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Genetic Divergence During Long-term Isolation in Highly Diverse Populations of Tropical Trees Across the Eastern Arc Mountains of Tanzania

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ABSTRACT

The Eastern Arc Mountains are believed to support some of the oldest tropical forest in the world. The current distribution of this forest is highly fragmented due to a combination of long-term effects of past changes in global climate and more recent deforestation. We sought to explore the hypothesized antiquity and long-term isolation of the Eastern Arc montane forests based on an assessment of the geographical distribution and interspecies similarity of chloroplast DNA sequence variation in five forest trees. Data were used to investigate regional patterns of diversity and population structure based on intraspecific phylogenies, and results were interpreted against hypotheses on ecosystem age and connectivity. Regional diversity was high, with up to 22 chloroplast DNA haplotypes being recorded within a species across the sampled populations. Geographical concordance of genetic and geographic structure was weak to absent in all species and there was little similarity of genetic structure between species. Haplotype sharing between mountain blocks was extremely limited. The generally weak phylogeographical structure, in conjunction with high regional diversity and genetic uniqueness of individual mountain forests does not support the assumption of widespread genetic connectivity of the mountain forests, indicating instead a pattern of past isolation and ongoing diversification. Our findings substantially add to understanding patterns of diversity in this region and lend weight to calls to use more sophisticated biodiversity assessments when setting regional conservation and research funding priorities.

Abstract in Swahili is available in the online version of this article.

Key words: Africa; biodiversity; conservation priority; genetic diversity; phylogeography; tropical forest.

High mountain flora can enable us to understand how geographical isolation and past climatic changes have shaped diversity within species. Intraspecific diversity and population structure are strongly influenced by the age of a mountain block and its environmental stability over time, such as mountain formation (Gübitz *et al.* 2005) and impacts of glacial–interglacial cycles (Hewitt 2004). While high latitudes experienced repeated extinction and recolonization events due to expansion of ice sheets during the Quaternary period, increased aridity reduced the extent of tropical forest and compressed tropical regions toward the equator. In contrast with more widespread extinctions in poleward regions, species in the tropics were able to persist through altitudinal migration and networks of topographically dependent refugia (Fjeldså & Lovett 1997a, Tzedakis *et al.* 2002, Hewitt 2004, Dimitrov *et al.* 2012).

This combination of long-term persistence of species with contemporary isolation is one of several hypotheses often cited as contributing to the high levels of tropical biodiversity (Fjeldså & Lovett 1997b, Gaston 2000, Rull 2006, Fjeldså & Bowie 2008, Dimitrov *et al.* 2012), which can also be reflected in high levels of intraspecific diversity (Hewitt 2004). Therefore, genetic data can provide additional tools to explore impacts of past environmental change (Hewitt 2004, Gübitz *et al.* 2005). For example, strong biogeographical barriers or past events often impact phylogeographical structure of ecologically similar species in a comparable way (Avise *et al.* 1987). Understanding the amount and distribution of genetic diversity within species can, therefore, allow us to understand the geological, climatic, and geographical factors that shape current vegetation distribution (Bermingham & Moritz 1998).

Across the Eastern Arc Mountains (EAM) of Tanzania and southeast Kenya, the combination of great geological age, topographic variation, and long-term climatic stability has been hypothesized as resulting in these mountains supporting some of the oldest tropical forest in the world (Hamilton 1981, Fjeldsa *et al.* 1997, Fjeldså & Lovett 1997a, Lovett *et al.* 2005). Recent pollen records from the EAM and neighboring mountains indicate that some montane areas have remained relatively moist, climatically stable, and consequently, covered in moist forest since around the Last Glacial Maximum (LGM) (Mumbi *et al.* 2008, Finch *et al.* 2009, Rucina *et al.* 2010, Schüler *et al.* 2011). Local topographic and moisture regimes play an important role in buffering vegetation composition against regional climatic changes—albeit that the

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ecosystem response is highly spatially variable (Mumbi *et al.* 2008, Rucina *et al.* 2010, Hampe & Jump 2011). However, this hypothesized general stability of the EAM forests contrasts with major changes to ecosystem composition and distribution in the broader region of East Africa due to lower CO_2 concentration and a colder and drier climate during the LGM (Jolly & Haxeltine 1997, Farrera *et al.* 1999, Cohen *et al.* 2007).

