

Defining spatial and temporal patterns of phylogeographic structure in Madagascar's iguanid lizards (genus *Oplurus*)

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Abstract

Understanding the remarkably high species diversity and levels of endemism found among Madagascar's flora and fauna has been the focus of many studies. One hypothesis that has received much attention proposes that Quaternary climate fluctuations spurred diversification. While spatial patterns of distribution and phylogenetic relationships can provide support for biogeographic predictions, temporal estimates of divergence are required to determine the fit of these geospatial patterns to climatic or biogeographic mechanisms. We use multilocus DNA sequence data to test whether divergence times among Malagasy iguanid lizards of the subfamily Oplurinae are compatible with a hypotheses of Pliocene–Pleistocene diversification. We estimate the oplurine species tree and associated divergence times under a relaxed-clock model. In addition, we examine the phylogeographic structure and population divergence times within two sister species of *Oplurus* primarily distributed in the north-west and south-west of Madagascar (*Oplurus cuvieri* and *Oplurus cyclurus*, respectively). We find that divergence events among oplurine lineages occurred in the Oligocene and Miocene and are thus far older and incompatible with the hypothesis that recent climate fluctuations are related to current species diversity. However, the timing of intraspecific divergences and spatial patterns of population genetic structure within *O. cuvieri* and *O. cyclurus* suggest a role for both intrinsic barriers and recent climate fluctuations at population-level divergences. Integrating information across spatial and temporal scales allows us to identify and better understand the mechanisms generating patterns diversity.

Keywords: biogeography, divergence time, Iguanidae, Oplurinae

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Introduction

The diverse and unique biota of Madagascar begs evolutionary explanation, and numerous biogeographic hypotheses have been proposed to account for the observed geographic patterns of species richness and diversity (Vences *et al.* 2009). While some hypotheses emphasize the contemporary factors that may limit distributions (e.g. climate, Pearson & Raxworthy 2009) or prevent gene flow (e.g. rivers-as-barriers, Martin 1972), others focus on the ways that historical fluctuations in

abiotic factors might have contributed to diversification (e.g. ecogeographic constraint, Yoder & Heckman 2006; riverine refugia, Wilmé *et al.* 2006; montane refugia, Wollenberg *et al.* 2008). Although species distributions coincident with proposed biogeographic regions (e.g. Wilmé *et al.* 2006; Pearson & Raxworthy 2009) and sister taxon pairs distributed to either side of a putative biogeographic barrier (e.g. Pastorini *et al.* 2003; Boumans *et al.* 2007) lend support to these hypotheses, current geospatial patterns alone are not sufficient to elucidate the mechanisms associated with divergence. Looking beyond patterns of geographic distribution at the timing of divergence events and associated demographic histories can help us to evaluate alternative

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hypotheses and tease apart current and historical processes.

Divergence times have been estimated to discriminate between vicariant and dispersal origins of endemic Malagasy clades (Nagy *et al.* 2003; Yoder *et al.* 2003; Yoder & Yang 2004; Noonan & Chippindale 2006; Poux *et al.* 2005, 2008; see Yoder & Nowak 2006) and, more recently, to test the plausibility of specific colonization routes (Townsend *et al.* 2011a). It seems intuitive that similar approaches should be applied towards understanding patterns of species diversity within these Malagasy clades; though as of yet, relatively few studies have estimated the timing of species diversification within Madagascar (but see Townsend *et al.* 2009).

The watershed hypothesis (Wilmé *et al.* 2006) is an attractive model that includes both temporal and spatial predictions. This biogeographic model suggests that Quaternary fluctuations in climate created a dynamic landscape with multiple refugia and, hence, the opportunity for allopatric divergence. The watershed hypothesis predicts that river drainage systems would have served as refugia during dry periods. Large rivers with high-elevation headwaters (from the high plateau) would have allowed for persistently suitable habitat along river corridors, thereby creating retreat-dispersion zones (RD). Smaller rivers would also have served as refugia defining isolated areas or centres of endemism (COE; see Fig. 1). Species distribution patterns for lemurs (Wilmé *et al.* 2006; Weisrock *et al.* 2010), geckos and chameleons (Pearson & Raxworthy 2009), and patterns of molecular divergence within multiple species of lizards (Boumans *et al.* 2007) are in line with the expectations derived from this hypothesis. However, the few studies for which interspecific divergence times have been estimated are incompatible with a Pliocene–Pleistocene time frame (e.g. Horvath *et al.* 2008; Townsend *et al.* 2009), suggesting that other mechanisms must have been at work.

An alternative hypothesis suggests that larger rivers do not serve as refugia, but instead constitute long-standing barriers to gene flow (Martin 1972). This hypothesis conversely predicts that rivers flowing from high-elevation headwaters to the coasts impede gene flow, thereby defining biogeographic regions of endemism in Madagascar. Studies of lemurs have shown in several cases that sister taxa are distributed on opposite sides of rivers (Pastorini *et al.* 2003; Olivieri *et al.* 2007), implying that the rivers serve as the biogeographic mechanism leading to (or at least maintaining) lineage divergence. For some taxa, such as sportive lemurs, even lower-order rivers within a biogeographic region serve as barriers to dispersal (Craul *et al.* 2007). These observations of rivers-as-barriers do not apply universally, however, and in taxa such as chameleons in the

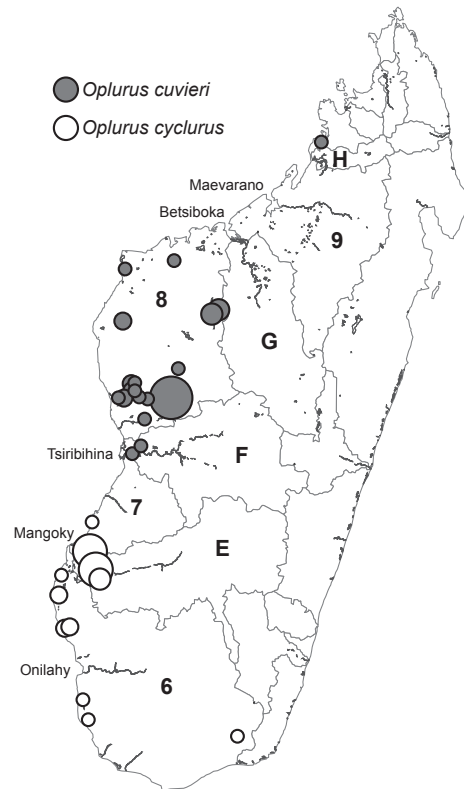


Fig. 1 Map of Madagascar with collection localities for *Oplurus cuvieri* (grey) and *Oplurus cyclurus* (white). Size of circles corresponds to sample size. Centres of endemism and retreat-dispersion zones as outlined by Wilmé *et al.* (2006) for western Madagascar are lettered and numbered, respectively.

genus *Brookesia*, rivers do not appear to influence the distribution of species (Townsend *et al.* 2009).

