

The discordance of diversification: evolution in the tropical-montane frogs of the Eastern Arc Mountains of Tanzania

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Abstract

Species with similar geographical distribution patterns are often assumed to have a shared biogeographical history, an assumption that can be tested with a combination of molecular, spatial, and environmental data. This study investigates three lineages of *Hyperolius* frogs with concordant ranges within the Eastern Afrotropical Biodiversity Hotspot to determine whether allopatric populations of co-distributed lineages shared a parallel biogeographical response to their shared paleoclimatic histories. The roles of refugial distributions, isolation, and climate cycles in shaping their histories are examined through Hierarchical Approximate Bayesian Computation, comparative phylogeography, and comparisons of current and past geographical distributions using ecological niche models. Results from these analyses show these three lineages to have independent evolutionary histories, which current spatial configurations of sparsely available habitat (montane wetlands) have moulded into convergent geographical ranges. In spite of independent phylogeographical histories, diversification events are temporally concentrated, implying that past vicariant events were significant at the generic level. This mixture of apparently disparate histories is likely due to quantifiably different patterns of expansion and retreat among species in response to past climate cycles. Combining climate modelling and phylogeographical data can reveal unrecognized complexities in the evolution of co-distributed taxa.

Keywords: Approximate Bayesian computation, comparative phylogeography, Eastern Arc Mountains, Ecological Niche Modelling, *Hyperolius*

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Introduction

While island chains or continental mountain ranges often experience similar geologic and climatic regimes, their biological inhabitants do not necessarily share the same evolutionary histories. These naturally fragmented and spatially structured systems provide an opportunity to investigate the patterns and processes of diversification by comparing evolutionary histories across multiple lineages. If phylogeographical structure is congruent across reciprocally monophyletic groups, the most parsimonious explanation is that faunas have

responded in concert to the same vicariant events or share dispersal paths (Zink 2002; Zink *et al.* 2002). Alternatively, co-distributed lineages may contain a variety of phylogeographical patterns implying that current communities are composed of lineages that have suffered past extinctions and/or have significant differences in dispersal ability (Zink 2002; Zink *et al.* 2002). Through the use of molecular and spatial analyses, comparative phylogeography can test for shared vicariance and dispersal histories among species and can interpret the influences of geologic, climatic, and spatial variables on emerging species (Bermingham & Moritz 1998; Arbogast & Kenagy 2001; Zink *et al.* 2002; Hickerson *et al.* 2006).

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Vicariant fragmentation into allopatric daughter lineages may result from a variety of geographical events including mountain-building (Ribas *et al.* 2007), changes in river courses (Pereira & Baker 2004), continental breakup (Gamble *et al.* 2007), sea-level changes (Vandergast *et al.* 2007), or shifts in the distributions of suitable habitat owing to climate change (Knowles 2000, 2001). Of these geographical events, climatically induced fragmentation of once continuous suitable habitat is believed to be a major driver of diversification in topographically rich landscapes (DeChaine & Martin 2005; Epps *et al.* 2006; Shepard & Burbrink 2009). During climate fluctuations, populations of species that closely track environmental conditions may experience alternating periods of isolation and connectivity as habitats scale and descend along elevation gradients in response to warming and cooling (Hamilton 1976; Hewitt 1996, 2004; Wiens & Graham 2005; Kozak & Wiens 2006). During climate cycles, the amount of reciprocal gene flow between discrete mountain blocks may be heavily influenced on the degree of isolation or connectivity associated with each climatic condition (Hewitt 1996, 2004; Wiens 2004). Examining the coincidence of major climate fluctuations to the spatial divisions and lineage splitting events among distinct lineages provides insight into how climate cycles shape the phylogeography of species. To investigate the impact of climate cycles on the phylogeographical patterns of current species found in restricted habitats, I compare the phylogeographical patterns of three lineages of montane reed frogs (*Hyperolius*) throughout the Eastern Arc Mountains (EAM) of Tanzania (Fig. 1) and use Ecological Niche Modelling (ENM—also called Species Distribution

Modelling or Environmental Envelope Modelling) to determine community-wide and lineage-specific responses to dry and wet climate conditions (Martinez-Meyer *et al.* 2004; Elith *et al.* 2006; Phillips *et al.* 2006; Elith & Graham 2009).

Comparing scenarios of concordance/discordance between lineages

Shared evolutionary history leaves a mark on co-distributed taxa either in mutual phylogeographical relationships, concordance of lineage-splitting events, or a combination of the two. If the spatial relationships of phylogeographical structure and the timing of divergence events are concordant between species, then they are assumed to share an evolutionary history and respond in concert to vicariant events (Fig. 2a—the ‘Shared History Scenario’). If, however, phylogeographical relationships are concordant but differ in the timing of lineage-splitting events, spatial relationships of occupied areas are probably the critical factor influencing diversification (Fig. 2b—the ‘Geographic Determinism Scenario’). Such asynchrony could arise from differential dispersal abilities across forming barriers, responses to different temporal events, or pseudocongruence (Cunningham & Collins 1994; Hunn & Upchurch 2001; Upchurch & Hunn 2002). A third possibility is that phylogeographical topologies differ, yet timing of divergence events is simultaneous (Fig. 2c—the ‘Time-Constrained Scenario’). This scenario suggests that while the route of colonization may be variable, the expansion and cessation of gene flow are temporally constrained (e.g., glacial cycles or global climate shifts). Finally, a

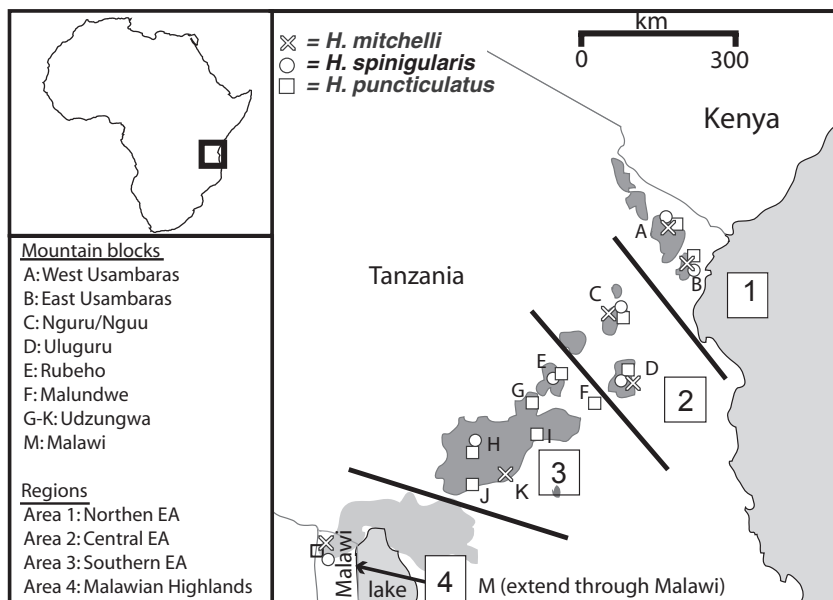


Fig. 1 Distribution of sampled localities of three frog species on a map of Eastern Arc Mountains in Tanzania and Highlands of Malawi. Mountain blocks containing populations of these lineages are marked with letters and major areas within this semi-linear system are denoted by numbers.