The EAM region has long been recognized as an area of global biological importance (ICBP 1992, Mittermeier *et al.* 1998, Stattersfield *et al.* 1998, Myers *et al.* 2000, Olson & Dinerstein 2002, Mittermeier *et al.* 2005) due to high levels of endemism in all hitherto researched taxonomic groups (Cordeiro *et al.* 2007). However, major gaps in our knowledge of the biodiversity of this region still exist, among which lies the near absence of studies conducted at the intraspecific level (Lawson 2010) (though notable exceptions exist: Kolehmainen & Korpelainen 2008, Lawson 2010, Measey & Tolley 2011, Dimitrov *et al.* 2012, Lawson 2013). Plant phylogeography in the EAM so far remains largely uninvestigated. Indeed, phylogeographic information more broadly is inadequate in East Africa (Beheregaray 2008) and the African continent as a whole remains very poorly understood from a phylogeographic perspective, with only 4 percent of studies reviewed by 2006 being based on African plants and far fewer still including a comparative plant phylogeographical component (Beheregaray 2008). However, several recent studies including the pan-African phylogeography of the Afromontane tree *Prunus africana* (Kadu *et al.* 2011), the *Acacia senegal* complex (Odee *et al.* 2012), and a comparative phylogeography of African savannah ungulates (Lorenzen *et al.* 2012) have highlighted East Africa as a highly important reservoir of diversity in the species investigated.

Palynological evidence (Mumbi *et al.* 2008, Finch *et al.* 2009) and dated phylogenies for chameleon species (Tolley *et al.* 2011) and African violets (*Saintpaulia* Spp.) (Dimitrov *et al.* 2012) occurring across the region give support for the antiquity of the EAM forests. However, recent studies of the distribution of genetic variation within animals of the EAM have identified complex phylogeographical patterns with little agreement between species (Lawson 2010, Measey & Tolley 2011). Consequently, we sought to determine if the distribution of genetic diversity within common tropical tree species shows similarly complex geographical relationships indicative of past processes and long-term isolation or much greater geographical concordance indicative of contemporary gene movement between mountains within the region. We also aimed to identify if data from tropical trees support the hypothesis of long-term persistence of the EAM forests (Lovett *et al.* 2005). Such information will aid our understanding of the geological history of the region and inform on the conservation and management of its biodiversity (Beheregaray 2008). Assisting in the prioritization of conservation effort is an especially important application of this study in the EAM given biased funding priorities recently identified for this region (Ahrends *et al.* 2011).

Predictions made from three alternative hypotheses of colonization age and contemporary isolation guided this study (Table 1). These predictions are broadly comparable with those derived from the Evolution in Isolation and Vertical Displacement hypotheses proposed to explain the species diversity and endemism in the Guiana Highlands (Antonelli *et al.* 2009, Desamore *et al.* 2010). We assume that each mountain block potentially represents a unique locality in that, although the environmental conditions of lowland habitats between mountain blocks present a barrier to the establishment of moist tropical forest taxa; excepting the effect of distance, there is no *a priori* reason to believe that barriers between particular mountain blocks should be more significant in biogeographic and evolutionary terms.

METHODS

The EAM comprise of a series of 13 isolated forest-capped mountain blocks (Platts *et al.* 2011) (Fig. S1) believed to have

TABLE 1. *Predicted genetic consequences of colonization and isolation scenarios across the Eastern Arc Mountains.*

Model	Hypothesis	Phylogeographic predictions
Recent colonization	Individual mountain blocks have been colonized or recolonized recently following loss of forest cover during Pleistocene	Within species: strong influence of geographical distance in shaping phylogeographic structure with geographically proximal populations being most genetically similar, low diversity at the regional scale, high genetic similarity between mountains. Between species: strong phylogeographical agreement
Ancient colonization + ongoing gene exchange	Forest predates Pleistocene and has persisted on individual mountain blocks with ongoing gene flow between mountains	Within species: strong influence of geographical distance in shaping phylogeographic structure with geographically proximal populations being most genetically similar, high diversity at the regional scale, high genetic similarity between mountains Between species: moderate phylogeographical agreement
Ancient colonization + long-term isolation	Forest predates Pleistocene, has persisted on individual mountain blocks and has remained genetically isolated in each mountain block	Within species: weak to absent influence of geographical distance in shaping phylogeographic structure with geographically proximal populations showing little to no more similarity than geographically distant ones, high diversity at the regional scale, little genetic similarity between mountains Between species: little phylogeographical agreement

been formed approximately 30 million years ago (Schlüter 1997), although estimates of their age vary (Iversen 1991). The geological origin and great antiquity of the Eastern Arc contrasts with nearby volcanic mountains, such as Meru and Kilimanjaro, which have their origin within the last 1–2 million years (Griffiths 1993, Schlüter 1997). The remaining tropical forests of the EAM encompass approximately 3500 km^2 of sub-montane, montane and upper montane forest, ranging in extent from 3 to 1353 km^2 per mountain block (Cordeiro *et al.* 2007), but together comprising less that 7 percent of the estimated original forested area (Myers *et al.* 2000).

