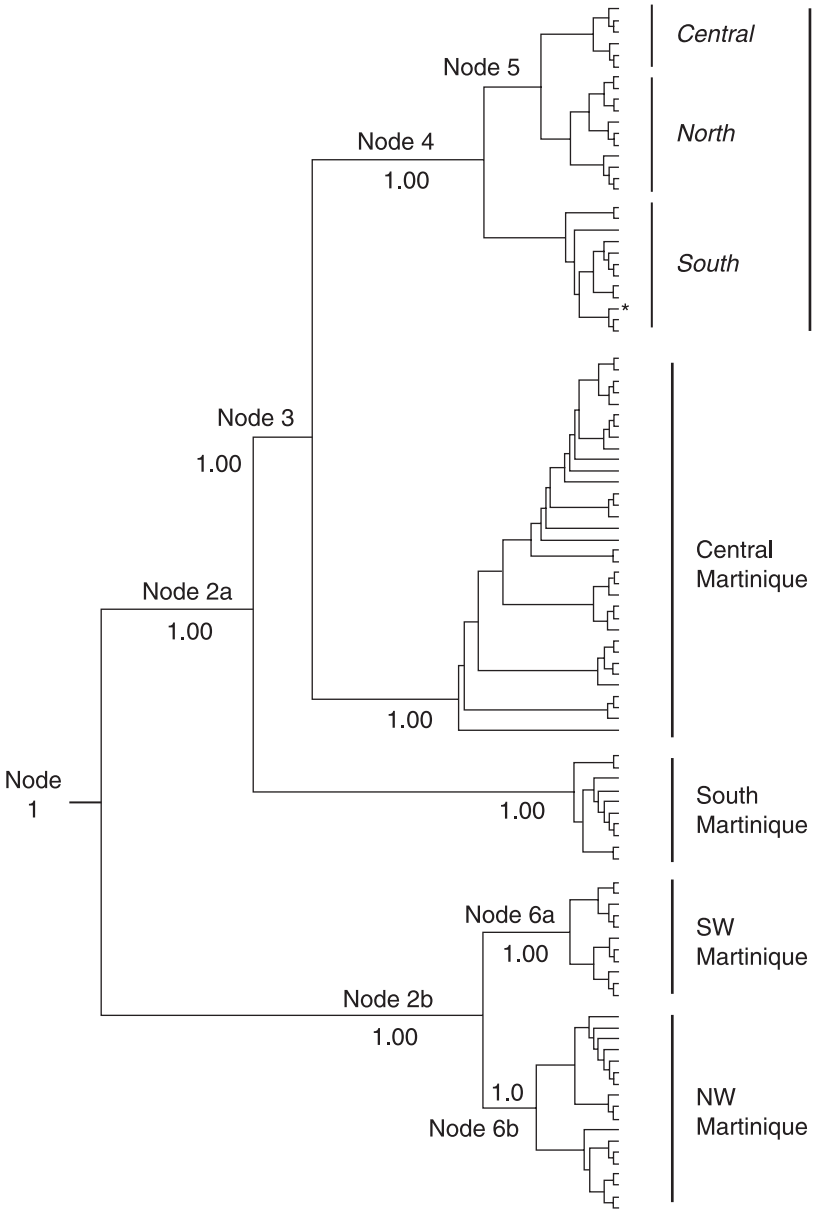

Fig. 2 Bayesian gene tree for the entire *Anolis roquet/extremus* complex. The main lineages are identified (nodes labelled 1–5) with their posterior probability. The Barbados samples have the same topology as in Fig. 3, but with the inclusion of a sample from the introduced St Lucia population (denoted by an asterisk) which is sister to sample 10a from southern Barbados. The further details of the relationships of the samples from Martinique are in Thorpe & Stenson (2003).

In the Barbadian sequences (Analysis 2), 250 sites were found to be variable and 120 sites were parsimony informative. MODELTEST 3.0 (Posada & Crandall 1998) identified the HKY85 + Gamma + I model for the phylogenetic reconstruction. The theoretical best value for the log likelihood (1 nL) was –3838.15 and A, C, G, T base frequencies were 0.305, 0.231, 0.136 and 0.328. The gamma distribution shape parameter was 0.935, the ti/tv ratio was 2.027 and the proportion of invariable sites, 0.516. The Bayesian (Fig. 3), ML and MP gene trees have fundamentally the same topology with well-supported deep nodes. There is distinct phylogeographical structure within Barbados, with three main lineages (northern, central and southern) together with some finer-scale phylogeographical structure (Fig. 3). There is minimal spatial overlap between haplotypes, but where two lineages abut geographically a population may have both haplotypes. The geographical structuring is significant, with the null hypothesis of no association with geographical proximity being rejected for the genetic distance ($r = 0.40$, $P < 0.0001$), patristic distance ($r = 0.47$, $P < 0.0001$), and clade membership contrast ($r = 0.41$, $P < 0.0001$). The Metropolis–Hastings simulations suggest only a modest positive growth rate for the Barbados populations ($g = 0.168$, $\Theta = 0.8695$).

