Abstract.—Madagascar’s flora and fauna are remarkable both for their diversity and supraspecific endemism. Moreover, many taxa contain large numbers of species with limited distributions. Several hypotheses have been proposed to explain this high level of microendemism, including 1) riverine barrier, 2) mountain refuge, and 3) watershed contraction hypotheses, the latter 2 of which center on fragmentation due to climatic shifts associated with Pliocene/Pleistocene glaciations. The Malagasy leaf chameleon genus *Brookesia* is a speciose group with a high proportion of microendemic taxa, thus making it an excellent candidate to test these vicariance scenarios. We used mitochondrial and nuclear sequence data to construct a *Brookesia* phylogeny, and temporal concordance with Pliocene/Pleistocene speciation scenarios was tested by estimating divergence dates using a relaxed-clock Bayesian method. We strongly reject a role for Pliocene/Pleistocene climatic fluctuations in species-level diversification of *Brookesia*. We also used simulations to test the spatial predictions of the watershed contraction model in a phylogenetic context, independent of its temporal component, and found no statistical support for this model. The riverine barrier model is likewise a qualitatively poor fit to our data, but some relationships support a more ancient mountain refuge effect. We assessed support for the 3 hypotheses in a nonphylogenetic context by examining altitude and species richness and found a significant positive correlation between these variables. This is consistent with a mountain refuge effect but does not support the watershed contraction or riverine barrier models. Finally, we find repeated higher level east-west divergence patterns 1) between the 2 sister clades comprising the *Brookesia minima* group and 2) within the clade of larger leaf chameleons, which shows a basal divergence between western and eastern/northern sister clades. Our results highlight the central role of phylogeny in any meaningful tests of species-level diversification theories. [Biogeography; Chamaeleonidae; phylogeny; Pleistocene glaciation; relaxed clock; speciation; Squamata.]

Once part of the southern supercontinent of Gondwana, Madagascar, is currently situated some 400 km off the southeastern shore of Africa and is famous for a remarkable flora and fauna that are increasingly threatened by loss of habitat and other human-induced changes. Madagascar’s last subaerial connections to Africa and India date to approximately 160 and 90 million years ago, respectively (Storey et al. 1995), although limited dispersal to and from other continents may have been possible over land bridges or small sea barriers in the Late Cretaceous or Paleocene (Briggs 2003; Noonan and Chippindale 2006; Van Bocxlaer et al. 2007; van der Meijden et al. 2007). This long history of isolation has contributed greatly to the remarkable degree of floral and faunal endemism of Madagascar (Goodman and Benstead 2003), which amounts to 100% in native amphibians and 92% in nonmarine nonavian reptiles (Glaw and Vences 2007).

In terrestrial vertebrates, the majority of species diversity corresponds to a limited number of endemic clades that colonized most of the available bioclimatic regions, including the eastern rainforests, central highlands, western dry deciduous forests, and southern arid spiny forests. There are several sister-taxon pairs that follow an east-west pattern and possibly arose by specialization to these different climatic conditions (the “ecographic constraint hypothesis”; see Yoder and Heckman 2006), from groups such as skinks, snakes, boophine treefrogs, and spiders (Nussbaum and Raxworthy 1998; Nussbaum et al. 1998; Vences and Glaw 2002, 2003; Wood et al. 2007). In other groups, the major evolutionary splits consistently separate a clade occurring roughly in the northern fifth of Madagascar from a more southern clade, with examples found in chameleons, geckos, skinks, and mouse lemurs (Yoder and Heckman 2006; Boumans et al. 2007). As a general pattern, the spatial distribution of species richness in some higher taxonomic groups may have been shaped by a latitudinal and altitudinal middomain effect (Lees et al. 1999). Nonetheless, even within the major bioclimatic regions, species-level local endemism is high, and a major goal of researchers has been to uncover the causes of this pattern (Goodman and Benstead 2003 and references therein; Vences et al. 2009).

Potential Climatic and Geographic Barriers

In their study of biogeographic patterns in the leaf chameleons (genus *Brookesia*) of northern Madagascar, Raxworthy and Nussbaum (1995) recognized 5 rainforest regions delimited largely by altitudinal differences and intervening dry forests and characterized by a high degree of endemism in these lizards. Raxworthy and Nussbaum (1995) hypothesized that Pleistocene climatic fluctuations had caused fragmentation of the rainforest, resulting in multiple allopatrically distributed sister-taxon pairs (hereafter referred to as the mountain refuge [MR] hypothesis). These conclusions were not based
on explicit phylogenetic hypotheses; sister-taxon pairs were assumed based on general morphological similarities.

Wilmé et al. (2006) analyzed Madagascar’s striking microendemism in the context of watersheds. Using museum data for more than 35,000 georeferenced land vertebrate specimens, they found that watersheds with low-elevation headwaters tended to define centers of endemism (COEs), whereas those with connections to the 3 highest summits in Madagascar (each at >2000 m) tended to contain more widespread species. Wilmé et al. (2006) reasoned that during periods of Late Tertiary and Pleistocene glacial maxima, aridification caused contraction of previously widespread mesic environments. Lower elevations were more severely affected than higher elevations, leading to fragmentation and isolation of low-elevation watersheds. In contrast, areas with riverine connections to high-elevation source waters were buffered from this effect and thus served as “retreat–dispersion watersheds” (RDWs), providing a means of refuge and recolonization during dry and wet cycles, respectively. The authors presented this model of Pliocene–Pleistocene fragmentation and refugia (hereafter referred to as the watershed contraction [WC] hypothesis) as the main generator of Madagascar’s famously microendemic biota. The Wilmé et al. (2006) study was based entirely on extant species distributions coupled with climatological, hydrological, and topographical variables and incorporated no phylogenetic data. Pearson and Raxworthy (2009) found some support for the WC hypothesis in lemurs and day geckos of the genus *Phelsuma* (a climatological gradient effect was also cited for these lizards), but once again relied on distribution patterns alone.

Finally, the presence of large rivers flowing from the highlands either toward the west or the east has also been discussed as a factor influencing species formation and microendemism (hereafter referred to as the riverine barrier [RB] hypothesis). In western Madagascar, these rivers coincide with the limits of distribution areas of species or phylogeographic lineages within species of lemurs and are therefore likely to constitute significant barriers to gene flow for these animals (Pastorini et al. 2003). A similar situation seems to exist in eastern Madagascar and may especially influence species occurring at low elevations where rivers are widest (Goodman and Ganzhorn 2004; Louis et al. 2005). For a more detailed overview of the processes inherent to the MR, WC, and RB hypotheses, see Vences et al. (2009).