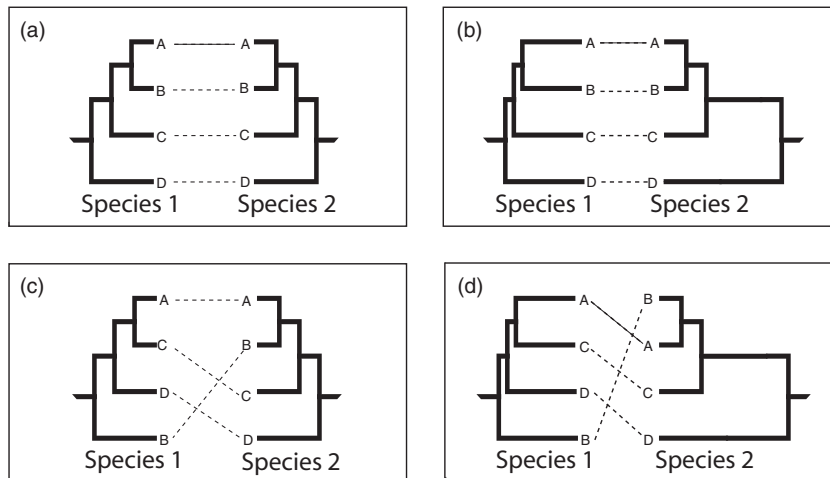


Fig. 2 Comparative phylogeography scenarios. a: Shared History, b: Geographic Determinism, c: Time Constrained, d: Random. Branch lengths proportional to time.

lack of correspondence in either spatial or temporal patterns would result if each lineage acts independently of others or if extinction has been frequent and random (Fig. 2d is one possible example—the ‘Random Scenario’). Congruence and discordance between different portions of phylogeographical trees may also exist resulting from a mixture of these four patterns. This study investigates the roles of vicariance, range expansions, climate cycles, and migration at the lineage and community levels that explain the diversity observed today.

Study species and area

The three lineages examined here are the sympatric African montane reed frogs (*Hyperolius*) from the EAM of Tanzania and the Malawi highlands (Channing 2001; Channing & Howell 2006) personal observations; amphibianweb.org; Fig. 1). Each lineage is comprised of multiple isolated populations throughout this fragmented montane system and the lineages appear to be of similar evolutionary age and regional geographical extent. Two of these lineages, *Hyperolius puncticulatus* also known as *Hyperolius substriatus*: (Pickersgill 2007); and *Hyperolius mitchelli* are recognized as single species, while *Hyperolius spinigularis* is a superspecies complex represented by a clade of three currently recognized species [*H. spinigularis*, *Hyperolius tanneri* (or *tannerorum*), and *Hyperolius minutissimus*]. Members of the *H. spinigularis* group show similar levels of interspecific morphological diversification to the other two lineages and clear species lines within the complex are hazy (Lawson 2010).

The EAM are fragmented mountaintops, each containing islands of forested habitats with highly endemic floral and faunal communities. The intervening lowlands are hot and dry with an entirely different

amphibian community (Poynton 2003). Floral and faunal diversification within the EAM is probably directly related to changes in environmental conditions through time that repeatedly fragment continuous forests and produce complex landscapes of heterogeneous habitats similar to those observed today. Paleoclimatic evidence from highland and lowland lake sediment and glacial cores in the East African region show that while temperature and moisture levels on mountaintops remained relatively stable through major climate cycles (Marchant *et al.* 2007; Mumbi *et al.* 2008), intervening lowlands experienced dramatic oscillations between wet forests and dry grasslands (Ashley 2001; Vincens *et al.* 2003; Osmaston & Harrison 2005; Trauth *et al.* 2005; Kiage & Liu 2006). These elevation-dependent climatic histories resulted in repeated shifts in forest connectivity (multiple vicariant events) that may have reduced the geographical range of once widespread taxa to isolated montane refugia before re-expansion during wetter climate periods (Iversen 1991). A number of recent studies investigating the distribution patterns of montane vertebrates in the Eastern African region have demonstrated the importance of fragmented landscapes in promoting speciation and phylogeographical structuring (Fahr *et al.* 2002; Bowie *et al.* 2004a; Carleton & Stanley 2005; Stanley *et al.* 2005; Huhndorf *et al.* 2007; Fjelds  & Bowie 2008; Taylor *et al.* 2009). Though all of these studies showed species-specific responses to climate cycles, a comparative study of co-distributed taxa can test for community-wide responses to climatic shifts.

Climate cycles in Eastern Africa and estimating lineage specific responses to alternate climate scenarios

Over the last 12 million years, climate cycles have significantly and repeatedly changed rainfall and temperature

patterns over the East African landscape. The final uplift of the Eastern Rift System ~9–12 Ma (Cohen *et al.* 1993; Lezzar *et al.* 1996) significantly decreased precipitation in the region causing aridification and fragmentation of once continuous forest (Sepulchre *et al.* 2006). This dry period lasted from 6 to 8 Ma (Leakey *et al.* 1996; Cerling *et al.* 1997; Pagani *et al.* 1999; Hay *et al.* 2002). The following period (1.5–5 Ma) was characterized by climate oscillations between wet and dry conditions, with a general trend towards aridification after 1.86 ± 0.44 Ma (Trauth *et al.* 2009). Between 1.8 and 4 Ma was a predominantly wet period (Cerling *et al.* 1988) punctuated by periodic dry cycles at 3.7–4, 2.95–3.2, and 0.9–1.1 Ma (Trauth *et al.* 2009). From 1.5 Ma to the present, climate cycle severity increased, resulting in extreme wet–dry cycles (pluvial–interpluvial) (DeMenocal 1995).