Although the watershed hypothesis and the rivers-as-barriers hypothesis were originally proposed to explain interspecific divergences, these mechanisms may also play a role in intraspecific differentiation. For example, although many interspecific divergences do not fit the temporal expectations for the watershed hypothesis, riverine refugia during recent climate fluctuations may have been important for the generating patterns of genetic divergence within species. Looking at more recent intraspecific divergence events can provide us with additional insight for understanding the processes that might underlie speciation in the more distant past.

The iguanid lizards of Madagascar are hypothesized to be Gondwanan relicts from ~90 Ma (Noonan & Chippindale 2006) belonging to the monophyletic subfamily Oplurinae. Neither of the two genera, *Chalarodon* nor *Oplurus*, is species rich, but there is accumulating molecular evidence for greater species diversity than currently described (Münchenberg *et al.* 2008). Madagascar iguanid lizards currently include the monotypic genus *Chalarodon* and six species of *Oplurus* (Glaw

& Vences 2007). *Chalarodon* is a terrestrial species frequently observed on sandy soil in open areas at forest edges and in dry and thicket forests. *Oplurus* is comprised of two distinct lineages: the spiny-tailed arboreal species (*Oplurus cuvieri* and *Oplurus cyclurus*) and the rock dwelling species (*Oplurus fierinensis*, *Oplurus grandidieri*, *Oplurus quadrimaculatus* and *Oplurus saxicola*) (Münchenberg *et al.* 2008; Gardner *et al.* 2011). Both species belonging to the first group frequent calcareous rock associated with thicket forest. Of these two species, *O. cuvieri* primarily occurs in the north-west and western regions of Madagascar at low-to-mid elevations, largely distributed in dry deciduous and transitional forests and very often arboreal, using calcareous rock and tree trunks as microhabitat. *Oplurus cyclurus*, in contrast, has a more south and south-western distribution found at low elevations and in drier habitats (e.g. spiny bush and thicket forest) (Glaw & Vences 2007; Rakotondravony & Goodman 2011).

We investigate the phylogenetic relationships among oplurine lizards and characterize the temporal pattern of divergence among species using multilocus sequence data to evaluate whether the timing of divergence is consistent with hypothesized Quaternary geoclimatic processes. In addition, we examine the temporal and spatial patterns of intraspecific genetic differentiation within *O. cuvieri* and *O. cyclurus* to test whether commonly invoked mechanisms for speciation might play a role at more recent scales and at the population level within species.

Methods

Sampling and molecular data collection

Chalarodon madagascariensis and six species of *Oplurus* (*O. cuvieri*, *O. cyclurus*, *O. saxicola*, *O. quadrimaculatus*, *O. fierinensis* and *O. grandidieri*) were collected from throughout Madagascar. Prior to fixation and preparation, muscle from each specimen was sampled and placed in EDTA buffer. Specimens were deposited at the Animal Biology Department collection room, University of Antananarivo, Madagascar, and at the Field Museum of Natural History, Chicago. Three individuals of *Chalarodon* were included to serve as the outgroup to *Oplurus*, and five *O. grandidieri*, one *O. fierinensis*, two *O. saxicola* and four *O. quadrimaculatus* were also included to verify the interspecific relationships and timing of divergence among *Oplurus* species. Population sampling included 34 individuals from 19 localities for *O. cuvieri* and 26 individuals from 11 localities for *O. cyclurus* (Fig. 1).

Whole genomic DNA was extracted with either the NucleoSpin kit or the Qiagen DNeasy kit. We used the

primers 16dR and tMet (Leaché & Reeder 2002) to amplify the complete mitochondrial NADH dehydrogenase 1 gene (ND1) and flanking regions. To amplify the exonic nuclear loci—the prolactin receptor (PRLR) and ubinuclein 1 (UBN1)—we used the primers PRLR_f1 and PRLR_r3, and UBN1_f1 and UBN1_r2, respectively (Townsend *et al.* 2008). PCR was performed in total volumes of 15 µL with 1 µL DNA template, 1× Buffer, 2 mM MgCl₂, 3.75 mM dNTPs, 2.5 µM of each primer and 0.375 U Jump Start *Taq* polymerase (Sigma). Cycling consisted of initial denaturation at 94 °C for 2 min, 35 cycles of 94 °C for 30 s, 58 °C (or 61 °C for UBN1) for 30 s, and 72 °C for 1 min followed by a final extension at 72 °C for 10 min. PCR was cleaned with ExoSapIT (USB). Sequencing was conducted with the same primers used in amplification and for ND1, an additional internal primer (ND1-INTR 5'-TAT TCT GCT AGG AAG AAW AGG GCG-3') (Schmitz *et al.* 2005) was used for sequencing to ensure high confidence in nucleotide calls. Sequences were visualized on an ABI3730xl and checked and edited by hand in Sequencher 4.8.

For the nuclear loci, PRLR and UBN1, we resolved the individual alleles for all heterozygous individuals. We used the programs SeqPHASE (Flot 2010) and PHASE (Stephens & Donnelly 2003) to estimate the posterior probability of haplotype pairs for each directly sequenced heterozygous sequence. Samples without well-supported computationally phased haplotypes (posterior probability, PP < 0.95) were subcloned to recover the individual haplotypes.

Sequence alignment

DNA sequence data were aligned by hand in MacClade 4.0 (Maddison & Maddison 2005), and annotation of 16s, tRNAs and the ND1 coding region was determined by alignment of fragments to the *Chalarodon madagascariensis* mitochondrial genome from GenBank (NC_012836). Alignment and annotation of codon positions for PRLR and UBN1 were straightforward.