**Brookesia Leaf Chameleons**

The chameleon genus *Brookesia* is an excellent group to test hypotheses of species-level diversification and microendemism on Madagascar. These lizards, which appear to form the sister taxon of all other chameleons (Rieppel 1987; Townsend and Larson 2002), constitute one of the largest Malagasy reptile groups (Raxworthy and Nussbaum 1995; Glaw and Vences 2007). Most *Brookesia* species have very small ranges (Raxworthy and Nussbaum 1995; Raselimanana and Rakotomalalana 2003), with almost half known essentially from single localities (Carpenter and Robson 2005). *Brookesia* can be divided into 3 main groups based on morphology. One is represented by just 2 species (*B. nasus* and *B. lolontany*) that are highly divergent from other *Brookesia* by both molecular (Raxworthy et al. 2002; Townsend and Larson 2002) and morphological measures (long snouts, laterally compressed bodies; Raxworthy and Nussbaum 1995). A second larger group is composed of approximately 18 species with more robust bodies and blunted snouts. The remaining 6 described species have taken the already diminutive body form of *Brookesia* to an extreme, with total lengths of about 28–45 mm, making them some of the world’s smallest vertebrate species (Glaw and Vences 2007). For ease of reference, these 3 groups will hereafter be referred to as the *B. nasus* group, the “typical” *Brookesia*, and the *B. minima* group, respectively.

**Hypothesis Testing**

In this study, we use *Brookesia* to test the temporal and spatial predictions of 3 species-level diversification hypotheses for Madagascar (MR, WC, and RB; Vences et al. 2009). The first step in this process is to infer a phylogeny of *Brookesia* to identify statistically supported sister-species pairs using DNA sequence data from multiple mitochondrial and nuclear protein-coding genes. Next, we use divergence dating to statistically test the temporal prediction of both the MR and the WC hypotheses that recent (Pleistocene or possibly Pliocene) climatic cycles are a major force promoting *Brookesia* species diversification.

Major climatic cycles have of course occurred repeatedly throughout the earth’s history, and each of these 3 general hypotheses make spatial predictions that can be tested independently of any temporal predictions (e.g., Miocene WCs could generate the same general fragmentation patterns as the proposed Pleistocene contractions). Specifically, the Wilmé et al. (2006) WC model states that contraction of mesic habitats within adjacent lowland watersheds during periods of glaciation created gaps in ancestral species’ distributions, leading to allopatric differentiation/speciation. Assuming stability in the relative geographic positions of populations, this model therefore predicts that sister species should generally occupy adjacent COEs or possibly a COE and an adjacent RDW (although the species in the RDW would be expected to have a wider geographic range). Because most of the major watersheds are composed of several smaller drainages, this model could also be construed to predict that sister-species pairs would occupy the same drainage. Likewise, the MR model of Raxworthy and Nussbaum (1995) predicts that sister taxa will tend to occupy montane forested areas separated by lower altitude dry forests. Finally, the RB model (Pastorini et al. 2003) predicts species’ ranges to be delimited by
major lowland rivers. To evaluate these hypotheses, we first reconstruct species ranges using a georeferenced database of *Brookesia* distribution records and adjust these ranges (when sample sizes permit) through environmental niche modeling. We then use simulations to test the fit of our data to the spatial predictions of the WC hypothesis, and we assess the qualitative fit of our data to the MR and RB hypotheses (all in a phylogenetic context).

Finally, each of the 3 hypotheses suggests predictions about the altitudinal distribution of species richness. The WC and RB models both predict a greater number of lowland species; low-headwater watersheds are more likely than high-headwater watersheds to become fragmented from their neighbors during periods of increased aridity, and rivers are widest at lower elevations and therefore more likely to constitute significant barriers to gene flow as altitude decreases. Conversely, the MR model predicts higher species diversity in montane areas because it is here that populations tended to become isolated during periods of forest contraction; lowlands were subsequently recolonized by some species. We use these predictions to evaluate relative support for these hypotheses in a nonphylogenetic context by correlation of species richness with altitudinal range.

**Materials and Methods**

**Taxa and Gene Regions Sampled**

A previous phylogenetic study (Townsend and Larson 2002) identified 9 clades with uncertain interrelationships that diverged from each other early in the history of Chamaeleonidae. Because current taxonomy places 2 of these major clades within the genus *Brookesia*, outgroup samples from all 7 non-*Brookesia* chamaeleonid clades were included in this study. Sampling within *Brookesia* was broad, including more than three-quarters of the named species as well as several undescribed forms, and species were sampled from multiple localities whenever possible. To facilitate divergence-dating calibrations, outgroup sampling included representatives of all major squamate lineages (Table 1).

All specimens were sequenced for 2 nuclear (*RAG1*, ∼1500 bp and CMOS, ∼800 bp) and 3 mitochondrial (*ND1*, ∼70 bp; *ND2*, ∼1035 bp; and *ND4*, ∼700 bp) protein-coding genes; we included several *ND4* sequences from Raxworthy et al. (2002) that expanded our taxonomic (3 species) or geographic (6 species) coverage. Our final *Brookesia* sampling covered approximately 28 species, including all but 2 of the named species. Museum/collection and GenBank numbers of specimens can be found in the online Supplementary Material (available from http://sysbio.oxfordjournals.org).

**Molecular Data and Phylogenetic Analyses**

Genomic DNA was extracted and amplified using standard protocols (see Townsend et al. 2004). Polymerase chain reaction products were sequenced with ABI 3100 PRISM™ automated sequencers, and contigs were assembled using Sequencher 4.1.2 (Gene Codes Corporation, Ann Arbor, MI). Primer sequences are given in Table 2. We used the Clustal algorithm (Thompson et al. 1994) implemented in the program DAMBE (Xia and Xie 2001) to align all sequences by their amino acid translations and adjusted alignments by eye using MacClade 4.03 (Maddison D.R. and Maddison W.P. 2000). Ambiguously aligned regions were excluded from all analyses (see Results section). Gaps were treated as a separate (binary) character partition in the MrBayes analyses and as missing data in the BEAST and maximum-likelihood (ML) analyses.

Partitioned Bayesian and likelihood methods were used to infer phylogenies. BEAST (Drummond et al. 2006) implements a Bayesian relaxed-clock method that allows the simultaneous estimation of topology and divergence times. The incorporation of a relaxed-clock constraint into the phylogenetic inference procedure has intuitive appeal, as it seems likely to model biological reality better than the 2 extreme alternatives of either a strict clock or no clock at all (i.e., the unrooted method of Felsenstein 1981). The relaxed clock is also expected to have greater statistical power due to the smaller number of estimated parameters relative to the unrooted method (Drummond et al. 2006). MrBayes v3.1.2 (Huelsenbeck and Ronquist 2001) and BEAST v1.4.6 (Drummond and Rambaut 2007) were used to conduct Bayesian analyses under unrooted and relaxed-clock models, respectively. The data were divided a priori into partitions by gene and codon position, with models chosen using the Akaike information criterion as implemented in MrModelTest (Nylander 2004). Analyses were performed under several a priori partitioning schemes, and the harmonic means of likelihood scores from the posterior distribution were compared using