While estimates for the timing and severity of climate fluctuations are available, they are not currently sufficient to provide spatially explicit climatic conditions across local landscapes or to help predict species habitat distributions through time. However, one proxy for modelling the shift in available habitat during dry cycles are paleoclimate reconstructions from the Last Glacial Maximum (LGM—21 000 years ago). Though the LGM is only the most recent extreme dry period in a long series of global climate cycles, by projecting current species habitat needs into this paleoclimatic setting, we can estimate the distribution of available habitat during dry climate cycles (or at least the relative proportions of the landscape that may have been habitable by these *Hyperolius* species). This provides a means to determine if distributions of suitable habitat shift in similar ways across lineages. Specific predictions about absolute connectivity are beyond the scope of such an approximation, but the LGM predictions can be used to identify lineage-specific responses to dry periods. If phylogeographical patterns are divergent, mismatched responses to alternate climate regimes may be a likely cause.

Materials and methods

Field collection

To collect locality data and obtain genetic samples, I sampled highland locations across the EAM on seven discrete mountain blocks (East Usambaras, West Usambaras, Nguru, Uluguru, Malundwe, Rubeho, Udzungwa) and the highlands of Malawi. The majority of *Hyperolius* individuals were collected during the Autumn rainy seasons of 2006, 2008, and 2009, when individuals aggregate at breeding pools (ranging from March–April in northern Tanzania to January–March in southern Malawi), with supplemental collections from

the Natural History Museum (NHM, London, UK) and the Museo Tridentino di Scienze Naturali, (MTSN, Trento, IT). *Hyperolius puncticulatus* was found on each of these mountain blocks, while members of the *Hyperolius spinigularis* species complex and *Hyperolius mitchelli* were found on a subset (Fig. 1). Voucher specimens are housed at the Field Museum (Chicago, USA), NHM, and MTSN.

Extraction and sequencing of DNA

A total sample size of 58 *H. mitchelli*, 111 *H. puncticulatus*, and 38 *H. spinigularis* are included in this study. Total DNA from liver and leg muscle tissue was extracted from freshly collected specimens preserved in 95% ethanol using the PUREGENE DNA Purification kit protocol (Qiagen, Valencia, CA, USA). The loci used in this analysis are the entire mitochondrial ND2 gene (NADH dehydrogenase subunit 2) and flanking tRNAs (~1500 bp), and the nuclear genes POMC (Pro-opiomelanocortin- ~590 bp coding) and C-myc (cellular myelocytomatosis proto-oncogene- 540 bp of exon 2, 500 bp of intron 2, and 300 bp of exon 3). Primers are listed in Appendix S1 (Supporting Information). POMC and C-myc are not known to be linked and are considered neutral in other studies of amphibians (Smith *et al.* 2005; Wiens *et al.* 2005).

PCR amplification and cycle sequencing were completed under standard conditions, and specifics are listed in the Appendix S1 (Supporting Information). Edited sequences of coding regions were translated into amino acids to verify the absence of frame shift indels and compared to published sequences using BLAST in Genbank to confirm identity. Though alignment was unambiguous in ND2, POMC, and coding regions of C-myc, MUSCLE (Edgar 2004) was used for both nuclear loci to determine the optimal alignment. The intron regions of C-myc were confidently aligned, but a short, 30-bp microsatellite region in the intron was removed from the analysis because of lack of confidence in the homology of base pairs. Gaps were coded as missing information. A single character was added to the end of the sequence indicating presence or absence of gaps using the program gapcode.py (Ree 2007).

A number of individuals were heterozygous for nuclear loci. POMC had in-frame tri-nucleotide indels, while C-myc contained indels in a number of locations in the intron. Length heterozygous individuals and individuals with very divergent alleles were cloned using the TOPO TA Cloning kit protocol (Invitrogen Corporation, Carlsbad, CA, USA) following standard procedures from (Carnaval & Bates 2007) to identify alleles. Colonies were sequenced from both directions with multiple internal primers. In addition, cloned

sequences were compared to the original sequences to eliminate clonally induced error. A number of sequences with particularly high heterozygosity were also cloned to help in phasing of the remaining, uncloned heterozygotes.

After cloning a number of individuals, we used the program PHASE ver 2.0.2 (Stephens *et al.* 2001; Stephens & Donnelly 2003) under default conditions [200 iterations (100 burn-in) with a thinning interval of '1'] to determine nuclear haplotypes of individuals that were heterozygous for more than one segregating site. Known haplotypes (from cloned and homozygous individuals) were used to train the PHASE algorithm to determine haplotypes of unknown phase. Loci with posterior probability >0.95 were accepted, and those of lower probability were evaluated multiple times with different seeds to identify the most stable reconstructions.

Models of sequence evolution and mutation rates

The models of nucleotide evolution were selected using the program MrModelTest 2 (Nylander 2004) with Akaike information criterion (AIC) scores (shown in Appendix S1, Supporting Information). Based on past analyses of mutational rates in amphibian ND2 sequences (Crawford 2003a), a substitution rate of 0.957%/lineage/my was used. Though C-myc has a published rate from studies by Crawford (2003a) and Crawford (2003b), this rate is largely untested in the amphibian literature. To re-estimate rate, I used the phylogeny of a Caribbean anuran radiation (Moen & Wiens 2008) as an independent estimate of C-myc substitution rate. This data set was also used to determine an estimation of POMC mutation rate. For this exercise, aligned sequences were obtained from the authors for their *Lophiohylini* (without *Osteopilus*) tree, and gene trees for each locus were estimated using BEAST 1.5.2 and constrained by their species-tree topology (constant mutation rate of 1). Each ultrametric tree from BEAST was then analysed in r8s (Sanderson 2003) with the DIVTIME method = lf [Langley–Fitch method (Langley & Fitch 1973, 1974)], algorithm = tn (truncated Newton method with bound constraints) with two different calibrated roots for the tree based on their two reported possibilities: 35.9 and 48.59 Ma. The mutation rate for C-myc for the exon region was calculated at 0.0724–0.0543%/lineage/my with an average of 0.0633%/lineage/my, and for POMC, 0.083–0.061%/lineage/my with an average of 0.072%/lineage/my. The ratio of substitution rates for ND2 (mitochondrial) to C-myc (nuclear) is ~15, close to the 16-fold increase found by Crawford (2003b) for these loci.

Sequences have been deposited in Genbank (Accession numbers for *H. spinigularis* super-species complex:

POMC = HM772141–HM772208, ND2 = HM772209–HM772247, C-MYC = HM772248–HM772301. Accession numbers for *H. mitchelli*: POMC = HM772302–HM772379, ND2 = HM772448–HM772496, C-MYC = HM772380–HM772447. Accession numbers for *H. puncticulatus*: POMC = HM772771–HM772968, ND2 = HM772677–HM772770, C-MYC = HM772497–HM772676. All Genbank submissions have associated voucher numbers and population identifiers. Voucher specimens are reported in Appendix S1 (Supporting Information).