STUDY SPECIES AND SAMPLING.—We identified an initial suite of 15 species for collection from forests across ten EAM based on their widespread distribution and ease of identification from sterile material. However, the density of individual species within the EAM forests was uniformly low and it was not possible to find all species across all sites sampled. Consequently, we selected the following five most extensively sampled species for further analysis: *Macaranga capensis* (Baill.) Benth. ex Sim (Euphorbiaceae) (47 individuals sampled), *Newtonia buchananii* (Baker f.) (Leguminosae, Mimosoideae) (25), *Ocotea usambarensis* Engl. (Lauraceae) (19), *Xymalos monospora* (Harv.) Warb. (Monimiaceae) (43), and *Zanthoxylum gillettii* (De Wild.) P. G. Waterman (Rutaceae) (19). Locations of survey regions are given in Table S1.

All of these species are native to Africa but none of them is endemic to the EAM. *Macaranga capensis* has been recorded over a large altitudinal range (305–3000 m) elsewhere but in the Eastern Arc, predominantly occurs as a pioneer species in lowland and submontane forests. The species bears a subglobose/bilobate drupe-like fruit, 0.3–0.5 cm long, 0.4–0.6 cm in diameter. *Newtonia buchananii* is frequently found in wet forest and near streams or ground water. Its altitudinal range (600–2300 m) encompasses lowland to montane forests. It bears pods 10–32 cm long and 1.3–2.5 cm wide containing winged seeds. *Zanthoxylum gillettii* occurs in submontane and montane forests and can also be found in wooded savannah, secondary forest, and bushlands. It bears a subglobose drupe-like fruit 0.4–0.6 cm in diameter. *Ocotea usambarensis* and *X. monospora* are generally found at higher altitudes (montane and upper montane forest). While *O. usambarensis* (900–2400, rarely to 3000 m altitude, bearing an ellipsoid drupe in a cup, 1 cm long, 0.6 cm in diameter), a valuable timber species, is typically restricted to forest habitat, the monotypic *X. monospora* (1600–2300 m, bearing an ovoid, fleshy 1–1.9 cm long drupe-like fruit) can also be found in forested grasslands. However, despite these broad habitat categorizations, none of these species is known to exist in the extensive savannah habitats that separate the individual mountain blocks of the EAM. While *N. buchananii* is dispersed primarily by wind, the other species investigated here are all predominantly primarily bird dispersed (N. Cordeiro and R. Gereau, pers. comm.).

We identified all trees and vouchers were deposited in the University of Dar es Salaam Herbarium (DSM). The position of each sampled tree was recorded using a hand-held GPS with an accuracy of 10–20 m. For each species and forest, we separated sampled trees by a minimum of 100 m, and sampled individuals through the collection of either a 10 cm^2 piece of leaf tissue or a similar sized cambium sample when leaves were inaccessible. Tissue samples were dried immediately in the field in fine-grained silica gel.

MOLECULAR METHODS.—In plants, the chloroplast genome is monoploid; it is transmitted by seed in most angiosperms. Investigating geographical variation in chloroplast DNA sequence polymorphisms can therefore enable us to identify how habitat availability and dispersal barriers influence seed movement over time. However, a drawback of investigating phylogeographic patterns using the chloroplast genome is that sequence variation is often low due to the non-recombining nature of this genome and smaller effective population size owing to its uniparental inheritance and cannot inform on gene flow by pollen. In this investigation, we were guided by the assumption that the hypothesized antiquity of the forests in this region should have resulted in the accumulation of diversity within species in parallel to its high species-level diversity, thereby allowing us to exploit geographical patterns in chloroplast DNA variation that should be assayable using widely used universal primers.

For each individual, we ground a 2 cm^2 tissue sample to a fine powder at 1500 vibrations/min using a Mixer Mill 301 (Retsch, Castleford, U.K.). DNA was then extracted and purified using a DNEasy Plant Mini kit (Qiagen, Crawley, U.K.) according to the manufacturer's instructions, with the exception that we eluted cambium samples in 2 × 50 μL volumes. We quantified DNA concentration using a Nanodrop ND 1000 Spectrophotometer (Labtech International, Ringmer, U.K.) using the manufacturers software and stored it at −20°C. Barcoding loci tested by Kress *et al.* (2005) were screened for use in this study. We chose four loci for sequencing and phylogenetic analysis based on maximum polymorphism and repeatable amplification; these were the (1) *trnC-ycf6* intergenic spacer, (2) *trnL-F* intergenic spacer, (3) *ycf6-psbM* intergenic spacer, and (4) *trnH-psbA* intergenic spacer. The primer sequences for each locus, as well as the loci genotyped for each species, are detailed in Table 2.