**Table 1. Nonchamaeleonid outgroup sampling for all phylogenetic analyses**

<table>
<thead>
<tr>
<th>Higher taxon</th>
<th>Representative taxon</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rhynchocephalia</td>
<td>Sphenodon punctatus</td>
</tr>
<tr>
<td>Squamata</td>
<td></td>
</tr>
<tr>
<td>Dibamidae</td>
<td>Dibamus</td>
</tr>
<tr>
<td>Gekkonidae</td>
<td>Gekko gecko</td>
</tr>
<tr>
<td>Xantusiidae</td>
<td>Xantusia vigilis</td>
</tr>
<tr>
<td>Scincidae</td>
<td>Mabuya</td>
</tr>
<tr>
<td>Cordylidae</td>
<td>Cordylus</td>
</tr>
<tr>
<td>Teiidae</td>
<td>Teiinae</td>
</tr>
<tr>
<td>Amphisbaenidae</td>
<td>Trogonophidae</td>
</tr>
<tr>
<td>Lacertidae</td>
<td>Lacertidae</td>
</tr>
<tr>
<td>Serpentes</td>
<td>Dinodon</td>
</tr>
<tr>
<td>Shinisauridae</td>
<td>Shinisaurus crocodilarius</td>
</tr>
<tr>
<td>Iguanidae</td>
<td>Liolepis</td>
</tr>
<tr>
<td>Uromastyca</td>
<td>Uromastyx</td>
</tr>
<tr>
<td>Leiolepidinae</td>
<td>Leiolepis</td>
</tr>
</tbody>
</table>

*a* All phylogenetic analyses included these outgroup taxa as well as all non-*Brookesia* chamaeleonid taxa from Figure 2.

*b* Compositetaxa with more than 1 species represented among the different gene partitions are called by the name of the most exclusive higher taxon possible.
Bayes factors to choose a final partitioning scheme (see Brandley et al. 2005).

We used the ML program RAxML (Stamatakis 2006) via its Web server (Stamatakis et al. 2008) to estimate a phylogeny and conduct nonparametric bootstrap analyses under the same partitioning scheme favored in the Bayesian analyses, except that all partitions were assigned a general time reversible model (currently the only option) with a proportion of invariable sites estimated, thus estimating branch lengths separately for each data partition. For simplicity, further references to the unrooted Bayesian, relaxed-clock Bayesian, and maximum-likelihood analyses will be referred to by the initials UB, RCB, and ML, respectively.

**Divergence Time Estimates**

In most cases, fossil constraints should place the maximum probability near the estimated age of the fossil, with probabilities dropping off rapidly for younger ages and more gradually for older ages (Benton and Donoghue 2007), effectively placing a hard bound on the minimum age and a soft bound (Yang and Rannala 2006) on the maximum age. A translated lognormal distribution models this situation well (Hedges and Kumar 2004; Drummond et al. 2006; Ho 2007) and was used for most of the calibrations in this study. Because overestimation of divergence times could lead to false rejection of Pliocene/Pleistocene speciation scenarios, we also ran analyses under a more conservative set of normally distributed fossil constraints in place of the lognormal distribution models. The most recent common ancestor (mrca) of these taxa is estimated to be 60 million years ago (Noguer et al. 1986), and the older of these dates served as a gamma-distributed maximum age constraint for the most recent common ancestors (mrca) of these taxa.

We were concerned about the effects that substitutional saturation at deeper levels might have on divergence time estimates, especially with regard to mitochondrial DNA (mtDNA) data (Hugall et al. 2007). However, the mtDNA data were needed to confidently resolve more recent branching points (see below), suggesting also that branch lengths within Brookesia would be better estimated by including mtDNA data. We therefore conducted analyses using 1) all data, 2) nuclear data plus first and second positions from the mtDNA data, and 3) nuclear data alone to test the robustness of our divergence time estimates. We also tested our fossil calibrations using the cross-validation method of Near et al. (2005), which identifies potentially problematic fossils that cause incongruent age estimates of other dated nodes in the tree.

All BEAST analyses were run for a sufficient number of generations to achieve an effective sample size of at least 200 for all estimated parameters, and 3 replicate runs were conducted for each analysis. Initial analyses were run without data to check the influence of the priors on the results. BEAST output was examined for evidence of proper mixing and convergence using TraceR (Rambaut and Drummond 2004), runs were combined using LogCombiner (part of BEAST package), and maximum credibility trees with divergence time means and 95% HPDs were produced using Tree Annotator (part of BEAST package). All BEAST files used are included in the online Supplementary Material.

### Table 2. Amplification and sequencing primers used in this study

<table>
<thead>
<tr>
<th>Direction</th>
<th>Location</th>
<th>Sequence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward</td>
<td>ND1</td>
<td>5′-CACTAAATACCTACCTATGAAA-3′</td>
<td>Macey et al. (1997)</td>
</tr>
<tr>
<td>Forward</td>
<td>ND1</td>
<td>5′-CGATCCGATATGACACARCT-3′</td>
<td>Kumazawa and Nishida (1993)</td>
</tr>
<tr>
<td>Reverse</td>
<td>tRNA&lt;sup&gt;Ala&lt;/sup&gt;</td>
<td>5′-AAAAATRTCTGRTGCATTCACTCAG-3′</td>
<td>Macey et al. (1997)</td>
</tr>
<tr>
<td>Forward</td>
<td>ND4</td>
<td>5′-TGACTACAAAAGCTCATGTGAGACC-3′</td>
<td>Raxworthy et al. (2002)</td>
</tr>
<tr>
<td>Reverse</td>
<td>tRNA&lt;sup&gt;Alu&lt;/sup&gt;</td>
<td>5′-CATTACTTTTCTTGAAITTCACCA-3′</td>
<td>Raxworthy et al. (2002)</td>
</tr>
<tr>
<td>Forward</td>
<td>RAG1</td>
<td>5′-TCTGAATGAAATTCAGACTTGTT-3′</td>
<td>Groth and Barrowclough (1999)</td>
</tr>
<tr>
<td>Forward</td>
<td>RAG1</td>
<td>5′-CCACTTTGAAAAATACTCCCTGA-3′</td>
<td>This study</td>
</tr>
<tr>
<td>Reverse</td>
<td>RAG1</td>
<td>5′-GTCACTACACAAAAATGTGATATGCTTGC-3′</td>
<td>This study</td>
</tr>
<tr>
<td>Reverse</td>
<td>RAG1</td>
<td>5′-GTGTCYACTGGTGATRTCACT-3′</td>
<td>Townsend et al. (2004)</td>
</tr>
<tr>
<td>Forward</td>
<td>CMOS</td>
<td>5′-ATTATGCAGTTCMCCTMTCC-3′</td>
<td>This study</td>
</tr>
<tr>
<td>Forward</td>
<td>CMOS</td>
<td>5′-TCTGGAATTTC1CCWCTCTTG-3′</td>
<td>This study</td>
</tr>
<tr>
<td>Reverse</td>
<td>CMOS</td>
<td>5′-GCTACCCACAGARTASAGTACA-3′</td>
<td>This study</td>
</tr>
</tbody>
</table>

Note: tRNA = transfer RNA.