Testing the different phylogeographical/evolutionary scenarios

Specific methods of comparative phylogeography are described later, but to outline the approach taken in this study I used a tree-based (chronogram) method to assess similarity across lineages and a population genetics method to assess synchronous splitting events between geographical regions. For the tree-based method, a multi-locus (mtDNA + two nuclear loci) chronogram from BEAST (described later) was used to test the three scenarios of concordance, (Fig. 3a–c). Using this tree, it is possible to determine whether population relationships within each lineage have the same topology (Geographic Determinism). It also provides estimates of the concentration of divergence events through time by estimating the timing of each lineage-splitting event (node) to evaluate the 'Time-Constrained' scenario. If the three lineages are identical in their phylogeographical relationships and the timing of phylogeographical splits are also synchronous, the lineages display a 'Shared History'.

If the results from the strict comparative phylogeography tests show asynchronous lineage splitting and/or discordant patterns, an analysis of diversification between representative mountain blocks along this linear geographical system may reveal concordance unrecoverable in the most complex system. For this analysis, I used the population genetics program msBAYES (Hickerson *et al.* 2007) on a multi-locus data set for a reduced number of mountain peaks (one mountain in each region of the system, only mountains with all three lineages; areas in Fig. 1). This approach incorporates all the variation possible in a multi-gene, multi-individual, multi-species, multi-area system, and allows comparison even though the distributions of the three lineages are not entirely congruent.

Genetic analyses—trees

Bayesian (MrBayes v. 3.1.2: Huelsenbeck & Ronquist 2001; Ronquist & Huelsenbeck 2003), Maximum Likelihood (Garli v.0.951: Zwickl 2006), and Parsimony

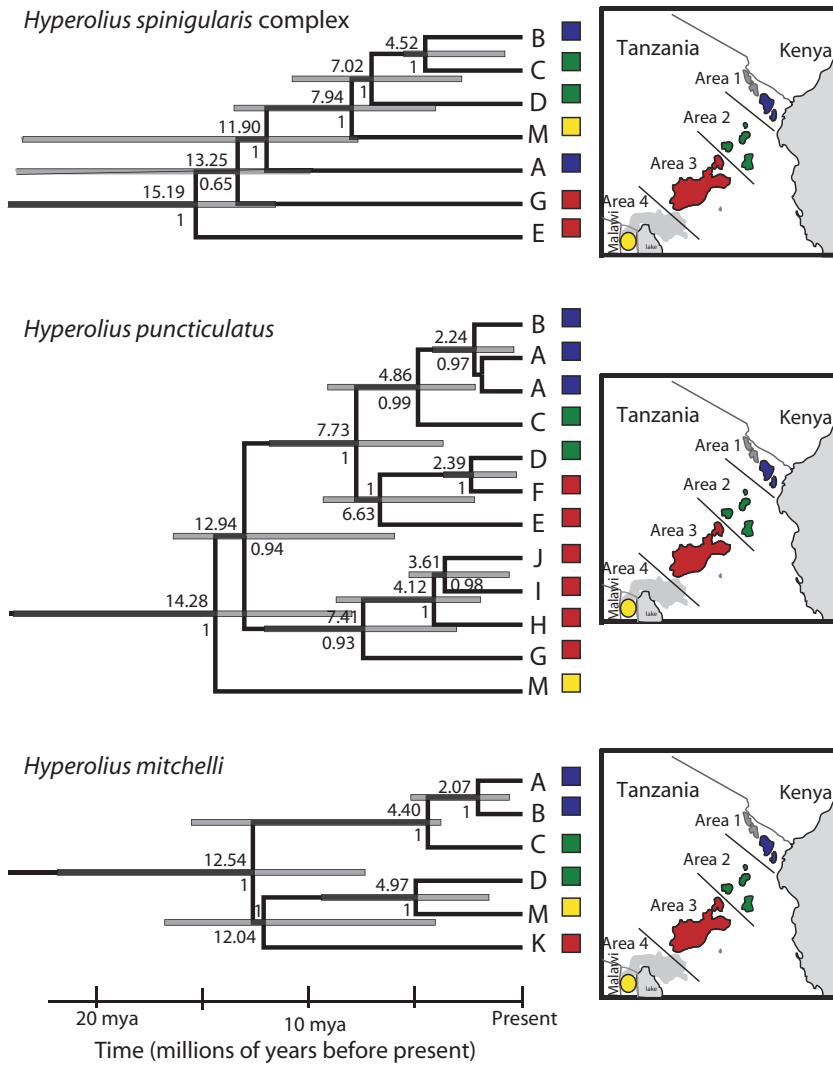


Fig. 3 BEAST birth–death multi-locus trees of each lineage including 95% posterior confidence intervals on the age of nodes. Branch lengths proportional to the age of time. Age (in millions of years) above nodes, posterior probability below nodes. Populations (tips of tree) correspond to mountain blocks in Fig. 1: A: West Usambaras, B: East Usambaras, C: Nguru/Nguu, D: Uluguru, E: Rubeho, F: Malundwe, G–K: Udzungwa, M: Malawi). Non-monophyly of populations within each area is shown through identifying general North/South placement along the Eastern Arc Mountains by coloured regions. Blue = Area 1 (Northern Eastern Arc), Green = Area 2 (Central Eastern Arc), Red = Area 3 (Southern Eastern Arc), Yellow = Area 4 (Malawian Highlands).

(PAUP*4.0b10: Swofford 2003) analyses were performed on individual genes in this data set to infer single-locus phylogenies.

To construct a chronogram using the multi-locus data set (mtDNA and one allele for each individual for each nuclear locus), I created a partitioned concatenated Bayesian chronogram with locus-specific mutation rates in BEAST v1.5.2: (Drummond & Rambaut 2007). One individual per population was used in the BEAST analysis with a Speciation: birth–death tree prior to avoid inflating branch lengths as a result of dense tips. I assigned each locus to the SRD06 model of molecular evolution and its pre-determined mutation rate with uncorrelated lognormal relaxed clocks (e.g. the ND2 region was assigned a mutation rate of 0.00957 mutations/million years, the C-myc region was assigned a rate of 0.0006334 mutations/million years, and so on). The analysis was run for 10 million generations. Of the

10 000 trees saved, 1000 were discarded as burn-in when computing the agreement subtree. The performance of each Bayesian run was assessed using Tracer (Rambaut & Drummond 2007) to insure proper mixing (ESS scores above 200). The chronogram from this analysis was used for all topological geographical comparisons and in investigating the concordance of divergence events.