Discussion

Barbados is an island with a maximum elevation of 340 m that is composed of a single precursor island and three

Table 1 Lineage divergence. The nodes for lineage divergence are numbered as in Fig. 2, where node 3 is the split between the Barbados populations and their sister lineage in Martinique and node 4 is the primary split within Barbados

Node	1	2a	2b	3	4	5	6a	6b
A. Uncorrected genetic distance	9.4	8.0	5.8	6.1	5.7	4.5	1.9	2.2
SE of G.D.	0.7	0.8	0.6	0.6	0.6	0.5	0.2	0.3
Est. div. time Ma (rate 1.05%**)	*	7.6	*	**5.8**	**5.4**	4.3	*	*
B. Corrected genetic distances	12.4	10.0	6.9	7.1	6.6	5.2	2.4	2.4
Est. div. time Ma (rate 1.28**)	*	7.8	*	**5.6**	**5.2**	4.1	*	*
C. Equivalent patristic divergence	14.0	11.6	7.5	8.3	8.0	6.2	2.6	2.6
SD P.D.	0.83	0.89	0.49	0.58	0.47	0.42	0.10	0.11
Est. div. time Ma (rate 1.40**)	*	8.3	*	**5.9**	**5.7**	4.4	*	*
D. NRPS Estimated divergence time	8.0†	7.2	3.8†	**6.1**	**5.4**	5.2	1.9†	1.9†
Confidence limits	—	7.2/7.2	—	6.0/6.2	5.4/5.5	5.1/5.2	—	—
E. PL Estimated divergence time	8.0†	7.3	4.1†	**6.1**	**5.3**	4.9	2.0†	2.0†
Confidence limits	—	6.8/7.6	—	5.4/6.7	4.3/6.0	3.9/5.7	—	—
F. Relative rate test *Z*	0.97	1.03	0.62	0.47	0.24	1.14	0.10	0.10
G. Relative rate test *P*	> 0.05	> 0.05	> 0.05	> 0.05	> 0.05	> 0.05	> 0.05	> 0.05

Divergence times in million years ago (Ma) are estimated from the geological times at the four nodes denoted by a single asterisk (1, 2b, 6a, 6b): the geological times for these nodes are 8.0, 6.0, 2.0, and 2.0 Ma, respectively. The mean rate of divergence using these four nodes is denoted by two asterisks. For the NRPS and PL estimates, the maximum time for the divergence of these four nodes (denoted by a cross) is constrained at 8.0, 6.0, 2.0, and 2.0 Ma, respectively. Rows are: (A) The mean pairwise uncorrected genetic distances (as percentage) between clades with standard error and estimated divergence time. (B) The mean pairwise corrected genetic distances (as percentage) between clades with estimated divergence time. (C) Equivalent patristic distance from a tree without clock-constraints and estimated divergence time. (D) Nonparametric rate smoothing estimates of divergence time with confidence limits. (E) Penalized likelihood estimates of divergence time with confidence limits. (F) Relative rates tests — the constancy of evolutionary rates between lineages is tested by a two-tailed normal deviate (*Z*) test, with (G) probabilities of the null hypothesis of no difference in rates.

coral rock terraces each progressively younger in age. Other Lesser Antillean islands are primarily volcanic in origin, but Barbados is sedimentary, being part of the accretionary prism above the subduction trace of the descending American and overriding Caribbean plates (Speed 1994). Geologists have indicated that although the accretionary prism is relatively ancient (50 Ma), the date of emergence of this precursor island above sea level is more recent, with its rise beginning before 650 000 years ago (Bender *et al*. 1979) such that its emergence is 'more or less a million years before present' (Speed 1994). This dating is achieved by assessing the age of overlying sedimentary rocks (Speed 1994).