<sup>a</sup>Mitochondrial forward and reverse primers extend the light and heavy strands, respectively.
Distribution Mapping and Diversity Analyses

Locality data for all Brookesia species of Madagascar were gathered from museum data, our own global positioning system readings, and pertinent literature. Single-species maps that almost fully agree with the data used herein are reproduced in Glaw and Vences (2007). Small sample sizes of locality records are not appropriate to obtain reliable estimates of potential distribution by modeling. For 9 species, more than 6 (range 7–39) locality records were available. For these species, we defined distributions by potential distribution models (pruned for overprediction), and for the rest of the species, we used point locality data.

For the models, we used 23 variables as predictors: potential evapotranspiration, yearly water balance (annual rainfall minus annual evapotranspiration), number of months with a positive water balance (a measure of drought), percentage of forest cover in 2000, and 19 climatic variables from the WorldClim database version 1.4 (Hijmans et al. 2005): annual mean temperature; mean diurnal temperature range; isothermality (monthly/annual temperature range); temperature seasonality (standard deviation across months); maximum temperature of warmest month; minimum temperature of coldest month; annual temperature range; mean temperature of wettest, driest, warmest, and coldest quarters; annual precipitation; precipitation of wettest and driest months; precipitation seasonality (coefficient of variation); and precipitation of wettest, driest, warmest, and coldest quarters.

We used Maxent v2.3 (Phillips et al. 2006) for distribution modeling, as it performs well in predicting species distributions (Elith et al. 2006), even when sample sizes are small (Hernandez et al. 2006). Analyses followed Vieites et al. (2008), using 1000 randomly selected data points across Madagascar as background pseudabsence data. All models had areas under the receiver operating characteristic curve (Hanley and McNeil 1982) higher than 0.7, suggesting that the models were good at discriminating between presence and absence sites (Fielding and Bell 1997). We applied a pruning algorithm to remove areas of overprediction from the mean model. This algorithm thresholds the Maxent output models using a user-defined probability of occurrence (t), defining a convex hull around all occupied regions, and buffering this hull by a given number of grid cells (b) and width (f), within which the model values are reduced until they reach zero. We used the following parameters: t = 40, b = 40, and f = 80, as they provide a conservative scenario to remove biogeographic overprediction areas (see Kremen et al. 2008).

To calculate values of spatial species richness and endemism, the distributional data (potential distribution models and point distribution data) were transformed into a grid cell data set and plotted on a one-quarter degree square grid covering the entire island. When more than 2 records were available per species, we drew a minimum convex polygon between localities, and grid cells within the polygon were considered to contain the species. We used Worldmap v. 4.20.18. (Williams 2002) to calculate species richness as the total number of species per grid cell. Endemism was calculated as a measure of range-size rarity, expressed as the percentage aggregated reciprocal range size for all species per grid cell.

Hypothesis Testing

Temporal concordance with the Wilmé et al. (2006) and the Raxworthy and Nussbaum (1995) Pliocene/Pleistocene speciation scenarios (WC and MR hypotheses, respectively) was statistically tested by comparing the most recent tail of the 95% HPD for each sister-species pair in our phylogeny to the oldest limits of these geological epochs. The RB hypothesis was not formulated with an explicit time frame and thus could not be tested in this manner.

The Wilmé et al. (2006) WC model predicts that sister species should generally occupy adjacent COEs or possibly a COE and an adjacent RDW. To test the fit of our Brookesia data to the spatial aspect of this model, we first plotted the distributions of all Brookesia sister-species pairs identified in our phylogenetic analyses onto the Wilmé et al. (2006) watershed map (Fig. 1) and counted the number of pairs matching the model’s predictions. Next, we randomly assigned each species from all sister-species pairs to one of the watersheds collectively occupied by them and counted the number of pairs fitting the model’s predictions. We repeated this 10,000 times to generate a null distribution of expected number of fits to the model if sister species are actually distributed randomly with respect to relative watershed positions. If less than 5% of the simulation rounds resulted in a number of matches equal to or

<table>
<thead>
<tr>
<th>Node</th>
<th>Relevant fossil</th>
<th>Median (95% CI) (million years ago)</th>
<th>TL zero offset (mean, SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rhynchocephalian-squamate</td>
<td>Indeterminate fossil</td>
<td>231 (225–301)</td>
<td>224 (2.0, 1.2)</td>
</tr>
<tr>
<td>Scincomorph-iganian</td>
<td>Balneilacerta</td>
<td>167 (162–204)</td>
<td>161 (1.8, 1.0)</td>
</tr>
<tr>
<td>Anguimorph-iganian</td>
<td>Pareira</td>
<td>167 (162–204)</td>
<td>161 (1.8, 1.0)</td>
</tr>
<tr>
<td>Lacertid-amphisbaenian</td>
<td>Hodchikula</td>
<td>102 (97–128)</td>
<td>97 (1.7, 1.4)</td>
</tr>
<tr>
<td>Teiid-(lacertid, amphisbaenian)</td>
<td>Phileto</td>
<td>116 (110–177)</td>
<td>110 (1.8, 1.3)</td>
</tr>
<tr>
<td>Furcifer polleni–Furcifer cephalolepis</td>
<td>N/A</td>
<td>6.3 (1.7–16)</td>
<td>0.0 (3.5, 2.0)</td>
</tr>
</tbody>
</table>

Notes: SD, standard deviation; N/A, not available.

*a* See text for references.

*b* Nonfossil calibration based on geologic data and modeled with a gamma distribution: zero offset (γ-shape, γ-scale) (see text).
Several factors make it impractical to test this MR hypothesis with our data using the statistical framework outlined above for the WC hypothesis. First, these AOE s comprise only a fraction of the land area of Madagascar (see Fig. 1), which would necessitate the exclusion of several sister-species pairs that are not confined to them (see Results section) and thus reduce statistical power. Also, there are multiple potential vicariant relationships among most of these areas (e.g., the northeastern region could have once been connected by suitable habitat to all the other AOE s), so the number of sister-species pairs consistent with the MR hypothesis under the null model (i.e., no real effect of forest contraction on speciation) would be high. Furthermore, as stated in Raxworthy and Nussbaum (1995), there is no clear biogeographic boundary between the northeastern and the eastern AOE s, making interpretation of relationships among taxa from these areas unclear. However, there is intuitive appeal to arguments that forest-fragment contraction promoted vicariant speciation in geologically complex northern Madagascar. We thus examine congruence of several potential examples with our new phylogenetic hypothesis (some of the proposed examples of Raxworthy and Nussbaum were flawed because they compared nonsister taxa; see Results section).

The RB hypothesis (e.g., Pastorini et al. 2003) predicts sister taxa to be separated by major rivers. A few Malagasy rivers can be unambiguously defined as “major” (e.g., the Betsiboka, Mangoro, and Mananara rivers) because they are quite long, with headwaters at relatively high elevations (Wilmé et al. 2006), and because previous works (e.g., Pastorini et al. 2003) have identified them as potential barriers for lemurs. However, for most rivers, an objective assessment of their potential as barriers would require combining parameters such as length, width, headwater elevation, and permanence. These factors, combined with the difficulty of determining the size and shape of the test areas, preclude a formalized test of the RB hypothesis. However, we examine the spatial relationship of Brookesia sister taxa with several of the largest rivers to look for any evidence of a major role in species-level diversification. Our geographic sampling within some species also allows some evaluation of the role that rivers might play in intraspecific genetic structuring within Brookesia.