Analyses were first rooted with *Kassina senegalensis* (member of Hyperolidae), *Hyperolius argus*, and *Hyperolius pictus*. In this larger topology, *H. argus* was external to the three focal lineages and was then used as an outgroup to a reduced data set of the three focal lineages. In this smaller data set, and in an ‘outgroup-free’ data set with midbranch rooting, the topologies of the within-lineage populations and the relationships between the three lineages did not change. The BEAST multi-locus chronogram was run without an outgroup

using mid-branch rooting, as this did not affect topology but improves branch length estimations.

Geographical determinism—concordance of species-tree topology

The similarity of phylogeographical relationships between the three lineages was assessed using the program TOPD-FMITS version 3.3 (Puigbo *et al.* 2007). Unrooted Bayesian maximum clade credibility trees (without branch lengths) of each lineage were used for this analysis. The TOPD-FMITS analysis was performed under default conditions, with 100 simulated trees as a null model. Populations not represented by all three lineages were pruned. To assess congruence, I calculated the nodal distance score between trees using the root-mean squared distance (RMSD) of the distance matrices. The RMSD is 0 for identical trees and increases as the trees become more dissimilar.

Time-constrained—concentration of lineage-splitting events through time

To address this hypothesis, I graphed the branching times of all within-lineage splits from the multi-locus BEAST tree to determine if any time points in history had greater than expected numbers of lineage-splitting events. All analyses were conducted in the statistical program R v 2.8.1 (packages ape, geiger, apTreeshape, and laser; R Development Core Team). Because these three lineages are not a monophyletic group, nodes prior to within-lineage diversification were removed. The observed diversification was compared to both a pure birth (yule) scenario and a birth–death scenario using AIC scores. In addition, I tallied lineage-splitting events in 500 000 years bins to qualitatively address concentration of splitting events. Time periods when lineage accumulation was outside the 95% confidence interval were compared to the paleoclimate record of climate fluctuations.

Simultaneous division between major areas

Genetic analyses—msBayes. The multi-locus version of the program msBayes (Hickerson *et al.* 2007) was used to test for simultaneous divergences between spatially neighbouring mountain ranges separated by lowland gaps. The Hierarchical Approximate Bayesian Computation (HABC) approach simultaneously analyses phylogeographical data sets in order to make cross-taxon inferences about biogeographical processes while allowing for uncertainty in the demographic differences within each taxon-pair (Hickerson *et al.* 2007; Hickerson & Meyer 2008). To structure the analysis, I divided the

Eastern Afromontane system (including the EAM in Tanzania and the Malawian highlands) into four areas that are roughly linear from the northern-to-southern extent of the EAM (area 1: East Usambaras, area 2: Uluguru, area 3: Udzungwa, and area 4: Malawian highlands; Fig. 1) and excluded all other populations from this analysis. The analysis was constructed to compare divergence times between geographically neighbouring communities across major habitat gaps (gap between Areas 1 and 2, Areas 2 and 3, and Areas 3 and 4 shown in Fig. 1) to determine if it was possible to rule out simultaneous divergences of all three lineages across each gap. The priorfile for this analysis relies on summary statistics from the data set and user-defined limits. The primary priors are the upper value of Theta [two times the largest value of Pi within the populations (scaled for mtDNA/nDNA rate differences)], Tau (upper limit is estimated through trial and error of data fit to the model), current migration (which was set to zero for these distant, non-molecularly overlapping localities), and an ancestral population size scaler (also set to zero to assume no significant change has taken place in overall population size through time). Each analysis was run for one million simulations with the 0.1% of simulations closest to describing the actual data saved as the model for these populations (accept/reject value = 0.001) using two parameters to assess the likelihood of simultaneous divergences in msBayes: Ψ (Psi; the number of tau classes: a parameter indicating the number of different divergence times. $\Psi = 1$ implies simultaneous divergence) and Ω (Omega; a dispersion index: the ratio of variance of divergence times to the mean). Bayes factors were used to determine the strength for rejecting a simultaneous divergence outcome at the cut-offs of $\Omega = 0.05$ and $\Psi = 1$. If the population analysis had $\Omega > 0.05$, or $\Psi > 1.499$, the analysis was repeated with subsets of the lineages to determine if any pairs were supported as simultaneous (following Hickerson & Meyer 2008).

Lineage specific reactions to dry climate cycles. ENMs (Elith *et al.* 2006) were created with Maxent version 3.3.0 (Phillips *et al.* 2004) using point locality information from collection localities (see Appendix S1 Supporting Information). Geographic Information Systems (GIS) climatic layers (30 arc-second resolution) were compiled from the WorldClim database (Hijmans *et al.* 2005) of the present and LGM conditions (LGM—21 000 years ago), and were clipped to the extent of Kenya, Tanzania, and Malawi in ArcGIS (ESRI v.9.3.1). The WorldClim bioclimatic variables represent precipitation and temperature variables (e.g., temperature of coldest month, temperature of warmest quarter, rainfall of the driest quarter, etc.). All runs were trained on the cur-

rent climate conditions with a convergence threshold of 0.00001, 1000 iterations, logistic output, and the 10% training presence threshold for outputs to create binary models of suitable/unsuitable habitats. These outputs were then projected onto climate models of the LGM. The program ENMtools (Warren *et al.* 2008) was used to evaluate similarity of the environmental conditions occupied for these lineages in current and dry (LGM) climates to determine current similarity of climatic requirements and the similarity of responses to dry climate scenarios. ASCII files of range distributions for each lineage were compared at both time points to determine niche similarity using the 'Niche Overlap' function. Statistical analyses of these similarities were performed using the 'Niche Identity Test' function to determine if lineages demonstrate significantly different ENMs compared to 100 ENMs with randomly assigned locality identities.

Results

Descriptions of genetic diversity within and between populations for each gene and lineage are shown in Table 1. Detailed population information and AMOVAs are available in the Appendix S1 (Supporting Information).

Geographical determinism scenario

The three *Hyperolius* lineages show widely divergent phylogeographical patterns (Fig. 3). *Hyperolius mitchelli* and *Hyperolius puncticulatus* have the most similar patterns, with a pruned nodal distance of 0.73, significantly more similar than random trees (95% confidence interval for random tree similarity = 1.60–1.27). The *Hyperolius spinigularis* complex falls on the 'very dissimilar' end of the 95% confidence interval in relation to *H. puncticulatus* and *H. mitchelli*. *Hyperolius puncticulatus* and *H. spinigularis* have a pruned nodal distance of 1.72, within the 95% confidence interval for random trees (1.83–1.31). *Hyperolius mitchelli* and

H. spinigularis have a pruned nodal distance of 1.55 (95% confidence interval 1.58–1.06), and are also not significantly similar.