For all loci, we performed PCR with a 1 min denaturing step at 94°C, a 1 min annealing step at 52°C, and a 1 min extension step at 72°C over 25 cycles. We purified amplified products using the QIAquick Gel Extraction kit (Qiagen) and performed sequencing in both the forward and reverse directions using the individual PCR primers (Macrogen Inc., Seoul, Korea). We repeated sequencing reactions that gave ambiguous or unclear sequence from the PCR stage. Sequences generated for this study have been deposited in GenBank under the accession numbers JX139119–JX139521.

STATISTICAL ANALYSES.—We created alignments for each chloroplast locus using Muscle 3.7 (Edgar 2004), which were then concatenated to form a single dataset for each species. The alignment lengths were: *M. capensis*, 1490 base pairs (bp); *N. buchannii*, 1240 bp; *O. usmabarensis*, 2579 bp; *X. monospora*, 1328 bp and *Z. gillettii*, 1196 bp. For each species, Modeltest 3.7 (Posada

TABLE 2. *Primer sequences for the four chloroplast loci used in this study (Kress* et al. *2005).*

Loci	Primer	Sequence	Species amplified
trnC-ycf6 intergenic spacer	trnC	5′-CCAGTTCAAATCTGGGTGTC-3′	*Macaranga capensis*, *N. buchannii, Ocotea usambarensis, Xymalos monospora*, *Z. gilettii*
	petN1r	5′-CCCAAGCAAGACTTACTATATCC-3′	
trnL-F intergenic spacer	trnL-Fe	5′- GGTTCAAGTCCCTCTATCCC-3′	*M. capensis*
	trnL-Ff	5′-ATTTGAACTGGTGACACGAG-3′	
ycf6-psbM intergenic spacer	petN1	5′-GGATATAGTAAGTCTTGCTTGGG-3′	*M. capensis*, *O. usambarensis*
	psbM2r	5′-TTCTTGCATTTATTGCTACTGC-3′	
psbA- trnH intergenic spacer	psbA3-f	5′-GTTATGCATGAACGTAATGCTC-3′	*N. buchannii, O. usambarensis, X. monospora*, *Z. gilettii*
	trnH-f	5′-CGCGCATGGTGGATTCACAATCC-3′	

& Crandall 1998) indicated that the General Time Reversible model (Rodríguez *et al.* 1990), incorporating a proportion of invariant sites and a gamma distribution to correct for among site rate variation (GTR+I+Γ) was the most appropriate nucleotide substitution model.

We subjected the concatenated alignments to phylogenetic analysis by maximum likelihood, using PhyML 2.4.4 (Guindon & Gascuel 2003), Bayesian inference with MrBayes 3.1.2 (Ronquist & Huelsenbeck 2003) and median-joining parsimony with Network 4.5.1.6 (Bandelt *et al.* 1999). We ran the MrBayes analyses using the GTR+I+Γ model and a four-category gamma distribution, with searches consisted of two parallel chain sets run at default temperatures, with a sample frequency of 10, until they had reached convergence (*i.e.*, average standard deviation of split frequencies equal to 0.01). The runs consisted of 500,000 generations, with the first 12,500 sampled trees being discarded as burnin before calculating posterior probabilities. We also performed the PhyML analyses using the GTR+I+Γ model, with a four-category gamma distribution, and bootstrapped with 1000 replicates. For the Network analysis, sequences were subjected to star contraction (Forster *et al.* 2001) and haplogroup networks were generated under default parsimony parameters estimated by the program. Nucleotide diversity within each species was determined using Watterson's estimator of θ (Watterson 1975) and π (Nei 1987) with DnaSP 5.0 (Librado & Rozas 2009). We compared genetic differentiation over all populations computed based on haplotype frequency (G_{st}), using the program PERMUT (http://www.pierroton.inra.fr/genetics/labo/Software/Permut; Pons & Petit 1996) with the equivalent parameters calculated taking into account the molecular distance between haplotypes, (N_{st}). To test for the existence of significant phylogeographic structure ($N_{st} > G_{st}$), we compared G_{st} and N_{st} using a permutation test with 1000 permutations. We excluded populations that included less than three individuals from the analysis to meet the assumptions of PERMUT.

RESULTS

The concatenated sequenced loci showed between 15 and 45 segregating sites within the chosen species, leading to the detection of generally high haplotype diversity within species across the region (*h*, 0.66–0.99; Table 3). The number of haplotypes detected varied from 22 in *X. monospora* to five in *N. buchananii*. *Newtonia buchananii* was the only species showing fewer than 12 haplotypes within the region. Individual haplotypes were generally restricted to an individual mountain block, with only *N. buchananii* and *M. capensis* showing frequent sharing of haplotypes between mountains (Fig. 1). Across all species, only eight haplotypes were shared between mountain blocks of a total of 71 haplotypes recorded across the five species investigated. Two species, *O. usambarensis* and *Z. gillettii*, exhibit significant phylogeographic structure as indicated by significantly greater N_{st} than G_{st}. In contrast, the values of N_{st} and G_{st} in *M. capensis, N. buchananii,* and *X. monospora* were not significantly different (Table 3).