The WC, RB, and MR hypotheses all make general predictions about relative species abundance at different altitudes. Following Wollenberg et al. (2008), we divided Madagascar into a coarser grid with cell sizes of $82 \times 63 \text{ km} = 5166 \text{ km}^2$ and used the ArcView extension “Endemity Tools” (provided by N. Danho) to calculate Brookesia species richness and corrected weighted endemism (Crisp et al. 2001) for each cell. We then tested for nonparametric correlation of these values with altitudinal range, defined as maximum–minimum elevations per grid cell (excluding grids with no occurrence of any Brookesia species).
RESULTS

Data Characteristics and Phylogenetic Analyses

Alignments were unambiguous across chameleons for all partitions. However, alignment of the 3’ ends of ND1 (∼70 bp) and ND2 (∼90 bp), as well as part of ND4 (∼60 bp), was problematic across more distant outgroups due to high levels of amino acid replacement and multiple indels. We therefore coded these regions as missing data for nonchamaeleonid taxa. Maximum-likelihood analyses of near-complete ND1 and ND2 data across several chameleon species found very similar evolutionary model parameter values for these 2 genes (results not shown). To avoid the statistical problem of low data/parameter ratios, the ND1 sequence (∼70 bp) was combined with the ND2 for partitioning purposes. For the same reason, data from the 3 transfer RNA genes separating ND1 and ND2 (and from which the loop regions were impossible to align confidently) were not used in this study. Bayes factor analysis on various partitioning schemes (2, 3, 6, 9, and 12 partitions) strongly favored the 12-partition scheme (nuclear and mtDNA genes each partitioned by codon position; Bayes factor ≥178 in all comparisons). All results reported here are from this partitioning scheme.

Within each of the ML, UB, and RCB analysis sets, phylogenetic results were broadly congruent across the different genomes and genes, with topological conflicts involving only poorly supported nodes (i.e., ML bootstraps <70%, Bayesian posterior probabilities [PPs] <95%). One exception to this pattern involves strongly conflicting nuclear and mitochondrial results for the placement of 1 specimen of Brookesia brygooi. This discrepancy is probably due to ancient mtDNA introgression, and the nuclear topology almost certainly reflects the true organismal history (see online Supplementary Material). Mitochondrial data for this specimen were therefore excluded in all further phylogenetic analyses. In general, the mtDNA data alone gave strong support for relationships within more terminal clades, but poor support for basal nodes, and the nuclear data showed the opposite pattern. All results presented here are from analyses of the combined data set (Fig. 2). This data set and trees from this study can be found at www.treebase.org under accession number SN4455.

The mrca of the B. nasus–B. lollantayi clade and all other Brookesia appears early in the history of the group (although monophyly of neither Brookesia nor this subclade is significantly supported; Fig. 2). The remainder of the genus is clearly monophyletic and is divided into 2 well-supported clades. One of these clades corresponds to the extremely miniaturized B. minima group. The other major clade, representing the typical Brookesia morphotype, consists of a series of 5 maximally supported subclades (one of which is a single species) whose interrelationships are strongly supported by the RCB analysis but less so by the UB and ML analyses (Fig. 2).

Referencing the numbered clades in Figure 2, Clade 1 contains Brookesia perarmata, Brookesia decaryi, Brookesia bonsi, and B. brygooi. These species are restricted to the dry deciduous forests of the west and/or southwest and together form the sister taxon of the remaining typical Brookesia. The eastern rainforest sister species Brookesia thereziensi and Brookesia superciliaris (Clade 2) and the northern rainforest species Brookesia ebenaui (Clade 3) are sister taxa to progressively less inclusive clades. Clade 4 contains several populations that show substantial sequence divergence from each other (11.1% average uncorrected ND4 divergence among specimens from distinct localities) but whose interrelationships are mostly poorly supported. The eastern rainforest species Brookesia thieli is paraphyletic, although basal divergences are poorly supported. One subclade of B. thieli appears to be the sister taxon of the morphologically distinctive Brookesia vadoni, suggesting the possibility of cryptic species within B. thieli. Finally, Clade 5 comprises several species (Brookesia antakaraana, Brookesia ambrensis, Brookesia valerieae, Brookesia griveaudi, and Brookesia stumpffi) largely restricted to evergreen rainforest of the north, northeast, and northwest (B. stumpffi is exceptional among Brookesia in its adaptation to both dry deciduous and evergreen forest). Brookesia valerieae is strongly supported by all analyses as the sister taxon of B. griveaudi. Brookesia stumpffi is weakly supported as the sister taxon of a strongly monophyletic B. antakaraana–B. ambrensis clade in the RCB analysis (Fig. 2). However, the other 2 methods find weak (ML, 54% bootstrap) to strong (UB, 96% PP) support for B. stumpffi as the sister taxon of the B. griveaudi–B. valerieae clade, with this larger clade as the sister group to the partially sympatric species pair B. antakaraana and B. ambrensis (Fig. 2). These latter 2 species exhibit very low levels of divergence from one another (0.6–1.3% uncorrected ND4 distance; see Fig. 2 inset) and may not be reciprocally monophyletic. There do appear to be 2 distinct morphologies in this lineage (see photos in Glaw and Vences 2007), but mtDNA data from several additional specimens suggest that either incomplete lineage sorting or mitochondrial introgression is a major factor at this site (results not shown).

Generally, the RCB method gave a more precise phylogenetic estimate than did the UB method (83% vs. 72% of nodes significantly supported in Fig. 2). The increase in support may be a consequence of the expected increase in statistical power inherent to the relaxed-clock approach. Alternatively, model misspecification may play a role in the difference.

Geographical Centers of Diversity and Endemism in Brookesia

For the 9 modelable species, all distribution models predicted known localities, although comparison with museum records indicated some overprediction. The pruning algorithm removed some (but not all) areas appearing to have suitable environmental niches for the species, but which were located far from the known distribution ranges. This suggests that nonautecological (e.g., biogeographical, community ecological) factors
Figure 2. Combined mitochondrial (ND1/ND2/ND4) and nuclear (RAG1/C莫斯) data, 12-partition relaxed-clock Bayesian (RCB) cladogram with maximum-likelihood (ML) phylogram insert. Analyses included all outgroup taxa, although for clarity, most nonchameleon taxa are not shown. Major clades are color coded, and major clades of “typical” Brookesia are numbered (see text). RCB/unrooted Bayesian (UB) PP >95% are denoted by asterisks (PP between 90% and 95% are shown as actual values) above branches, and ML bootstrap values >50% are shown below branches. Black diamond indicates alternate attachment point for Brookesia stumpffi clade in the UB and ML analyses. Dashed terminal branches mark individuals represented by ND4 data only.
are needed to explain the restricted microendemic distribution of some species. *Brookesia* distributions span the island, although they are absent from many large areas in the central highlands and the south. Three main centers of diversity (Fig. 3a) are found in the northeast, north, and northwest, respectively. These areas are also COEs, with the highest percent values of range-size rarity (Fig. 3b). However, there are several areas, mainly in the west and northwest regions of the island, with a high degree of endemism corresponding to several species only known from single locality records.