Interspecific relationships and divergence times

Fossil calibrations or locus specific substitution rates are necessary to estimate divergence times on molecular phylogenies. There are no fossil calibration points within oplurine lizards, but multiple calibration points are available within the iguanian clade (see Townsend *et al.* 2011b). Thus, to estimate divergence times among oplurine species, we first estimated the mean and standard deviation of the substitution rate for the oplurine clade at ND1 using complete ND1 sequences from 20 iguanian and one lacertid lizard from GenBank (Table S1, Supporting information) and one *Chalarodon*

and two *Oplurus* species newly sequenced here. We used MAFFT within SeqView to align ND1 nucleotide sequences by translated amino acid and subsequently used MrModeltest (Nylander 2004) to determine the appropriate nucleotide substitution model at each codon position under the Akaike information criterion (AIC) (Table 1). We used three calibration points with log-normal priors following Townsend *et al.* (2011b; Iguania—mean = 1.2, SD = 1.9, offset = 70; Acrodonta—mean = 1.35, SD = 1.9, offset = 46.2; Pleurodonta—mean = 1.8, SD = 1, offset = 70). Townsend *et al.* (2011b) used two alternate root ages for the calibration of the Iguania clade; thus, to be most conservative in testing for congruence with Pleistocene divergence, we used the more recent of these calibration points. We analysed the ND1 alignment in BEAST 1.6.2 (Drummond & Rambaut 2007) assuming an uncorrelated log-normal relaxed-clock model and a Yule speciation tree model. After checking for mixing within runs and convergence among runs, we used logCombiner to merge two final independent runs (30 million steps recorded every 1000 steps) after discarding the first 3 million steps as burnin. We additionally conducted analyses without data to verify the priors. The oplurine clade and associated substitution rates and branch lengths were extracted from the posterior distribution of trees using ape 2.7–3 (Paradis *et al.* 2004) to calculate the posterior for the oplurine ND1 substitution rate.

We simultaneously estimated the multilocus species tree and species divergence times for the oplurines in *BEAST (Heled & Drummond 2010). The potential for confounding effects of much denser sampling within *O. cuvieri* and *O. cyclurus* was eliminated by analysing two alternate data sets, each with a subset of *O. cuvieri* and *O. cyclurus* sequences (Table S2, Supporting information). DNA substitution models for each partition were determined in MrModeltest (Table 1) and runs assumed a birth–death speciation model and an uncorrelated log-normal relaxed-clock model. The prior for the ND1 substitution rate was specified as the posterior substitution rate from the fossil-calibrated analysis; uniform priors were used for the three additional clock partitions (ND1 flanking, PRLR and UBN1). Final runs for each data set were each 30 million steps with trees saved every 1000 steps. The majority rules species tree was calculated and annotated in TreeAnnotator after discarding the first 3001 trees.

Phylogeography of *O. cuvieri* and *O. cyclurus*

Haplotype networks for each locus were constructed for *O. cuvieri* and *O. cyclurus* under a parsimony framework in TCS 1.2 (Clement *et al.* 2000) to visualize the relationships among allelic diversity and geographic locality. Separate networks were constructed for each of the two species at mtDNA because of high sequence

Table 1 Substitution models used for data partitions in coalescent-based analyses, including estimation of (A) the ND1 substitution rate, (B) the species tree and associated divergence times, (C) the intraspecific mitochondrial phylogenies and divergence times and (D) changes in population size within watershed zones with extended Bayesian skyline plots

	A.	B. Species Tree		C. Within species		D. E BSP		
	Iguania	Subset A	Subset B	<i>Oplurus cuvieri</i>	<i>Oplurus cyclurus</i>	<i>Oplurus cuvieri</i> Zone 8	<i>Oplurus cyclurus</i> Zone 6	<i>Oplurus cyclurus</i> Zone E
ND1 flanking		GTR + G	GTR + G	HKY + I	HKY + I	HKY + I	HKY + I	HKY
ND1 position1	GTR + I + G	SYM + I	SYM + I	K80	SYM			
ND1 position2	GTR + I + G	HKY + I	HKY + I	HKY	F81			
ND1 position3	HKY + G	GTR + I + G	GTR + I + G	GTR	GTR + G			
ND1 all coding positions						HKY + G	GTR + I	HKY
PRLR position1		HKY	HKY					
PRLR position2		HKY + I	HKY + I					
PRLR position3		HKY	HKY					
PRLR all positions						HKY	F81	GTR
UBN1 position1		K80	K80					
UBN1 position2		HKY	HKY					
UBN1 position3		SYM + G	SYM + G					
UBN1 all positions						HKY + I	HKY + I	HKY + I

ND1, NADH dehydrogenase 1; PRLR, prolactin receptor; UBN1, ubinuclein 1.

divergence, but single networks including both species were constructed for both PRLR and UBN1.

To get a general idea of the putative timing of intra-specific divergence within *O. cuvieri* and *O. cyclurus*, we estimated the mtDNA gene genealogy and nodal divergence times. We first used the likelihood ratio test to check for adherence to a molecular clock at noncoding and coding partitions of the mtDNA sequence data for each species. We rejected a molecular clock at the coding ND1 locus for *O. cyclurus*. Thus, phylogenetic and divergence time analyses in BEAST (Drummond & Rambaut 2007) assumed an uncorrelated log-normal relaxed clock for the coding regions in *O. cyclurus*; non-coding *O. cyclurus* and both regions of *O. cuvieri* assumed a strict molecular clock. Partitioned Bayesian coalescent analyses were run under models of constant population size and exponential growth. We used the posterior distribution of the substitution rate at ND1 for both *O. cuvieri* and *O. cyclurus* from the oplurine species tree analyses to specify a normal prior on substitution rate for these intraspecific analyses. Mixing and convergence were examined in Tracer. Final runs consisted of 100 million steps sampled every 10 000 generations. Gene trees were summarized on the majority rules consensus topology in TreeAnnotator after discarding the first 1001 trees as burnin. Bayes factors were used to compare support for the alternative coalescent population growth models.

Tests of alternative phylogeographic hypotheses

The rivers-as-barriers and watershed hypotheses predict reciprocal monophyly of populations to either side of major rivers, and within delineated COE and RD zones, respectively. We examined the posterior support for geographically delineated clades in our intraspecific analysis of phylogenetic relationships and divergence times.

Incomplete lineage sorting because of recent divergence and/or large population sizes can obscure phylogeographic patterns. Thus, we used coalescent simulations when possible to test support for alternative hypotheses. Small sample sizes of *O. cuvieri* south of the Tsiribihina River and within Zone F precluded formal coalescent-based tests of alternative hypotheses for *O. cuvieri*. We examined the geographic distribution of haplotypes and the phylogenetic support for intraspecific groups to assess support for these hypotheses.