The molecular phylogenies for all five species showed moderate to strong support (*i.e.*, ≥50% maximum likelihood bootstrap percentages (mlBP), ≥0.70 Bayesian Inference posterior probabilities (biPP)). There was also broad agreement between the median-joining parsimony networks and the likelihood phylogenies (Figs. 2A–E and S2A–E), with only one strongly supported difference (≥75%mlBP, ≥0.97 biPP) being present in the likelihood phylogenies. The median-joining parsimony network for *Z. gillettii* recovered the South Pare sequences as a monophyletic group; in contrast, the both the maximum likelihood and the Bayesian Inference phylogenies showed the South Pare sequences to be closely related, but paraphyletic with strong support (77%mlBP, 0.98 biPP).

Genetic differentiation between populations is generally high and consistently higher when assessed by N_{st} (0.44–0.98) rather than G_{st} (0.03–0.90) (Table 3). However, the five tropical tree species investigated here did not show consistent patterns in the geographical structuring of their genetic diversity. In general, the overall picture is one of little agreement between haplotypes and their geographical origin. Based on assessment of the haplotype median-joining parsimony networks (Fig. 2A–E), *M. capensis*, *N. buchananii,* and *O. usambarensis* appear to show strongest concordance between sampling location and genetic structure, with individuals sampled from individual EAM mountains generally clustering together. Indeed, there is also an indication of more regional structuring such that more eastern EAM mountains (*e.g.*, the Usambara mountains) generally cluster together, with Nguru and Nguu either clustering with these eastern mountains or

TABLE 3. *Summary statistics for genetic diversity within species across the Eastern Arc Mountains of Tanzania.*

Species	N	No. haplotypes	No. segregating sites	Haplotype diversity	Nucleotide diversity (π/θ)	N_{st} (±SE)	G_{st} (±SE)
Macaranga capensis	47	12	21	0.853	0.0032/0.0035	0.929 ± 0.061	0.897 ± 0.062
Newtonia buchananii	25	5	15	0.658	0.0044/0.0031	0.978 ± 0.014	0.848 ± 0.083
Ocotea usambarensis	19	17	31	0.988	0.0040/0.0041	0.643 ± 0.138	0.025 ± 0.027***
Xymalos monospora	43	23	45	0.771	0.0036/0.0080	0.440 ± 0.127	0.299 ± 0.089
Zanthoxyllum gillettii	19	14	31	0.850	0.0095/0.010	0.776 ± 0.196	0.292 ± 0.247***

Significance values for Permut tests by asterisks (P < 0.001).

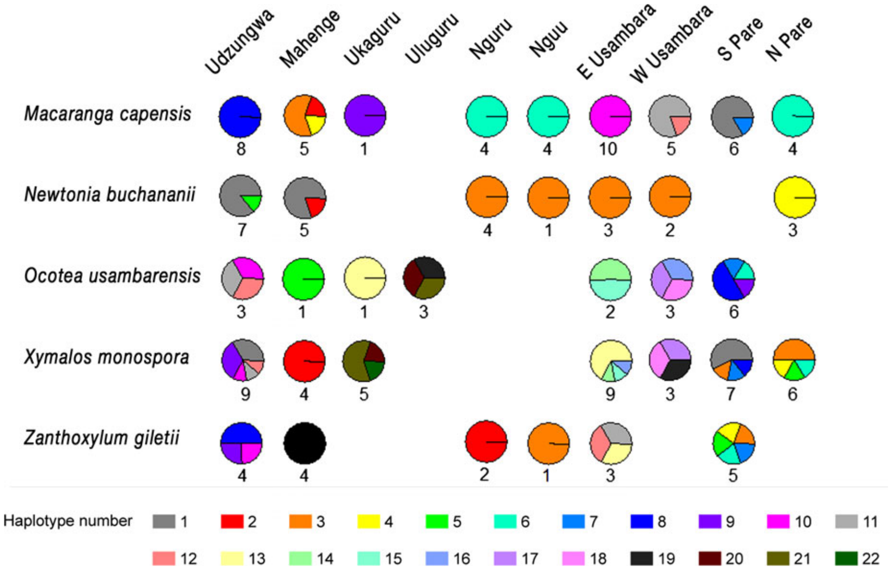

FIGURE 1. Geographical distribution of arbitrarily numbered chloroplast DNA haplotypes across five tropical trees of the Eastern Arc Mountains. Sample size is given below the chart for each species and mountain combination.

forming a separate cluster, and more westerly mountains such as Mahenge and Udzungwa forming a further group.