The 3 main clades recovered in our phylogenetic analyses (Figs. 2 and 4) show distinct biogeographic patterns. The basally diverging *B. nasus* clade contains 2 species, *B. nasus* in the southeast and *B. lolontany* in the north, suggesting an old north-south connection. The *B. minima* clade shows the highest degree of endemism; most of the 11 species are restricted to very small areas in the north and north-central regions. The third and largest clade (typical *Brookesia*) spans most of the island, although centers of both diversity and endemism in this clade are in the north. Within this clade, *B. brygooi*, *B. bonsi*, *B. decaryi*, and *B. perarmata* (all western species; Clade 1, Fig. 2) form the sister taxon of the remaining species, none of which are restricted to the western forests.

**Timing of Diversification in Brookesia**

The RCB 12-partition analysis places the basal split within *Brookesia* at approximately 72 million years ago (95% confidence interval [CI] from ~63 to ~81 million years ago; Fig. 4), which is possibly older than any divergence within non-*Brookesia* chameleons (see Supplementary Material). Generally, divergences between recognized species groups and even sister taxa appear to be quite deep. Within the typical *Brookesia* clade, aside from the problematic *B. ambreensis–B. antakarana* complex, the most recent sister-species split (*B. bonsi–B. decaryi*) has a 95% CI extending no more recently than the Middle Miocene (Fig. 4). Within the *B. minima* clade, mean estimates for sister-taxon divergences are even older (the most recent divergence time mean is at the Eocene–Oligocene boundary), although the 95% CIs within the 2 clades overlap. Thus, we can confidently reject the temporal component (i.e., Pliocene–Pleistocene time frame) of both the MR (Raxworthy and Nussbaum 1995) and the WC (Wilmé et al. 2006) hypotheses.

Additional analyses using alternative calibration distributions and data sets had little effect on divergence time estimates. Analyses performed with normally distributed calibrations (with standard deviations arbitrarily set at 10% of the mean) gave average intrachameleon divergences about 10% more recent than those using lognormally distributed calibrations. Likewise, analyses that excluded mtDNA third codon positions and all mtDNA data gave respective divergence estimates on average about 15% and 18% more recent than the full-data analysis. In all these analyses, *Brookesia* sister-taxon divergences remained solidly Miocene in age, and thus, our above conclusions are unaffected.

Using the method of Near et al. (2005), we found no significant incongruence among our fossil calibrations.
Figure 4. Chronogram from the 12-partition (ND1/ND2, ND4, CMOS, and RAG1, each partitioned by codon position) RCB phylogenetic analysis. Time units on scale bar in millions of years ago. See Supplementary Materials for exact divergence time means and 95% CIs for all nodes. Maps above chronogram illustrate patterns of species richness (quantified as numbers of species present within one-quarter degree square grid cells) for the 3 main clades (1 = Brookesia nasus group, 2 = Brookesia minima group, and 3 = “typical” Brookesia group).
with one exception; the root calibration (representing the rhynchocephalian/squamate split) was a significant outlier ($P = 0.017$). Following Near et al. (2005), this calibration should be discarded as potentially misleading. Exclusion of this calibration point draws all extrachamaeleoid divergences to markedly more recent dates, and the estimated root age varies from approximately 170–150 million years ago. However, fossil rhynchocephalians are known from multiple Laurasian and Gondwanan localities by the Late Triassic (Evans et al. 2001). Thus, even allowing for reasonable error in fossil dates, Middle to Late Jurassic estimates for the Rhynchocephalia–Squamata split are clearly untenable (see Marshall 2008 for further discussion of the potential pitfalls of excluding outliers). We therefore favor the results from the full constraints analysis. That said, we note that even in analyses performed without the root constraint, in no case did the 95% HPDs for *Brookesia* sister-taxon divergences reach the Pliocene.

**Fit of Spatial Hypotheses**

We compared the distribution of *Brookesia* with the 12–15 COEs recently proposed by Wilmé et al. (2006) (Fig. 1). Of approximately 30 *Brookesia* species, 18 are limited to single COEs (3 of these are also found in 1 RDW), 9 are found in 2 or more separate COEs (1 is also found in an RDW), and 2 additional species are limited to single RDWs. However, these numbers are difficult to interpret for several reasons. First, *Brookesia* distributions are heavily skewed toward the northern end of the island, and certain COEs are occupied by disproportionate numbers of species. For example, 22 of 30 species (73%) are distributed across just 6 COEs, and 7 of these species are restricted to a single COE (S. Bemarivo/N. Mangoro [2] on the eastern and northeastern coast) (Fig. 1). Furthermore, the level of microendemism within *Brookesia* is extreme: 18 of 30 species are essentially known from single localities (Glaw and Vences 2007). Thus, most species of *Brookesia* will be confined to single COEs regardless of the mechanism of their isolation. But perhaps most importantly, simple tabulations of species counts across proposed COEs ignores phylogeny completely.

To test for spatial concordance with the WC hypothesis, we first plotted the distributions of the 10 strongly supported (PP > 95%) *Brookesia* sister-species pairs identified in our phylogenetic analysis (i.e., 20 species, see Fig. 2, also Supplementary Table S1) onto the Wilmé et al. watershed map (Fig. 1). Although none of these sister-species pairs are strictly confined to adjacent COEs, *Brookesia tuberculata* is found on Montagne d’Ambre, which straddles COEs 1 and 12, and its sister species *Brookesia* sp. “Montagne de Francais” occupies COE 1. Two sister-species pairs (*Brookesia exarmata* [8]/*Brookesia dentata* [G; also 9] and *Brookesia* sp. “Betampona” [2]/*Brookesia ramannantsoai* [B]) follow the adjacent COE/RDW pattern (Figs. 1 and 2). However, neither of these RDW residents is a wide-ranging species, as the Wilmé et al. (2006) hypothesis would predict. Nonetheless, accepting these 3 pairs as potential fits to the model, we performed 10,000 rounds of random allocations of the 20 relevant species (i.e., those belonging to sister-species pairs) to the watersheds collectively occupied by them and found that 12% of the simulation rounds allocated the members of 3 or more sister-species pairs to adjacent watersheds (i.e., $P = 0.12$). When allopatric taxa occupying the same watershed were included as potential matches in the simulations (there were none of these in the real data), $P = 0.32$. Thus, we found no support for the spatial component of the WC hypothesis.