We tested both the rivers-as-barriers hypothesis and the watershed hypothesis for *O. cyclurus* with parametric bootstrap. For each spatial model, we tested five different divergence times (0.1, 0.5, 1, 2 and 3 Ma) intended to bracket Pleistocene differentiation and the population divergence in our intraspecific trees. To

parameterize models, we first estimated population-specific θ across all three loci under a Bayesian framework in IMA (Hey & Nielsen 2007). We set $t = 0$ to mimic a single panmictic population, and multiple independent runs were conducted to ensure convergence among runs. Final analyses set $q1 = 10$ and consisted of 5 000 000 steps following an initial burnin of 500 000 steps. We calculated the diploid autosomal N_e for each population from θ using the geometric mean of the locus specific substitution rates for *O. cyclurus* from *BEAST and assuming a likely generation time of 2 years (A. Mori, personal communication). The northern population under the watershed hypothesis had only one individual; thus, we assumed an N_e of 500 000, approximately two-thirds and one-fifth N_e for the central and southern populations, respectively. For each population model, we simulated 1000 gene trees at each locus for each of five divergence times in *ms* (Hudson 2002) and used *Seq-Gen* (Rambaut & Grassly 1997) to simulate DNA sequence data on gene trees given the best-fit model of sequence evolution for the empirical data. For each simulated DNA sequence data set, the maximum-likelihood tree was estimated in Garli 2.0 using two search replicates with search cut-offs of 20 000 steps and 0.001 likelihood units. Slatkin and Maddison's *S* statistic (Slatkin & Maddison 1989) was calculated for each tree given the hypothesized population tree in Mesquite 2.7.5 (Maddison & Maddison 2011), and one-sided tests were used to determine the probability of each model given the empirical data.

The watershed hypothesis predicts population expansion within COE and RD zones. Thus, we additionally tested for support for demographic expansion within zones delineated by Wilmé *et al.* (2006) at each locus for both *O. cuvieri* and *O. cyclurus*. We used Arlequin 3.5 (Excoffier & Lischer 2010) to estimate Fu's F_S (Fu 1997) and Tajima's *D* (Tajima 1989a,b) and determine their significance through 1000 coalescent simulations under a null hypothesis of population stability and selective neutrality. We additionally examined extended Bayesian skyline plots (Heled & Drummond 2008) in BEAST across all three loci for evidence of population expansion within each COE and RD zone assuming a strict molecular clock. Runs were partitioned by ND1 flanking regions, ND1, PRLR, and UBN1 and were run for 10 million generations sampled every 1000 with the first 10% discarded as burnin.

Results

We recovered 1310 bp of mitochondrial DNA, including the entire ND1 gene (963 bp), tRNAs (Leu, Ile, Gln), and partial 16s and tRNA-Met. Across all samples, 892 sites were invariable, and 375 of the variable sites were

Table 2 Number of unique alleles and haplotype diversity (π) at each locus for *Oplurus cuvieri* and *Oplurus cyclurus*

Locus	<i>Oplurus cuvieri</i>		<i>Oplurus cyclurus</i>	
	# alleles	π	# alleles	π
mtDNA	24	18.433	19	29.329
PRLR	11	0.813	19	7.489
UBN	16	2.747	28	3.221

ND1, NADH dehydrogenase 1; PRLR, prolactin receptor; UBN1, ubinuclein 1.

parsimony informative. For PRLR, we recovered 541 bp, of which 469 sites were constant and 45 sites were parsimony informative. We collected 720 bp of UBN1, 639 sites were constant, and 59 were parsimony informative. Haplotype diversity was higher in *O. cyclurus* at all three loci, but this was most pronounced at ND1 and PRLR (Table 2).

The posterior distribution for the substitution rate of ND1 for oplurine lizards from the fossil-calibrated analysis had a mean of 0.013876 substitutions \times site⁻¹ \times myr⁻¹ (standard deviation = 0.00474) corresponding to sequence divergence rate of approximately 2.8% myr⁻¹. This is within the range of divergence rates reported for mitochondrial loci in other squamates (Brandley *et al.* 2011; Bryson *et al.* 2012).

Interspecific relationships and divergence times

Bayesian phylogenetic analysis of one mitochondrial and two nuclear loci in *BEAST resolved interspecific relationships within the oplurines with strong support (Fig. 2). The posterior mean substitution rate for ND1 was 0.0178 substitutions \times site⁻¹ \times myr⁻¹; the posterior mean substitution rates for ND1 flanking regions, PRLR and UBN1 were 4.92×10^{-3} , 5.45×10^{-4} and 5.03×10^{-4} substitutions \times site⁻¹ \times myr⁻¹, respectively (3.6%, 0.98%, 0.11% and 0.10% sequence divergence myr⁻¹). Posterior distributions for species divergence times for the two subsets of data are largely overlapping, but with broad 95% credible intervals. The most recent common ancestor between *Chalarodon* and these *Oplurus* occurred in the Eocene, Oligocene or perhaps the late Miocene. The remaining divergence times primarily fall within the Miocene with the exception of *O. fieriensis* and *O. grandidieri* that diverged more recently, between 1.0 and 5.6 Ma.

Phylogeography of *O. cuvieri* and *O. cyclurus*

Parsimony networks revealed strong geographic structuring of haplotypes at the mitochondrial locus, but substantial sharing of haplotypes among localities at the

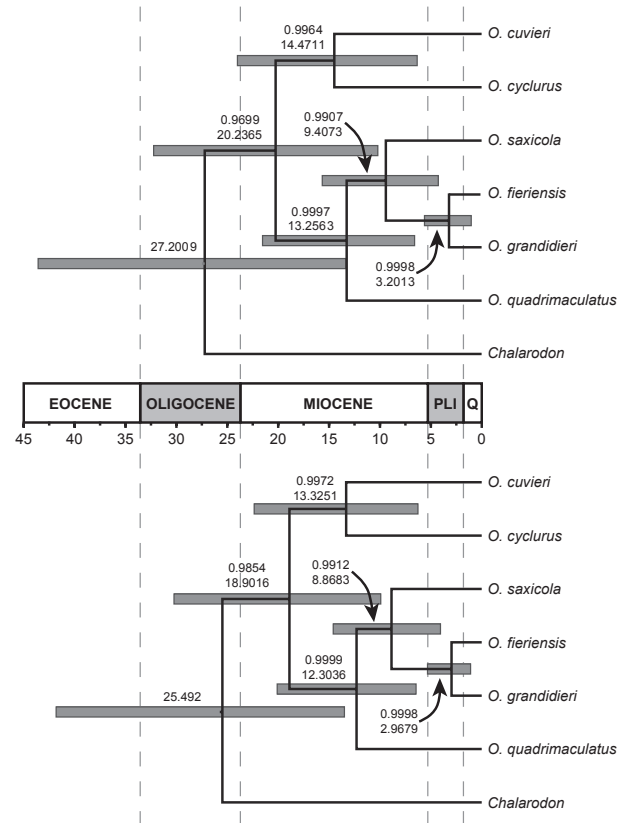


Fig. 2 Species tree and divergence times for oplurine lizards. The results from analysis of two subsets of taxa are above (Subset A) and below (Subset B) the timescale bar. Nodal support and mean divergence time are indicated above and below each node, respectively. Grey bars represent the 95% CI for divergence times.