However, when the genetic similarity between geographically proximate samples is looked at in more detail, it becomes apparent that this regional structure is often superficial. For example, samples of *M. capensis* from North and South Pare occur in widely separated locations in the network for this species, while in *X. gillettii*, nearby populations of the South Pare and East Usambara mountains are found in widely separated locations in the network for this species. This same pattern is seen when the maximum likelihood phylogenies are considered (Fig. S2A–E), with samples from within mountain blocks generally clustering together, while the genetic relationships between mountains lack a clear geographical pattern. This geographical discord is particularly evident in *X. monospora* and *Z. gillettii*, for which genetic relationships between sampling locations appear almost randomized.

DISCUSSION

GEOGRAPHICAL STRUCTURING OF GENETIC DIVERSITY.—There is variable but generally weak geographical structure of genetic variation at the regional (Tanzania-wide) scale across the five species investigated in this study. However, structuring at the local level (high similarity between neighboring samples and sometimes neighboring mountain blocks) is sometimes present. N_{st} is significantly greater than G_{st} in only *O. usambarensis* and *Z. gillettii,* indicative of significant phylogeographic structure in these species, but not in *M. capensis*, *N. buchananii,* and *X. monospora* (Table 3). In combination with the generally high genetic divergence between populations of each species (Table 3), these results indicate that genetic drift has been more influential in structuring diversity across the region rather than legacies of past colonization events. Further insight can be gained by considering the above results alongside haplotype networks and maximum likelihood (ML) phylogenies and the haplotype distributions shown in Figure 1.

The haplotype median-joining parsimony networks (Fig. 2A–E) and the ML phylogenies (Fig. S2A–E), both show a pattern of grouping of samples from within mountain blocks or from neighboring blocks (*e.g.*, Mahenge and Udzungwa or East and West Usambara), but then a lack of structure at the regional scale, with neighboring groups of mountains having no clear genetic relationship between them. Where nearby mountain blocks do cluster together, the groupings of mountain blocks are often not intuitively sensible. Critically, geographically proximate mountains are often genetically divergent at fundamental levels of the ML trees, while individuals sampled from a particular mountain block are not always monophyletic. For example, in *N. buchananii*, populations from East and West Usambara cluster with the neighboring South Pare, but are separated from the neighboring North Pare population at a fundamental level of the network and ML tree (Figs. 2B and S2B).

Despite the general absence of clear geographic structuring of genetic diversity across the species investigated, the distribution of individual haplotypes across the region (Fig. 1) demonstrates that there is very little haplotype sharing between mountains, except in *N. buchananii* and *M. capensis*. In the other three species studied, it is common for individual haplotypes to be restricted to a particular mountain block, with up to five haplotypes reported from any one mountain. These results imply that, based on chloroplast haplotypes, there is little to no seed-mediated gene flow between individual mountain blocks such that, as far as the chloroplast genome is concerned, each supports isolated and now independently evolving populations (Guajardo *et al.* 2010). In *M. capensis* and *N. buchananii*, however, haplotypes are shared between neighboring mountain blocks potentially indicating more effective recent gene movement between mountains in these species (Fig. 1). Indeed, the haplotype distributions shown in Figure 1, when combined with the lack of geographical concordance of the haplotype networks (Fig. 2) and ML phylogenies (Fig. S2) demonstrates that there is no coherent signal across the five species reported here for either convincing phylogeographic structure of the chloroplast genome or effective ongoing gene movement. Given that the chloroplast genome is non-recombining, these patterns suggest that, rather than being the result of ongoing seed movement between neighboring populations, the lack of shared haplotypes between neighboring mountains, combined with high haplotype diversity and geographically discordant structure is more representative of the long-term isolation of these populations (Guajardo *et al.* 2010).

The general discord in genetic structure at the comparative level does not implicate any specific event at the regional scale as having a lasting impact of the structuring of diversity across species (Avise *et al.* 1987). Interspecific differences might, in part, be ecologically based. The five study species are predominantly bird dispersed with the exception of the mainly wind dispersed *N. buchananii* (N. Cordeiro and R. Gereau, pers. comm.). While these broad dispersal categories do not readily explain the observed genetic patterns, more detailed information on the type of dispersal vector (species) and its interaction with the landscape would increase our ability to interpret spatial genetic patterns in light of dispersal attributes (Lehouck *et al.* 2009b). Regrettably, for the majority of our study species such detailed information is not available. However, while the patterns that we identified may partly be exacerbated due to the recent disappearance of dispersal agents as a consequence of forest fragmentation and degradation (Cordeiro & Howe 2003, Cordeiro *et al.* 2009, Lehouck *et al.* 2009a), such recent processes would be highly unlikely to lead to the spatial segregation of haplotypes that we have reported.