Unlike the WC hypothesis, the MR hypothesis of Raxworthy and Nussbaum (1995) was formulated with respect to 5 AOEs in the north and east of Madagascar (see Materials and Methods section and Fig. 1), as opposed to watersheds covering the entire island. Of the 10 *Brookesia* sister-species pairs from our phylogeny (Fig. 2), only a subset of these taxa are distributed in these areas and therefore useful for evaluation of this hypothesis. Specifically, the species pairs *B. antakarana*–*B. ambreensis*, *B. griveaudi*–*B. valeriae*, *Brookesia karchei*–*Brookesia peyrierasi*, and *B. tuberculata*–B. sp. “Montagne de Francais” are wholly confined to these AOEs. Of these pairs, *B. griveaudi* (northeast) and *B. valeriae* (northwest) both inhabit forests less than about 900 m altitude and are absent from the higher altitude (>1600 m) forests of the intervening Tsaratanana massif. As proposed by Raxworthy and Nussbaum (1995), these areas may once have been connected by an east-west corridor of even lower altitude forest that was lost during a period of aridification. Tsaratanana itself is home to *B. lolontany*, likely sister taxon of the only other *Brookesia* species with populations known to inhabit this altitudinal range, *B. nasus* of southeastern Madagascar. Although none of the other species pair distributions are consistent with this spatial model, 2 interesting higher level patterns within the *B. minima* group clade are potential fits. First, *B. minima* is restricted to the northwest, whereas its sister clade (*B. tuberculata* + *B. sp. “Montagne des Francais”) is restricted to the Montagne d’Ambre region. Second, the clade formed by these 3 species is found only to the west of Tsaratanana, whereas its sister clade (*B. cf. karchei* + *B. peyrierasi*) is found only to the east of Tsaratanana.

Finally, a comparison of *Brookesia* distribution and phylogeny with major rivers yields no clear support for the RB hypothesis. The Betsiboka River in western Madagascar, which has been shown to be a major barrier for several lemur populations (Pastorini et al. 2003), may possibly lay between the sister species *B. exarmata* (to the southwest) and *B. dentata* (to the northeast). However, our *B. dentata* is the only known specimen from Ankaranfantsika National Park, which actually straddles the river. At any rate, *B. exarmata* is confined to the Tsingy de Bemaraha approximately 300 km (and several more rivers) away to the southwest. Similarly, *B. bontsi* is found at a single locality (Namoroka) about 75 and 100 km to the west of the large Mahavavy and Betsiboka rivers, respectively, and its sister species
B. decaryi is known only from Ankarafantsika. Once again, the highly restricted and disjunct distributions of these species make any inference of a river barrier as the isolating mechanism highly speculative. No other sister-species pair (or higher level sister-clade pair) from our phylogeny is divided by what might reasonably be considered a major river.

River barriers can of course play a role in intraspecific genetic structuring (e.g., Pastorini et al. 2003; Kozak et al. 2006; Lemmon et al. 2007), and there are a few Brookesia species with distributions that span large rivers (B. nasus, B. superciliaris, and B. thieli in the east; B. stumpffi and possibly B. ebenau in the north; and B. brygooi in the west). To fully study the phylogeographic effects of these rivers will require much more thorough sampling. However, our sampling within some species does allow at least some preliminary comments. The western B. brygooi has an extensive distribution, including populations on both sides of the Betsiboka River. However, the deepest split within B. brygooi separates a population to the south of the river from multiple populations spanning both sides of the river. Likewise, the distribution of B. stumpffi spans the Sambirano, Mahavavy, and Mananjeba rivers (among others) in northwestern Madagascar. Although the deepest split does separate a population to the south of the Sambirano River from several more northern populations, relationships among the remaining populations do not support the Mahavavy and Mananjeba rivers as barriers to gene flow. Finally, B. nasus is found both to the north and to the south of the large Mananara River in southeastern Madagascar. However, an individual from the northernmost population is nested within a clade of individuals from populations to the south of the potential barrier. See Supplementary Material for figures illustrating these scenarios. Thus, although further sampling could reveal a more complex pattern (possibly also involving smaller rivers), our data do not suggest a major river effect on genetic structuring within any of these species.

Species richness of Brookesia was negatively correlated with the minimum altitude ($P < 0.05$) and positively correlated with the maximum altitude ($P < 0.001$) in each grid cell. Species richness and corrected weighted endemism of Brookesia furthermore showed a significant correlation with altitudinal range per grid cell ($P < 0.001$ and $P < 0.05$, respectively). These results are not congruent with the RB and WC hypotheses, both of which predict a higher species richness and endemism at lower elevations where rivers are widest and where river basins forming COEs have their headwaters. In contrast, the results are consistent with the MR hypothesis, which predicts more species will be restricted to montane regions where there is a higher probability of isolation during dry periods.

**DISCUSSION**

Our ability to evaluate past processes that produced current patterns of organismal distribution and diversification is constrained and biased by the relative availability of climatological and geological data from different periods in the earth’s history. In general, Pleistocene climatic changes (specifically, glaciation cycles that have predictable effects on sea level, aridity, and temperature) are much better understood than those from the Pliocene, Miocene, and earlier (Wells 2003). This may explain their prominence in many glaciation-related diversification theories. Results from phylogeographic studies have challenged the importance of the most recent Pleistocene and Holocene glaciations as major drivers of species-level diversification for several groups (Klicka and Zink 1997; e.g., Avise et al. 1998; Rull 2008). In Madagascar, the existence of Pleistocene glacial activity and dynamic vegetational shifts has been documented (Burney 1995; Vidal-Romani et al. 2002), but well-studied examples indicating the dependence of species diversification on these processes are lacking.

Most phylogeny-based biogeographical studies of Madagascar have centered on the relative importance of vicariance and dispersal as the source of Madagascar’s many endemic higher taxa (Yoder and Nowak 2006 and references therein). Given the long-standing interest in Madagascar’s high level of microendemcity, it is surprising that only a few phylogenetic studies have explicitly estimated sibling-species divergence times (e.g., Vences et al. 2002; Raxworthy et al. 2008; Wirta et al. 2008). To our knowledge, none of these studies have found species-level divergence times compatible with Pleistocene–Pliocene radiations. Other mtDNA studies of various groups including geckos, skinks, spiders, and lemurs (Yoder et al. 2000; Vences et al. 2002; Schmitz et al. 2005; Wood et al. 2007; J ackman et al. 2008) have reported genetic divergences that are likewise incompatible with widespread Pleistocene–Pliocene speciation, using standard rates of mtDNA sequence evolution (but see Wirta and Montreuil 2008 for a possible Pliocene radiation of beetles). Thus, although well-sampled phylogenies of many more groups are needed, existing evidence (including this study) suggests that recent climatological cycles have had relatively minor effects on levels of species richness and associated microendemcity on Madagascar.

Theoretical considerations (e.g., Hewitt 1996) and empirical examples from other groups have suggested the importance of earlier glaciation cycles on the North American and European flora and fauna (Veith et al. 2003; Ayoub and Riechert 2004; Hewitt 2004; Kadereit et al. 2004; Turgeon et al. 2005). Unfortunately, historical species distributions in Madagascar have been very difficult to determine due to the paucity of Tertiary fossil deposits earlier than the Quaternary (Wells 2003). This has in turn greatly limited the ability to infer paleoclimates and historical vegetation cover that might help explain existing complex microendemic distribution patterns. Nonetheless, assuming relative stability of drainage systems and topographical features, resultant phylogenetic patterns could be very similar between earlier and more recent climatic cycles. An evaluation
of the competing hypotheses thus depends largely on our knowledge of current distribution, habitat, and phylogeny.