nuclear loci, suggesting that the nuclear loci may reflect incomplete lineage sorting (Fig. 3). Mitochondrial haplotypes corresponded with geographic expectations for both species, and the average pairwise divergence within *O. cyclurus* (2.17%) was nearly twice that for *O. cuvieri* (1.27%). In *O. cuvieri*, we recovered several common and geographically widespread haplotypes at PRLR and UBN1 with multiple closely related haplotypes in much lower frequency. In contrast, we did not find any particularly common nuclear alleles among *O. cyclurus*.

Population divergence times within *O. cuvieri* and *O. cyclurus* were comparable under the constant population and exponential growth models (Fig. 4, Fig. S1), and Bayes factors did not support one model over another. For the sake of simplicity, we refer to divergence time estimates from the constant population model. For both species, intraspecific divergences occurred recently, within the last two million years. Within *O. cuvieri*, divergence between the three samples from the northern coast and all other populations occurred 0.88–2.85 Ma. Among these remaining

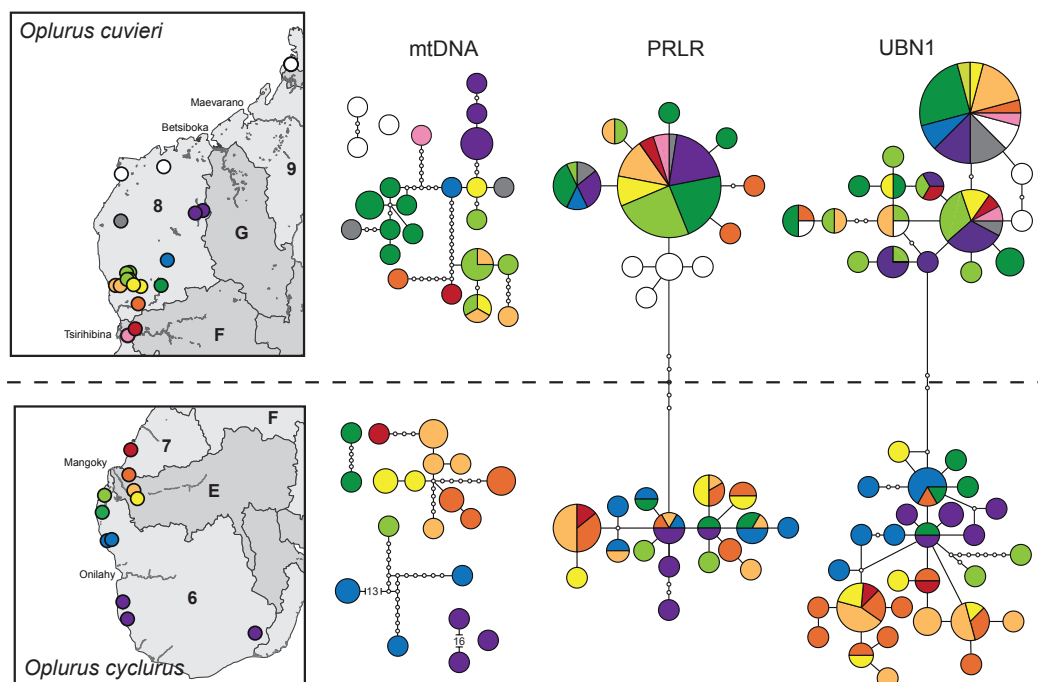


Fig. 3 Haplotype networks for mtDNA, prolactin receptor (PRLR) and ubinuclein 1 (UBN1). Map of localities and associated haplotype networks are indicated for *Oplurus cuvieri* in the top portion of the figure and for *Oplurus cyclurus* in the bottom portion of the figure. The size of each circle indicates the relative frequency of that haplotype. Colours within haplotype circles correspond to the sampling localities on the respective map for each species.

samples, there is support for two clades that last shared a common ancestor 0.27–0.98 Ma. Clades largely correspond to individuals west and east of the Tsingy de Bemaraha; though, they are not reciprocally monophyletic with respect to collection locality (Fig. 4a). Phylogenetic analysis of mtDNA in *O. cyclurus* reveals three clades with divergence among them estimated to have occurred 0.64–1.94 Ma. The mean node ages of the northern, central and southern clades are 0.21, 0.48 and 0.69 Ma, respectively (Fig. 4b).

Coalescent simulations to test alternative demographic hypotheses in *O. cyclurus* did not unequivocally support either the watershed or rivers-as-barriers hypothesis (Table 3). We were unable to reject either hypothesis at any divergence time for mtDNA simulations, while we could reject divergence at 0.5–3 Ma under both hypotheses at PRLR. UBN1 simulations rejected 1–3 Ma divergence models under the rivers-as-barriers hypothesis, but none of the watershed models. The only two models that were congruent across all loci were the rivers-as-barriers and watershed models with divergence at 100 Ka.

Tests of population expansion

Tajima's D and Fu's F_S were significantly negative at PRLR for the COE defined group for *O. cuvieri*

(Table 4). For Zone 6 individuals of *O. cyclurus*, Tajima's D at UBN1 and Fu's F_S at both nuclear loci were significant. Fu's F_S at UBN1 was also for Zone E. Extended Bayesian skyline plots show increasing population size since the past in all three populations (Fig. 5). However, the credible intervals also become increasingly broad, and although median population size increases, we cannot rule out population stability given the broadly overlapping CI through time.

Discussion

We recovered strong support for the interspecific relationships among these oplurine lizards (Fig. 2) using a multilocus species tree approach and found a topology consistent with previous studies based on concatenated 16s and *c-mos* sequences (Münchenberg *et al.* 2008). Within *Oplurus*, we recover a monophyletic arboreal, spiny-tailed and round-bodied group that includes *O. cuvieri* and *O. cyclurus*, and a monophyletic saxicolous group that is normal-tailed with a dorsal ventrally flattened body (*O. grandidieri*, *O. fierimensis*, *O. saxicola* and *O. quadrimaculatus*).