The present molecular phylogenetic studies indicate that the endemic Barbadian species, *Anolis extremus*, is nested deep within the Martinique populations of the *Anolis roquet* complex, and suggest that the ancestors of the Barbadian species colonized Barbados from the 'central' region of Martinique, compatible with Thorpe & Stenson (2003). The mean corrected genetic and patristic divergences between the Barbados and central Martinique lineages are 7.1% and 8.3%, respectively. Under accepted clock rates the extent of this divergence is incompatible with colonization of Barbados directly from central Martinique within the last million years (the suggested time of emergence of Barbados). The hypothesis that the precursor population spent the intervening period (between diverging from central Martinique population and the recent emergence of Barbados) on an island, from which it has subsequently been eradicated, predicts little divergence or phylogeographical structure within Barbados even though there could be substantial divergence between the Barbados lineage and its sister lineage on Martinique. However, this study (Table 1) clearly shows that the extent of divergence between lineages within Barbados is high (8.0% patristic divergence) and very close to the divergence between Barbados and central Martinique lineages (8.3% patristic divergence). Moreover, the lineages within Barbados have a distinct phylogeographical structure. Consequently, the 'intervening island' hypothesis can be rejected as it appears that *A. extremus* precursor started to diverge *in situ* on Barbados soon as it 'left' central Martinique. Similarly, one can reject the hypothesis that the extent of divergence and phylogenetic structure within Barbados is the result of multiple colonization (e.g. three separate colonization events for the three main lineages). Anoles of this series occur on all the southern Lesser Antilles, and are relatively well studied phylogenetically, with several studies (Creer *et al*. 2001; Thorpe & Stenson 2003) showing the Barbados/Martinique complex

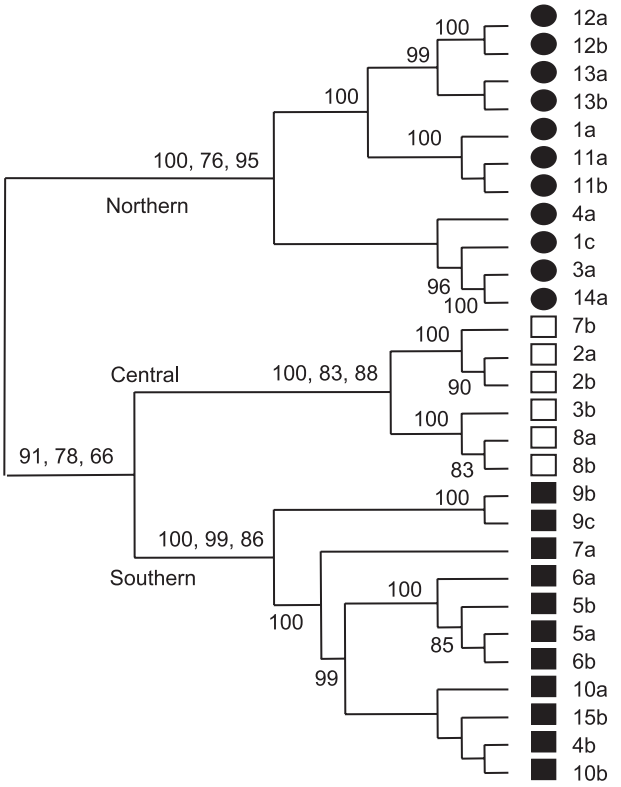

Fig. 3 Bayesian gene tree showing three main lineages for Barbados samples. The terminal nodes are sequences for individual specimens from the numbered locality in Barbados (Fig. 1). The lineages are coded by the symbols used in Fig. 1. Posterior probabilities for nodes are given where they are over 80% followed by the ML and MP bootstrap support (for basal nodes).

to be monophyletic. Moreover, Martinique has been exhaustively sampled for phylogeographical analysis of this complex (Thorpe & Stenson 2003). Consequently, multiple colonization of Barbados would be revealed by phylogenetic analysis of the entire Barbados/Martinique complex if it had occurred. Other, factors make multiple colonization untenable. These include the lack of candidate islands or any geological evidence for the subsequent submergence of past islands (this is not a conveyer belt system such as Hawaii or the Galapagos Islands). Even if there were such candidate islands, it is not tenable that the colonization sequence, time and geography could produce the phylogenetic and phylogeographical infrastructure that exists, where sister taxa are geographically closest and levels of within lineage divergence fit so well with divergence levels at subsequent nodes. However, all these are predicted from a single colonization with *in situ* differentiation.

In converting the divergence to time before present, this study employs four geological calibration points based on volcanic emergence of Martinique precursor islands and regions in the younger arc. These calibration points are within the phylogeny of the *A. roquet/extremus*, rather than from more distant relatives and so do not compromise the optimized model of evolution (Posada & Crandall 1998) employed in the phylogenetic analysis. There is close agreement among the wide range of methods used to estimate the time of divergence. The divergence times (Table 1, node 3) suggests a colonization of Barbados 5.6 to 6.1 Ma and divergence within Barbados (Table 1, node 4) from 5.2 to 5.7 Ma. These dates (and their confidence limits) are dramatically earlier than the geological date suggested for the emergence of Barbados. For the reasons given in the introduction, a comparison of precise molecular clock rates may be inappropriate, but broad comparison is possible. While the four geological dates used for calibration can only give the earliest date of colonization, the molecular clock rates they suggest for each method (*c*. 1–1.4%) are compatible with that of poikilothermic vertebrates in general (Martin & Palumbi 1993; Johns & Avise 1998; Avise 2000), and small lizards in particular. Studies of other Lesser Antillean anoles and island lizards using cytochrome *b* (and mtDNA genes with comparable rates) have suggested fairly similar rates of genetic divergence (Gubitz *et al*. 2000; Malhotra & Thorpe 2000a; Creer *et al*. 2001; Thorpe 2002; Thorpe & Stenson 2003), i.e. a tendency to be in the mid to lower region of the 1–2% per Myr band. Hence, comparison with other studies indicates that the rates suggested by these four calibration points are predictable and realistic.