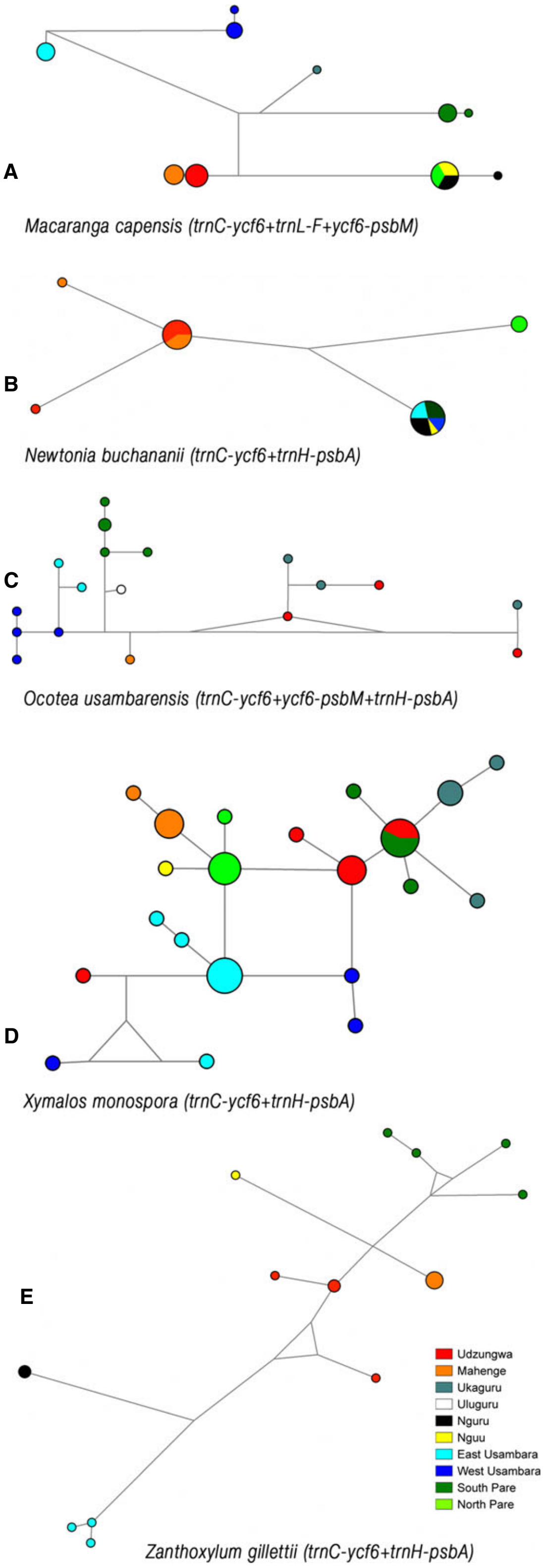

FIGURE 2. Median-joining parsimony networks, based on the GTR+I+Γ nucleotide substitution model of: (A) *Macaranga capensis*; (B) *N. buchannii*; (C) *Ocotea usambarensis*; (D) *Xymalos monospora;* and (E) *Zanthoxyllum gillettii*. Node size is proportional to the frequency of the node.

We stress, however, that these results should be interpreted with caution due to low sample sizes restricting the power of some analyses. High species diversity in these forests is associated with low individual density of the species we investigated, thereby restricting the population sizes that we were able to analyze. Consequently, we do not seek to compare levels of diversity between populations but rather the structure of that diversity across populations. Although we believe that our study represents an important first step in understanding the genetic structure of species across the Eastern Arc forests, estimates of structure will, however, be subject to greater error as a result of low population sizes.

RECENT RANGE FRAGMENTATION VERSUS LONG-TERM ISOLATION.—Previous studies assessing the amount and distribution of genetic diversity across naturally fragmented plant populations have commonly focussed on postglacial fragmentation (*e.g.*, Wesche *et al.* 2006, Bossuyt 2007, Kuss *et al.* 2008, Medrano & Herrera 2008). In these studies, habitats have suffered substantial recent changes in environmental conditions during alternate interglacial and glacial periods such that current plant distributions are likely to be of recent origin (Holocene). While many plant phylogeographic studies are believed to demonstrate signals from distributions predating the Quaternary (*e.g.*, Andrianoelina *et al.* 2006, Ayele *et al.* 2009, Desamore *et al.* 2011, Garcia *et al.* 2011, Molins *et al.* 2011, Santos-Gally *et al.* 2012), there is generally no evidence of (or reason to hypothesize) long-term isolation of individual populations.