Mean node ages within the species tree are congruent with Miocene and Oligocene divergence events. However, our estimate for the *Oplurus/Chalarodon* split is slightly older than that estimated by the Townsend

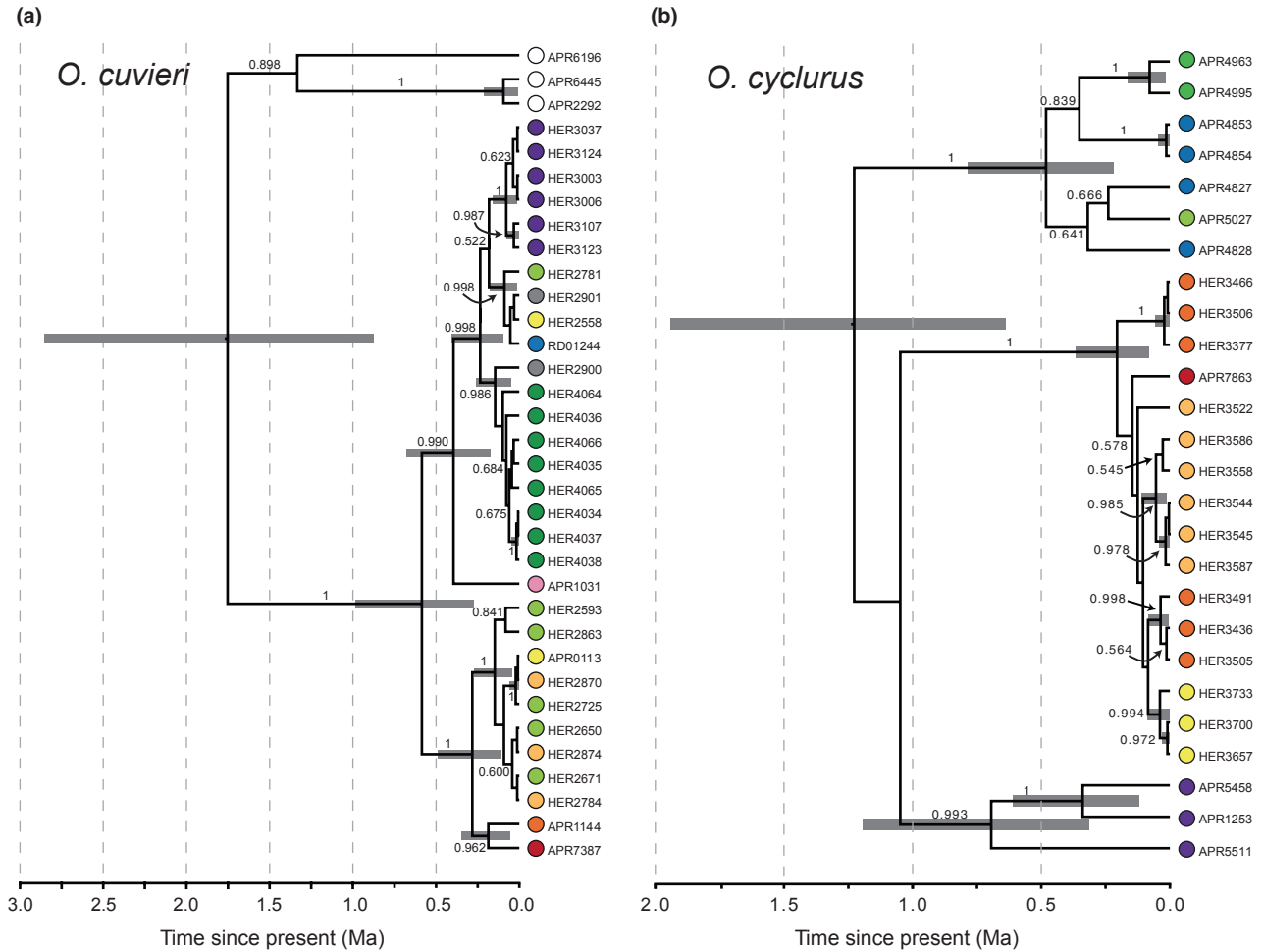


Fig. 4 Mitochondrial gene trees for (a) *Oplurus cuvieri* and (b) *Oplurus cyclurus* with divergence times estimated in BEAST under a coalescent model of constant population size. Colours at the tips correspond to colours on the maps of Fig. 3. Posterior probabilities of monophyly >0.50 are indicated at the nodes. For nodes with support >0.90, grey bars represent the 95% HPD for divergence time.

Table 3 Probability of Slatkin and Maddison’s *S* statistic greater than or equal to *S* for the empirical data under each population divergence model. Test significance at $\alpha = 0.05$ is indicated in bold

Model—Gene	100 Ka	500 Ka	1 Ma	2 Ma	3 Ma
Rivers-as-barriers					
mtDNA	0.999	0.922	0.849	0.772	0.821
PRLR	0.273	0	0	0	0
UBN1	0.973	0.107	0.032	0.023	0.020
Retreat dispersion					
mtDNA	1	1	1	1	1
PRLR	0.985	0	0	0	0
UBN1	1.0	0.934	0.887	0.852	0.875

PRLR, prolactin receptor; UBN1, ubinuclein 1.

et al. (2011b) analysis of 29 concatenated nuclear genes for Iguania. Fossil-calibrated divergence time estimations based on mtDNA alone can result in an underesti-

mation of the mean rate of evolution across the tree (Ho *et al.* 2005; Brandley *et al.* 2011; Lukoschek *et al.* 2012). Thus, it is possible that the substitution rate estimated from our analysis of iguania ND1 sequences is low, resulting in inflated estimates of divergence times in subsequent phylogenetic analyses both among oplurines and within species. Partitioned data analyses, such as those employed in our fossil-calibrated analysis, can help to reduce this error (Brandley *et al.* 2011). The ND1 substitution rate we estimate is within the range of general mitochondrial divergence rates assumed for recent intraspecific studies of other vertebrates (e.g. Leaché *et al.* 2007; Galbreath *et al.* 2009; Bryson *et al.* 2012) and similar or greater than estimates from fossil-calibrated relaxed-clock analyses for lizards (Brandley *et al.* 2011; Bryson *et al.* 2012; respectively). In addition, it is greater than mitochondrial substitution rates for sister taxon pairs of turtles and lizards assuming a strict molecular clock (Zamudio & Greene 1997), suggesting

Table 4 Tajima's D and Fu's F_S for populations delineated by the watershed model and corresponding P -value determined by coalescent simulations under the null hypothesis of population stability and selective neutrality. Significant results are indicated in bold