Four patterns are apparent from these data: (i) No lineages within a species/species-complex (Neither sister-taxa nor distantly related clades) occupy the same highland (complete allopatry), (ii) *H. puncticulatus* and *H. mitchelli* are the most similar in their phylogeographical patterns, (iii) The Malawi Rift is only sister to the East Arc in one lineage (*H. puncticulatus*), and (iv) None of the major areas (Areas 1–4) contain monophyletic groupings within the three study lineages. Taken together, the montane *Hyperolius* of East Africa have a very complex evolutionary history.

Time-constrained scenario and comparison to paleoclimatic history

The Lineage Through Time (LTT) plot of the multi-locus data set (Fig. 4; confidence intervals shown for birth–death null distribution) is step-like with rises at approximately 12–13, ~6.5–7.5, 4–5, and 2–2.4 Ma. The slope of the LTT plot is significantly different from the pure-birth null expectation of exponential lineage accumulation (Cramer-von Mises test: $W_2 = 2.091$ $P < 0.01$, Anderson–Darling test: $A_2 = 1.735$ $0.01 < P < 0.025$), and a birth–death model had the highest AIC score and a significant Likelihood Ratio Test when compared to pure-birth and a 2-rate model. The two sections of the LTT curve that fall outside of the 95% confidence interval of birth–death trees occurred at 12 Ma and just before 2 Ma where they are greater than expected. Lineage-splitting events do not appear to correspond to major, extended dry cycles (Fig. 4, grey bars).

Simultaneous division between major areas

In testing for simultaneous divergence across spatial gaps, Bayes factor analysis of Psi (Ψ) and Omega (Ω) for each of the three splits yielded support for non-simultaneous divergence across gaps, though the

Table 1 Summary data for genes and species

Lineages	Sample Size	ND2 (PiX/PiXY)	POMC (PiX/PiXY)	C-myc (PiX/PiXY)	Characters (#) (ND2/POMC/C-myc)	P I characters (ND2/POMC/C-myc)
<i>Hyperolius mitchelli</i>	58	5.24/109.5	1.78/4.23	8.17/11.11	1151/652/1308	208/22/51
<i>Hyperolius spinigularis comp.</i>	38	1.97/59.59	5.05/7.65	1.59/16.89	1150/652/1308	184/47/58
<i>Hyperolius puncticulatus</i>	111	3.54/52.47	7.30/10.95	2.40/4.11	1152/652/1308	174/51/49

Under each genes is listed the pairwise distance within populations (PiX)/pairwise distance between populations (PiXY) within each lineage for each locus sampled. Also listed are samples numbers, number of characters, and number of parsimoniously informative characters. Pairwise differences for each locus within and between populations were performed with the program ARLEQUIN v. 3.11 (Excoffier *et al.* 2005).

POMC, Pro-opiomelanocortin.

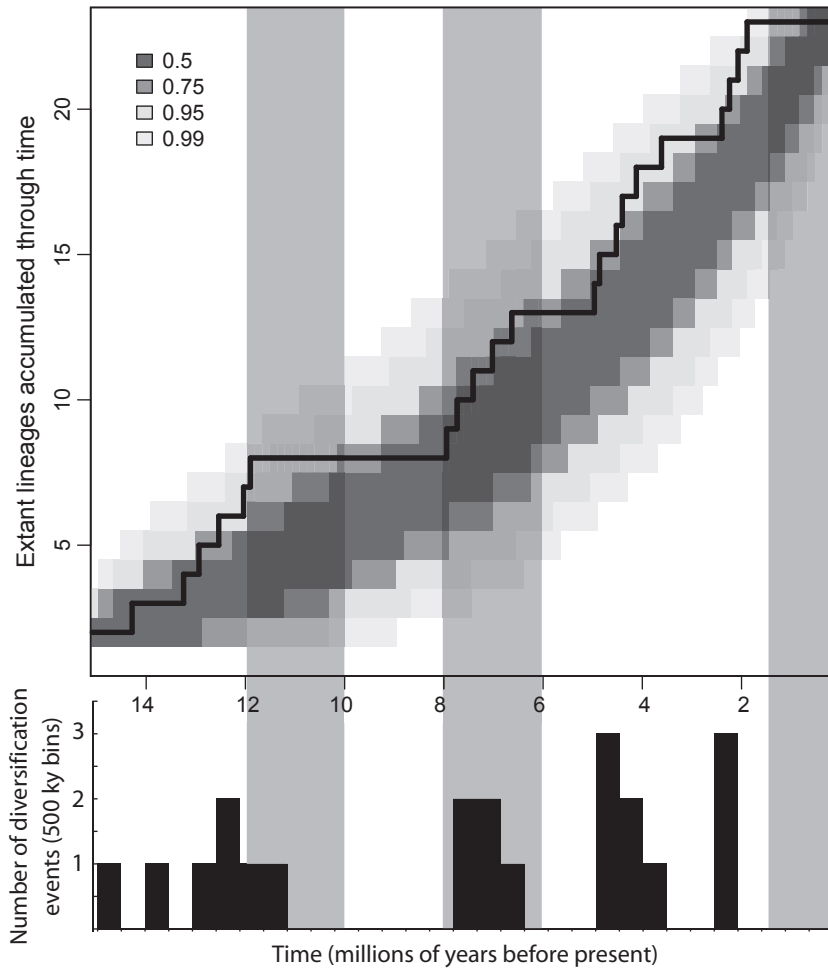


Fig. 4 Top: Lineage through time plot and confidence intervals of trees generated under a birth–death model of lineage accumulation (confidence interval limits defined according to inset legend). Nodes prior to intra-specific diversification were removed. Bottom: Histograms of lineage divergence events in 500 000 years bins. Grey bars correspond to extended periods of dry climate.

strength of the support was mixed (Table 2). In each case, the most well-supported scenario showed two lineages as simultaneous divisions across the gap, and the third at a different time point (Table 2). Additionally, the lineage pairs that showed simultaneous divergences were not consistently the same. Some congruence of evolutionary history exists, though the pattern is not consistent between all members.

Ecological niche models; dry climate and current conditions

Though current approximations for the distribution of suitable habitat for the three *Hyperolius* lineages are nearly identical (Fig. 5—top row, Table 3—top), their ‘refugial’ distributions during dry climate conditions show that each lineage has a unique habitat distribution (Fig. 5—bottom row). All lineages show large reductions in habitat suitability throughout central Tanzania during the LGM, producing isolation between northern and southern populations. Additionally, significant reductions in habitat would increase

the likelihood of local extinctions, adding a randomizing force in extant phylogeographical patterns. Though *H. mitchelli* suffers the same reduction of habitat in central Tanzania as the other two lineages, it is also able to utilize submontane and coastal forests as they become suitable in the dry climate scenario. This greatly increases its predicted range relative to today, even as the other two lineages retreat into refugia (Fig. 5—bottom row, Table 3—bottom). This increase in habitat is primarily throughout Northern Tanzania and Kenya, though *H. mitchelli* also uniquely retains sizable areas throughout Malawi.