Watershed Contractions, Mountain Refuges, and Riverine Barriers

Our test of the spatial component of the Wilmé et al. WC hypothesis is potentially biased in that phylogenetically closer species might be expected to have distributions geographically closer than more distantly related species. This could result in a nonrandom association between sister taxa and adjacent watersheds, even if WC was not the cause of their separation. Thus, our failure to reject a random watershed/sister-taxon association, combined with a negative correlation between altitude and species richness, strongly suggests that this mechanism cannot explain current Brookesia distributions. On the other hand, because species-level divergences in this group are relatively deep (Late Eocene to Late Miocene), there is the possibility that extinctions, climate/habitat changes, and range expansions have obscured patterns that would support this hypothesis. These are problems inherent to the study of any older radiation (Losos and Glor 2003). Unless new relevant fossils (including those indicative of past climates) are discovered, the first 2 problems are largely unavoidable. However, Brookesia may be less prone than many groups to the last potential problem due to the probable low vagility of these leaf litter inhabitants.

The Raxworthy and Nussbaum (1995) MR model, although restricted to northern Madagascar, seems to fit to our data reasonably well. Our study reveals that some of the sister-taxon pairs originally cited in favor of this model (B. ambreensis/B. valerieae and B. stumpffi/B. griveaudi) are actually not sister taxa but only more distantly related. Nonetheless, the Tsaratanana massif and associated montane areas do separate 2 sister taxa as well as 2 multispecies clades, and another clade is split between the Northwest and Montagne d’Ambre regions. We noted earlier that for any given forest fragment, multiple potential vicariance relationships often exist. This fact may account for some of the apparent fit to this model. However, independent support for this model is provided by the significant positive correlation of altitude and altitudinal range with species richness and endemism, which is the pattern expected if most speciation occurs by fragmentation and isolation in montane forest fragments.

Rivers appear to have played a substantial role in the diversification of several lemur groups (Pastorini et al. 2003; Goodman and Ganzhorn 2004; Louis et al. 2005), some frogs (Vieites et al. 2006), and possibly even some chameleons of the genus Furcifer (Raselimanana and Rakotomamalala 2003). However, this does not seem to be true for leaf chameleons, at least at the species level and above. It seems unlikely that individual Brookesia disperse over large distances, and they certainly cannot swim across rivers. However, given their small size and leaf litter microhabitat, they could quite plausibly float across rivers on debris washed away during heavy rains.

Higher Level Phylogenetic and Biogeographical Patterns

Wells (2003) hypothesized that the northward drift of Madagascar from its pre-Paleocene position south of the subtropical arid zone precipitated the aridification of most or all the island, causing the loss or great reduction of much of the existing biodiversity. Later entry into the “trade wind” zone of southeasterly winds (most likely sometime in the Eocene) brought rains to the east coast, causing the formation of the eastern humid forests. According to Wells (2003), this increased moisture, combined with flow of the warm southern equatorial current through the widening Mozambique Channel, also led to the formation of the western deciduous forests. Correlating this scenario with our dated phylogeny allows some speculation on drivers of diversification and distribution at higher levels within Brookesia. Specifically, aridification may have fragmented the ancient B. nasus clade into disjunct high montane areas, with expansion of B. nasus into lower altitudes of the eastern rainforest during the Eocene–Oligocene. Most diversification within both the B. minima and the typical Brookesia clades occurred during this same period and was thus likely tied to the same expansion of mesic habitats. The striking size disparity between members of these 2 clades, combined with largely overlapping distributions, suggests some sort of niche partitioning (most likely prey size and/or microhabitat usage), but data supporting or refuting this hypothesis are lacking.

Our geographical analyses confirm that northern Madagascar is the center of species richness and endemism of Brookesia (Fig. 3), as postulated by Raxworthy and Nussbaum (1995). However, within the clade of typical Brookesia, the most basal splits do not divide taxa restricted to the north. Instead, the first 2 splits separate a western and eastern clade, respectively, from the remainder, which itself contains mostly northern and a few more nested eastern species. This pattern suggests ancient east-west dispersal or vicariance with subsequent diversification within these areas and a later diversification in the north. Although the B. minima group is speciose, it is split at its base into a north-restricted clade and a more southern clade. Thus, hypotheses of northern or southern origins for this group are equally parsimonious. However, within the southern clade, there is a clear basal split between 1 western (dry deciduous) and 2 eastern (rainforest) groups. Interestingly, the repeated east-west splits within Brookesia represent a type of within-landmass biome shift in one of the lineages, a phenomenon that was found to be quite rare in a recent global-wide study of plants (Crisp et al. 2009).

In summary, higher level diversification within Brookesia was quite possibly tied to an Eocene–Oligocene shift to more mesic habitats on Madagascar, and our analyses support a substantial role for rainfall fragmentation.
(especially in the elevationally heterogeneous north) in species-level diversification. A true test of any model purported to generally explain the complex patterns of diversity in the Malagasy fauna will require at least a combination of distributional and current climatic data such as that provided by Wilmé et al. (2006) and a series of well-supported phylogenies (with reasonably accurate estimates of divergence times) from a variety of taxonomic groups. As paleoclimatological and paleo-vegetational reconstructions become available, they will also be very valuable because Malagasy terrestrial fossil deposits from the Tertiary are virtually nonexistent.

SUPPLEMENTARY MATERIAL
Supplementary material can be found at http://www.sysbio.oxfordjournals.org/.

FUNDING
This work was supported by grants from the Deutsche Forschungsgemeinschaft and the Volkswagen Foundation to M.V. and F.G. and by U.S. National Science Foundation grants (postdoctoral bioinformatics fellowship DBI-0204451 to T.M.T., Tree of Life NSF 1-10135 grant to D.R.V., and Tree of Life grant EF 0334923 to Tod Reeder).

ACKNOWLEDGMENTS
We are grateful to Daniel Scantlebury for assistance in the lab and to Edward Louis of the Henry Doorly Zoo’s Center for Conservation and Research for providing collecting opportunities for T.M.T. in Madagascar. Akira Mori generously provided important additional tissue samples. Franco Andreone, Parfait Bora, Kathrin Glaw, Fabio Mattioli, Roger Daniel Randrianarina, and Jasmin E. Randrianirina assisted M.V., F.G., and D.R.V. during fieldwork. Katharina C. Wollenberg helped with the geographical analyses. Scott Kelley gave helpful Python coding advice. This research was carried out in the framework of collaboration accords with the Département de Biologie Animale, Université d’Antananarivo, and the Association Nationale des Aires Protégées ANGAP. We are indebted to the Malagasy authorities and an anonymous reviewer gave helpful comments on earlier versions of the manuscript.

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Received 19 January 2009; reviews returned 3 April 2009; accepted 21 September 2009

Associate Editor: Marshal Hedin