		Tajima's D	P -value	Fu's F_S	P -value
<i>Oplurus cuvieri</i>					
mtDNA	Zone 8	-0.98300	0.17500	-1.76831	0.26000
PRLR	Zone 8	-1.72534	0.02300	-6.28101	0.00100
UBN1	Zone 8	-0.32511	0.42700	-4.64664	0.04800
<i>Oplurus cyclurus</i>					
mtDNA	Zone E	-0.58163	0.30500	-0.28326	0.43800
PRLR	Zone E	0.04030	0.56200	1.74469	0.80800
UBN1	Zone E	-0.52616	0.34200	-7.37246	0.00100
mtDNA	Zone 6	-0.09809	0.50400	0.79504	0.57400
PRLR	Zone 6	-1.31930	0.09500	-5.20318	0.00300
UBN1	Zone 6	-1.67811	0.03100	-8.31482	0

PRLR, prolactin receptor; UBN1, ubinuclein 1.

that our analyses do not grossly underestimate the rate of mitochondrial evolution. To place the mean divergence time for other oplurine speciation events within the Pliocene–Pleistocene, substitution rates would need to be at least two to four times faster than what we estimated. While further study of rates of evolution given fossil-calibrated phylogenies is warranted, our results point to differentiation among oplurines occurring prior to the Pliocene–Pleistocene epoch.

Although climatic fluctuations associated with Pleistocene glaciation are consistent with diversification in some vertebrate groups (Wilmé *et al.* 2006; Pearson & Raxworthy 2009), our results from oplurine lizards add to growing empirical support for the observation that for many vertebrate groups, speciation events occurred long before Pleistocene. This trend compromises the general applicability of the watershed hypothesis for explaining patterns of vertebrate species diversity in Madagascar. The estimated Oligocene and Miocene divergence times we recover contradict the model of Wilmé *et al.* (2006); though, the divergence of *O. fierinensis* and *O. grandidieri* is a possible exception. Even so, this does not eliminate a role for past climatic changes in Madagascar as a driver of species diversification. Zachos *et al.* (1997) found signatures of climatic oscillations at the Oligocene–Miocene boundary. The Oligocene and Miocene divergence times estimated for oplurine lizards are similar to those reported for other Malagasy vertebrates (e.g. Horvath *et al.* 2008; Poux *et al.* 2008; Townsend *et al.* 2009, 2011a). For instance, divergence times for sister taxon pairs within the chameleon genus *Brookesia* (Townsend *et al.* 2009) and node ages for Malagasy tenrecs (Poux *et al.* 2008) and within true lemur and mouse lemur clades fall within

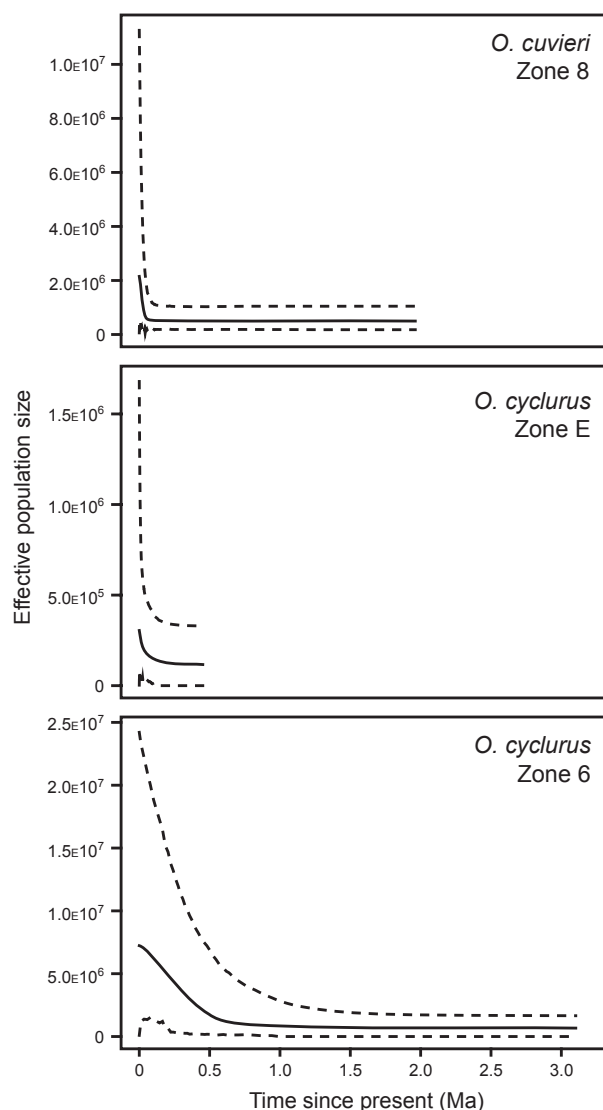


Fig. 5 Extended Bayesian skyline plots of effective population size through time in groups delineated by centres of endemism and retreat-dispersion zones of Wilmé *et al.* (2006). Mean N_e are indicated with solid lines, and the lower and upper bounds of the 95% HPDs are indicated with dashed lines. Plots have been adjusted, so that they each share the same x-axis.

the early- to mid-Miocene (Yoder & Yang 2004; Horvath *et al.* 2008). The climate fluctuations of the more distant past may have contributed to diversification in some groups; though, this hypothesis is difficult to test given the long period of time during which other uncorrelated processes could have shaped species boundaries and genetic diversity.

The two species for which we have population sampling, *O. cuvieri* and *O. cyclurus* are sister to one another and primarily occur north and south of the Tsiribihina River, respectively. This river could have played a role in the initial divergence of these species

or in the maintenance of species boundaries. However, both species are currently found in some areas beyond those sampled in this study (Münchenberg *et al.* 2008), indicating that the Tsiribihina River has not been an absolute or persistent barrier to movement in either species.

Intraspecific differentiation

Population genetic divergence within each species occurred much more recently, within the last 2 million years (Fig. 4). Although these are general estimates based on mitochondrial sequence data, they are broadly consistent with the temporal expectations for climate-based mechanisms of differentiation. We found little support for the watershed hypothesis in intraspecific divergences within either *O. cuvieri* or *O. cyclurus*. While we were unable to test geospatial patterns of genetic divergence explicitly with coalescent simulations in *O. cuvieri* given that we had so few samples from outside of Zone 8 (Fig. 1), COE and RD zones did not constitute well-supported clades in phylogenetic analysis of mitochondrial sequence data. Finally, we lack strong and consistent support for demographic expansion within the species across multiple tests. While extended Bayesian skyline plots suggest recent population expansion among Zone 8 individuals, estimates of population size through time have broad credible intervals such that we cannot exclude the possibility of population stability through time. In coalescent simulations of *O. cyclurus*, we were unable to reject the watershed model with population divergence at 100 Ka. However, similar to *O. cuvieri*, we fail to find unequivocal support for population expansion in both single locus (Tajima's D and Fu's F_S) and multilocus tests (extended Bayesian skyline plots). Despite the temporal congruence of intraspecific divergences with the watershed hypotheses, demographic expectations underlying this hypothesis are not met.