Discussion

The extent and distribution of suitable habitat of each lineage during dry climate cycle conditions accurately matches spatial phylogeographical relationships within each lineage. This is much more accurate than predictions made on current habitat approximations. General responses to dry climate scenarios also predict extreme habitat reductions for at least some areas of all three

Table 2 Timing and concordance of divergence events between major areas from HABC analysis in msBayes

Split	Psi	BF	Omega	BF	Time (Ma)	Members of split
Area 1/Area 2	2	Non 14.74	0.68534	Non 396.06	1.340 11.150	2 (<i>spinigularis</i> and <i>puncticulatus</i>) <i>mitchelli</i>
Area 2/Area 3	2	Non 5.90	0.20802	Non 39.64	2.560 7.819	2(<i>mitchelli</i> and <i>puncticulatus</i>) <i>spinigularis</i>
Area 3/Area 4	1–2	Non 3.76	0.07165	Non 7.63	4.022 6.971	2 (<i>mitchelli</i> and <i>puncticulatus</i>) <i>spinigularis</i>

HABC, Hierarchical Approximate Bayesian Computation.

Most likely number of divergence events (Ψ Psi) and degree of concordance of divergence times (omega Ω) are shown along with Bayes factor (BF) support of non-simultaneous divergences for each measure.

lineages, which likely underlie the conflicting spatial and genetic signals.

Environmental Niche Modelling predictions for current and the dry climate scenario appear to closely match expectations for all three lineages from intraspecific trees and migration estimations, except in the relationship between populations A and B in the *Hyperolius spinigularis* complex. *Hyperolius spinigularis* and *H. puncticulatus* have very similar ecological niche envelopes and likely respond similarly to periods of dry climate. The distribution of suitable habitat for *Hyperolius mitchelli* during dry periods is unique for this system, showing

expansions in the northern edge of its range and sustained suitability in the southern end (while showing dramatic reductions in central regions). This deep spatial divide is reflected in its phylogeographical pattern.

HABC analysis of areas that appear to have remained stable through time showed a great deal of congruence: two of three lineages show concordant divergences between areas straddling major gaps (Table 2). This supports the hypothesis that local extinctions and subsequent recolonizations of more ephemeral montane habitats may have structured some of the discordance in phylogeographical structure.

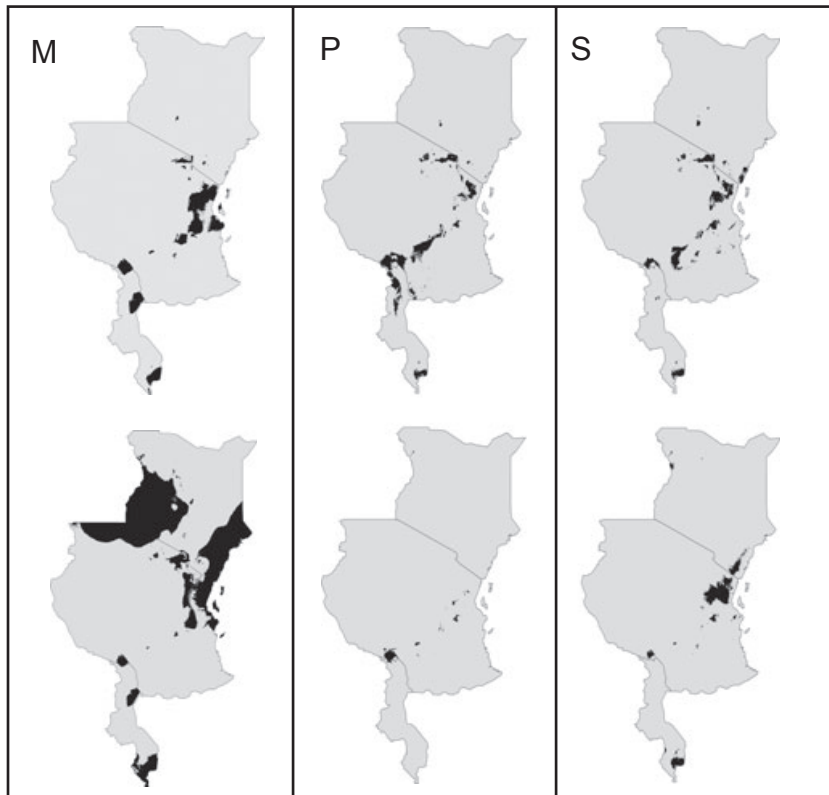


Fig. 5 Maxent environmental niche envelopes for each lineage under current (top row) and Last Glacial Maximum (dry) climate conditions using 10% training omission thresholds. M = *Hyperolius mitchelli*, P = *Hyperolius puncticulatus*, S = *Hyperolius spinigularis* complex.

Table 3 Niche overlap between lineages (Ecological Niche Modelling tools), D summary statistics above the diagonal, I summary statistics below

	<i>Hyperolius spinigularis</i>	<i>Hyperolius mitchelli</i>	<i>Hyperolius puncticulatus</i>
Current			
<i>H. spinigularis</i>	–	0.99*	0.99*
<i>H. mitchelli</i>	0.93*	–	0.98*
<i>H. puncticulatus</i>	0.94*	0.92*	–
LGM			
<i>H. spinigularis</i>	–	0.92*	0.99*
<i>H. mitchelli</i>	0.85*	–	0.85*
<i>H. puncticulatus</i>	0.95*	0.91*	–

Top: current climate conditions. Bottom: Last Glacial Maximum (LGM) distributions.

*Distributions are significantly more similar than random.

In addition to different ecological niches, there are alternative explanations for phylogeographical differences between these three lineages. For example, these frogs may have originally colonized this highland system from different source populations. The deepest nodes within each lineage take place in different geographical areas, which could be because of different geographical origins for each lineage; as previously shown for other taxonomic groups in these highlands [e.g. Sheppardia Forest Robins (Voelker *et al.* 2010), chameleons (Matthee *et al.* 2004), and frogs (Blackburn & Measey 2009)]. Completion of a detailed *Hyperolius* phylogeny (Lawson, in preparation) and identification of the geographical locations of sister lineages will inform this hypothesis.