Long-term isolation has been reported, however, from several phylogeographical studies of tropical trees species. For example, *Camellia taliensis* shows very high haplotype diversity at the species level (h = 0.84) and extremely high divergence between populations (G_{st} = 0.99). The authors propose that this pattern of chloroplast haplotype variation may potentially result from long-term population isolation as a consequence of tectonic activity that led to the uplift of the Qinghai-Tibetan Plateau. Similarly, in the tropical dry forest tree *Caesalpinia echinata,* long-term isolation between regions is believed to have led to the very high divergence witnessed between populations of this species (Phi_{st} = 0.91) (Lira *et al.* 2003). However, as with *Camellia taliensis* and in contrast with our findings, *Caesalpinia echinata* shows very low diversity within populations, with only two of seven populations showing more than one chloroplast haplotype.

DIVERGENCE DURING LONG-TERM ISOLATION IN THE EAM.—In a recent phylogeographical study of *Saintpaulia*, which is largely confined to moist forest habitats in the EAM and neighboring coastal forests, the authors found strong evidence for long-term climatic stability of the EAM and the existence of montane refugia fostering isolation of populations on individual mountain blocks and subsequent divergence and species radiation (Dimitrov *et al.* 2012). These findings reported by Dimitrov *et al.* (2012) strongly parallel the radiation at the intraspecific level for the tree species that we investigated here. Phylogeographical study of starred robins (*Pogonocichla stellata*) across the EAM and wider East African region, similarly found little evidence for ongoing gene flow within the EAM (Bowie *et al.* 2006). Long-term isolation of the moist tropical forest of individual mountain blocks in the EAM is also held responsible for the diversification and retention of chameleon (*Kinyongia*) lineages across the EAM (Tolley *et al.* 2011). In a recent study of three lineages of African montane reed frogs (*Hyperolius* spp.) from the EAM, Lawson (2010) found complex phylogeographical relationships of present day populations of these taxa and related these to different ecological requirements of these co-distributed lineages. Lawson (2010) points out that even in lineages that might be expected to have a similar response to past vicariance events, such as the montane tropical forest taxa investigated here, their evolutionary histories may be widely divergent, in a large part due to the additional complexity introduced by their naturally fragmented mountain habitats. Subsequent phylogeographic study on the endemic spotted reed frog (*H. substriatus*) has reinforced this pattern of strong and complex phylogeographic structure that, while geographically concordant within the EAM does not correspond neatly with the physical distribution of individual mountain blocks within the wider East African region (Lawson 2013). These findings are echoed by Tolley *et al.* (2011), who advise that any attempt to form a hypothesis that synthesizes the diversity and endemism of the EAM and wider East African region must be based on diverse and multiple taxa.

This is the first comparative study of intraspecific diversity of tropical moist forest tree species in the EAM. Our findings emphasize the complexity of the biogeographic patterns of this region that was previously noted by Lawson (2010) and Tolley *et al.* (2011). Our results, based on chloroplast DNA sequence variation, show that no single geographical barrier within the EAM has been important in shaping present day intraspecific diversity in the region's montane moist tropical forest trees. Consequently, whereas diversity is high within tree species across the EAM and each mountain is highly genetically distinct, no individual mountain or group of neighboring mountains stands out as exceptional across all of the species investigated. Rather, and echoing the conclusions of the studies into EAM taxa reported above, we find strongest support for the hypothesis that, across the species that we investigate, most populations have suffered long-term isolation, such that populations are highly divergent and show little evidence of recent gene movement between mountains.

IMPLICATIONS FOR RESEARCH AND CONSERVATION PRIORITIES.—Recent analysis has shown a likely bias in the identification of regional patterns of biodiversity and consequent conservation prioritization within the EAM due to unequally distributed funding for biodiversity research (Ahrends *et al.* 2011). At the intraspecific level, our study demonstrates high uniqueness of most populations across all of the mountains and species assessed. This pattern highlights that the distribution of biodiversity across the region is likely to be more complex than the simple assumptions used to drive recent research. Therefore, conservation funding priorities should be based on a more even geographical distribution of studies, including those conducted at the intraspecific level and across diverse taxa, if they are to be more effective in understanding and conserving the biodiversity of this highly diverse region.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

TABLE S1. *Approximate locations of sampling regions for trees included in this study according to Eastern Arc Mountain block.*

FIGURE S1. The Eastern Arc Mountains in relation to neighboring highland areas. Sampling locations are marked with circles.

FIGURE S2. Maximum likelihood phylogenies of *M. capensis*, *N. buchannii*, *O. usambarensis*, *X. monospora*, and *Z. gillettii*.

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