In *O. cuvieri*, some haplotype relationships are congruent with expectations under the rivers-as-barriers hypothesis. For instance, two individuals 22 km apart on either side of the Tsiribihina River are distantly related, suggesting that this river may serve as a biogeographic barrier. Patterns of divergence among the three northern coast samples are also congruent with the Betsiboka and Antsohihy Rivers serving as barriers as proposed by Martin (1972). Based on the heterogeneity among habitat types in these regions, these populations may represent distinct ecotypes. However, as with any simple correspondence between genetic divergence and putative barriers, this apparent pattern may be the result of limited sampling and underlying isolation by distance (Irwin 2002) and not necessarily

vicariance. Further sampling and study would clarify the mechanisms underlying this observed pattern of divergence.

We also found some evidence that rivers may act as barriers to dispersal in *O. cyclurus* with all loci supporting a divergence at 100 Ka, and with mitochondrial and UBN1 data also supporting greater divergence times (Table 3). Coalescent simulations were not able to discern between the watershed and rivers-as-barriers models; however, we find support for recent divergence at nuclear loci, perhaps occurring more recently than suggested by phylogenetic analysis of mtDNA alone (Fig. 4). Ho *et al.* (2005) and others have found that mitochondrial substitution rates may be particularly high for recent divergences (<1–2 mya). If the substitution rate we applied is low (as discussed above), particularly for distal nodes, the nuclear loci may more accurately reflect the true intraspecific divergence times. While it is beyond the scope of this study, exploration of the discrepancies between nuclear coalescent analyses and mtDNA-only phylogenetic analyses and an assessment of which more accurately reflects reality is needed.

There is some support for the rivers-as-barriers and watershed hypotheses for intraspecific divergences, but it is clear that other biogeographic factors have also shaped the patterns of differentiation within each species. Within *O. cuvieri* we found strong support for a north-western clade and a primarily western clade consistent with an earlier study (Münchenberg *et al.* 2008). We also found evidence for a third mitochondrial clade consisting primarily of individuals east of the Tsingy de Bemaraha, a karstic plateau running north to south. Phylogenetic analyses and parsimony networks suggest that differentiation occurred on either side of this physical barrier, possibly with some migration between populations (see Chan *et al.* 2011). Incidentally, the third clade corresponds approximately with the Bongolava plateau, a long mountain chain running parallel to the Bemaraha plateau but composed of a different substratum.

In contrast to previous phylogenetic analyses of sequence data that did not detect any phylogeographic structure (Münchenberg *et al.* 2008), our phylogenetic analysis of ND1 data from *O. cyclurus* recovered three well-supported and divergent clades (Fig. 4b). The three clades correspond largely with geographic structure; though, the relationship among the clades is unresolved. Within the northern clade, mitochondrial haplotypes correspond strongly with geography, with individuals from the same locality having identical or similar alleles. This is in contrast to the coastal clade, wherein individuals from the same locality have divergent mitochondrial alleles. This latter result suggests that current and historical processes influencing population connectivity and genetic diversity in the northern portion of the range,

north of the Mangoky River, are different from those in the coastal range. Such large divergence among individuals at the same locality in some *O. cyclurus* populations could, for example, reflect isolation in multiple refugia with subsequent connectivity, but greater geographic and population-level sampling is necessary for population genetic and coalescent-based analysis of historical dynamics. As a cautionary note to over interpretation of these results, we must acknowledge that population sampling is far from ideal, given the remote areas and difficult terrain that characterize much of the geographic distribution of these lizard species.

Conclusions

Interspecific and population genetic differentiation lie along a continuum, and the mechanisms underlying divergence may be important at multiple spatial and temporal scales. Rivers have been found to define species boundaries in some groups (e.g. Pastorini *et al.* 2003; Craul *et al.* 2007; Olivieri *et al.* 2007), and we find some, albeit weak, evidence here that they may additionally influence connectivity at the population level. Riverine refugia during dry periods of the Pliocene–Pleistocene did not influence speciation and were not the sole mechanisms underlying intraspecific differentiation in the taxa studied here. However, we cannot exclude the possibility that climatic fluctuations were important to both inter- and intraspecific processes. Glacial cycles of the Miocene and Oligocene, for example, may have influenced speciation among oplurines. Likewise, the distribution and abundance of particular microhabitats important to local population dynamics and persistence in both species may have fluctuated throughout the Quaternary, thereby influencing isolation and connectivity in oplurines and, hence, population genetic diversity and differentiation.

Diversification hypotheses that apply to multiple taxa are attractive because they present potentially widespread and pervasive mechanisms in biogeography and evolution. However, in this study, we did not find clear support for the rivers-as-barriers or watershed hypotheses. Rather, the data suggest that multiple and alternative processes have shaped species and population genetic diversity among these Malagasy lizards. Continued efforts to reveal ancient climatic conditions will enhance our ability to postulate cause and affect relationships between species diversification and geoclimatological events.

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Data accessibility

DNA sequences: GenBank accessions JQ941139–JQ941391.

Phylogenetic data for species tree and intraspecific gene tree analyses: TreeBASE accession no. 12644.

Full nucleotide alignments for oplurine mtDNA, PRLR, and UBN1 sequences and XML input files for analyses conducted in BEAST and *BEAST: DRYAD entry doi:10.5061/dryad.27q72.

Supporting information

Additional supporting information may be found in the online version of this article.

Fig. S1 Mitochondrial gene trees for (a) *Oplurus cuvieri* and (b) *Oplurus cyclurus* with divergence times estimated in BEAST under a coalescent model of exponential population growth. Colours at the tips correspond to colours on the maps of Fig. 3. Posterior probabilities of monophyly > 0.50 are indicated at the nodes. For nodes with support > 0.90, grey bars represent the 95% HPD for divergence time.

Table S1 GenBank samples included in fossil-calibrated estimate of ND1 substitution rate.

Table S2 Subsets of samples used for intraspecific phylogeny and divergence time estimates.

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