Difficulties have been encountered in extrapolating rates in one taxon to another due to differences in generation time, body size and clutch size (Martin & Palumbi 1993). However, the Barbados lineage is nested inside the Martinique lineages and the life history, size and general biology of the Barbadian anole are not atypical of solitary anoles in general, and are the same as those of its sister lineage in Martinique. Even given the need for caution, the magnitude of the discrepancy between the rate predicted by the geological times and the actual extent of divergence (both between the Barbados lineage and the sister lineage, and within Barbados) is too great to be explained away by normal variation in rates observed in poikilothermic vertebrates (Avise 2000) let alone among members of the same species complex. Moreover, relative rates tests show no difference in rates of molecular divergence between, and within, the Barbados and Martinique lineages. Metropolis–Hastings simulations allow an estimation of effective population size if the mutation rate is assumed. If the mutation rate is derived from assuming Barbados emerged 1 Ma (and assuming a realistic mean generation time of 1 year), then the mutation rate is high and the estimation of effective population size gives an unrealistically low value of one female per 165 m^2. However, an assumption of a 1% to 2% per Myr rate compatible with rates observed in *A. roquet* in Martinique and other small lizards, gives far

more realistic estimates of effective population size on Barbados. Consequently, we find no evidence to suggest that the rate of cytochrome *b* divergence in Barbadian anoles is atypical.

Both the emergence time of Barbados suggested by Speed (1994), and the calibration of the molecular clock that contradicts it, rely on geological times.

Dramatic origin of a volcanic island, as seen in the Lesser Antilles, can be dated with K-Ar dating (Briden *et al*. 1979). The period of volcanicity, is relatively short for any given eruption and the oldest K-Ar date allows one to choose between periods of volcanicity if they exist, and give the earliest date of origin. One point on an island or peninsular may be able to do this and this is the procedure employed with the four geological calibration points used to date the phylogenetic lineages. However, dating emergence when there is gradual uplift may be much more difficult. The uplift producing the emergence may take place over a long time and the actual emergence cannot be timed. It is done indirectly by looking for overlying sedimentary rocks. One can never be sure that a part of the island was not exposed, when the sedimentation took place in another part.

Examples exist of radiations being older than the current islands in an archipelago. The conveyer belt system (Fleischer *et al*. 1998) of Galapagos and Hawaii lend themselves to this. In this situation a chronological series of islands may arise such that new islands emerge while older islands are eroded to submergence. Even though there are concerns about clock methods in some proposed examples (Baldwin & Sanderson 1998), it is then quite possible for a radiation that has started on the oldest islands to colonize new islands as they emerge, so that the phylogeny dates from the oldest submerged islands rather than the current islands. Hence, the intergeneric Galapagos iguana radiation is thought to predate the oldest current island (Rassmann 1997), and numerous Hawaiian radiations have been suggested as predating the oldest current island, including begonias (Clement *et al*. 2004), asters (Kim *et al*. 1998), *Drosophila* (Thomas & Hunt 1991), and apiaceans (Vargas *et al*. 1999). However, none of these examples of a radiation predating current Galapagos or Hawaiian islands, is comparable to the situation in the Barbadian anole as this involves intraspecific phylogeographical differentiation within a single island. Indeed, when one takes a case such as the Galapagos iguanas, although the intergeneric radiation may predate the oldest current island (Rassmann 1997) the differentiation of a species within island (and even between islands in this case) does not (Rassmann *et al*. 1997).

Consequently, in this study, there is a real and unusual contradiction between the molecular clock assumptions of cytochrome *b* evolution in Barbadian anoles and the suggested geological time of emergence. All approaches have their limitations and assumptions. The calibration of the molecular clock in this study is dependant on the accuracy of the four geological calibrations of the molecular phylogeny (even though the subsequent rate is typical of the type of organism), and is based on only one mitochondrial gene (albeit all of it). Similarly, the suggested geological time of emergence of Barbados may not be accurate. This suggests that further studies of the phylogeography of the Barbadian fauna and the geological timing of the emergence of Barbados are called for.

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This paper forms part of an ongoing study of the causal factors influencing speciation and geographical variation in Lesser Antillean and Canary Island lizards led by Professor R. S. Thorpe. Dr C. E. Pook is interested in molecular phylogenetics and phylogeography of reptiles and Ms D. L. Leadbeater worked on this project as part of her MSc studies in Bangor.