Though the LTT plots indicate that one major splitting event corresponds to a dry climate cycle, two important events (three of the four, Fig. 4) occur during wet and variable time periods, not traditionally identified as important for driving speciation in montane habitats (e.g., Bowie *et al.* 2004a; Bowie *et al.* 2004b; Roy *et al.* 1998, 2000; Voelker 1999). This correspondence may result from under-appreciated processes driving speciation such as dispersal through ephemeral wet corridors. Alternatively, our understanding of the paleoclimate record for this region may be inaccurate. Whatever the cause, these diversification times are a poor fit for the paleoclimate data of this region.

Methodological assumptions

Inherent to any study concerning divergence dating are assumptions built into the estimation of divergence times. While this study uses rates of molecular evolution that are carefully calibrated, differences in speciation/mutation rate between lineages likely introduces

error into these analyses. Another important assumption to be considered is that LGM climate reconstructions can serve as a proxy for evaluating responses to periods of dry climate. While ideally we would have detailed climate maps throughout history, until global climate models for deeper time periods exist, this combination of area-specific paleoclimate data and recent palaeontological wet/dry estimates provide an initial approach to understanding evolutionary responses to climate pressures. Once models of deeper time climate shifts are available, more precise models of connectivity and extinction-risk will be available.

LTT plots are also a potential source of error, because identifying time periods exceeding expected lineage accumulation is reliant on point estimates of divergence times. It is widely understood that point-estimates are much less informative than confidence intervals for depicting the age of each node. While the histogram of divergence events included earlier allows for some measure of uncertainty in the exact age of diversification events, this analysis should ideally include a test that incorporates posterior probabilities of the age of each node into a LTT test.

Intraspecific divergences, species definitions, and ENM

The divergences within each of the three lineages are deep, raising the question of whether to treat populations within each lineage as separate species. *Hyperolius mitchelli* and *H. puncticulatus* have divergences as old as species splits within *H. spinigularis*, yet unlike the *H. spinigularis* complex, it is difficult to draw concrete lines between populations in these lineages based on morphology, habitat preference, or call. Additional study of morphology, ecology, and vocalizations may provide stronger arguments to consider populations within these lineages as independent species.

Treating the *H. spinigularis* complex (*Hyperolius minutissimus*, *Hyperolius tanneri*, and *H. spinigularis*) as a unit in this analysis is a potential source of error, particularly in reconstructing ENM distributions for this lineage. Though these three species form a strongly supported monophyletic group (Lawson 2010), the distribution of *H. minutissimus* is limited to the Udzungwa and Rubeho Mountains (Area 3, Fig. 1), which has a slightly different climate from the rest of the montane areas where *H. tanneri* and *H. spinigularis* are found (Schjötz 1975, 1982, 1999). Though combining two habitat types into one model is a valid concern in any lineage with deep internal nodes, spatial autocorrelation may make this shift appear more severe than experienced by the focal lineages. Populations of *H. puncticulatus* and *H. mitchelli* are also found in the same mountain block (Area 3, Fig. 1), and if locality records from Area

3 are removed for any of these lineages, Maxent models fail to recognize the Udzungwas and Rubehos as suitable habitat compared to the rest of the range. Predictions for all three lineages are then confined to Area 1 and 2. Each lineage appears to occupy a slight habitat shift in the Udzungwa Mountains, possibly because of spatial autocorrelation because climate conditions from northern Tanzanian montane areas do not exist in the Udzungwa Mountains.

Biodiversity with the EAM—past, present, and future

The highlands of East Africa harbour some of the most unique and vulnerable biodiversity on the planet, and as a result they are one of Conservation International's 'Biodiversity Hotspots' (Myers *et al.* 2000). Though of great biological importance, major gaps in our understanding of Eastern Arc fauna exist at multiple levels of organization. This includes incomplete species distribution records, the near total lack of intraspecific studies, and geographical sampling biases that skew or obscure diversity patterns. Though severely under-sampled compared to Northern Hemisphere system, a few studies have been completed in the fragmented highlands of Tanzania and Malawi that can be compared to these amphibian patterns. A study of an endemic Eastern Arc shrew (*Sylvisorex howelli*) showed the same phylogeographical pattern as *H. puncticulatus* for the subset of the range that *S. howelli* occupies with similar mtDNA divergence levels (Stanley & Olson 2005). African montane forest robins (*Pogonocichla stellata*) show a phylogeographical pattern similar to *H. mitchelli*, though the time scale is an order of magnitude younger (Bowie *et al.* 2006). *Arthroleptis xenodactyloides* (another frog from this region) also shares some similarities in phylogeographical patterns to these lineages (Blackburn & Measey 2009), though it is found in a number of localities in addition to those where these lineages live. These additional regions are drier and more deforested than current Eastern Arc Areas and/or on islands off the coast, and thus appear to not support the *Hyperolius* species in this study. *Arthroleptis xenodactyloides* shows admixture between populations corresponding to A and B (Fig. 1), and these populations are divergent from a clade of populations corresponding to D, M, and K. This split is similar to *H. mitchelli*, though the relationships within D, M, and K are different. *Arthroleptis xenodactyloides* appears to be able to generalize into submontane forests, must as *H. mitchelli* can, which likely underlies the similarity in their distribution patterns. Taken together, the vertebrate fauna of the EAM are a mix of similarities and differences. Additional population-level studies are necessary in order to understand the

geologic and environmental forces underlying the accumulation of biodiversity in the EAM.

Conclusions

This study, combined with other patterns within the EAM, presents strong evidence that the evolutionary history of contemporary lineages of the EAM is complex and most often lineage-specific, probably because of different ecological niche requirements of these convergently co-distributed lineages. Just as oceanic islands often show a mixed history of *in situ* diversification, colonizations, and extinctions, fragmented mountain chains offer an ideal model for demonstrating the process of diversification across a fragmented landscape (Knowles 2000; Cadena *et al.* 2007).

The results from this study combine phylogeographical, spatial, and ENM analyses to better understand the geographical and environmental processes underlying current distribution patterns in the EAM fauna. This study of three congeneric frog lineages with similar but varying current niches and overlapping ranges show that even in lineages that a priori would be predicted to have a high probability of corresponding reactions to vicariance events, their evolutionary histories may be widely divergent. This study and others (e.g., Bowen and Avise 1990 and Zink 1996) show that biogeographical and evolutionary histories of species within fragmented areas are often surprisingly complex and require detailed comparative analysis to fully reveal the determinants of their current distribution.

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This article forms part of the PhD research of Lucinda Lawson investigating how the landscape and environment that organisms experiences influences their evolution, specifically in East African sky-islands. She is primarily interested in methods of integrating multi-disciplinary data into biologically and spatially relevant evolutionary reconstructions.

Supporting information

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

Appendix S1 Voucher specimen information, population level diversity estimates, primers, mtDNA gene trees, and demographic estimates.

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