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# Evolutionary relationships, species delimitation and biogeography of Eastern Afromontane horned chameleons (Chamaeleonidae: *Trioceros*)

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ABSTRACT

The Eastern Afromontane Region (EAR) contains numerous endemic species, yet its reptile diversity remains relatively poorly understood. We used molecular data to examine species diversity of the Sub-Saharan chameleon genus *Trioceros*. In particular, we focus on establishing species boundaries for taxa with disjunct distributions across the fragmented mountains of the EAR, including *T. affinis*, *T. balebicornutus*, *T. deremensis*, *T. harennae*, *T. tempeli* and *T. werneri*. We applied three species-delimiting approaches, General Mixed Yule-Coalescent (GMYC), a Bayesian implementation of the GMYC, and Bayes Factor Delimitation to estimate species diversity. Using a dated phylogeny, we also examined spatial and temporal diversification patterns in *Trioceros*. We found strong congruence between different species delimitation approaches, with all methods suggesting that species diversity is currently underestimated. In particular, *T. werneri* consists of at least four candidate species (i.e. species awaiting description) with some mountain ranges (Uluguru and Udzungwa) having potentially more than one species. Most interspecific divergences between extant *Trioceros* lineages are estimated to be >5 Mya, consistent with a Pliocene origin of the endemic montane fauna, as exhibited in other taxonomic groups. Multiple, overlapping geographic events (climate and/or geomorphological changes) might account for speciation patterns in *Trioceros* given the dating results.

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## 1. Introduction

A number of areas in Sub-Saharan Africa are notable for high species richness and endemism (Mittermeier et al., 2004). One such area is the Eastern Afromontane Region (EAR), which contains numerous endemic species (Burgess et al., 2004), exemplified by many vertebrate groups (Dinesen et al., 2001; Burgess et al., 2007; Davenport et al., 2013). Despite the recognized importance of the biodiversity of the EAR, this region remains poorly studied for various taxa, and new species are frequently documented (Burgess et al., 2007), including the discovery of a new genus of primate as recently as 2005 (Jones et al., 2005; Davenport et al., 2006). A sharp increase in nominal species reflects the general underestimation of biodiversity in this region, advanced recently by more thorough geographic sampling and new methods for delimiting species (e.g. Demos et al., 2014; Dimitrov et al., 2012; Huhndorf et al., 2007; Loader et al., in press; Mlambo et al., 2014; Voje et al., 2009). DNA-based approaches have revealed the presence of many 'cryptic' species overlooked by morphological estimates (e.g. Gehring et al., 2012). However, the appropriate use of such species-delimiting methods is currently debated (e.g. Ceccarelli et al., 2012; Monaghan et al., 2009; Vieites et al., 2009) and determining species diversity in biodiverse areas, including the EAR, is still relatively incomplete across most groups of organisms.

DNA-based species delimitation can be carried out using several methods, most of which require a phylogeny of the taxonomic group in question. For example the General Mixed Yule-Coalescent (GMYC) method identifies the point of transition between a coalescent and a speciation branching pattern on an ultrametric phylogeny (Pons et al., 2006). Although this method has proven useful for

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rapid biodiversity assessments in mega-diverse groups (e.g. Monaghan et al., 2009), it has been found in certain cases to be highly dependent upon the tree-building method and parameters used (e.g. Ceccarelli et al., 2012). Because the GMYC method also relies only on a single consensus tree, it is more prone to phylogenetic error and it has been found to return dissimilar estimates of species numbers compared to other methods in some cases (e.g. Miralles and Vences, 2013). To counter this problem, a Bayesian implementation of the GMYC was developed (bGMYC; Ried and Carstens, 2012) that samples over the posterior of the output trees. Another alternative to GMYC based species delimitation approaches is the Bayes Factor species Delimitation method (BFD, Grummer et al., 2014). BFD analysis relies on the coalescent species tree algorithm (for a review see Degnan and Rosenberg, 2009) to test different hypothesis of "species groups" defined by the user (i.e. different *BEAST runs, each with individuals placed in alternative groupings). For each potential "species group" hypothesis, the marginal likelihood estimates (MLE) are compared by path sampling (PS; Lartillot and Philippe, 2006) and stepping-stone sampling (SS; Xie et al., 2011) analyses. The appropriate use or not of different species delimitation methods (GYMC, bGYMC, and BFD) is still debated, and thus researchers are currently being encouraged to use several different methods and compare consistencies between various approaches (Carstens et al., 2013; Miralles and Vences, 2013; Satler et al., 2013).

The squamate reptile family Chamaeleonidae comprises 200 named species found primarily across the African continent, Madagascar and other Indian Ocean islands, and some parts of Eurasia (Tilbury, 2010; Tolley and Menegon, 2013). As with many other reptile groups, chameleons are incompletely understood, primarily due to lack of baseline field survey information and taxonomic work (Böhm et al., 2013) but progress is being made (e.g. Branch et al., 2014; Fisseha et al., 2013; Gehring et al., 2012; Glaw et al., 2012; Tilbury et al., 2006; Tilbury and Tolley, 2009a; Tolley et al., 2006; Townsend et al., 2009). These contributions have resulted in a sharp increase in the number of recognized chameleon species, with 51 described in the last two decades. This contrasts with the previous 250+ years of taxonomic work on chameleons, beginning with Linnaeus (1758), during which the rate of species discovery averaged approximately six per decade (Tolley and Herrel, 2013b).

The horned chameleons (*Trioceros* Swainson, 1839) of Afrotemperate forest (*sensu* Poynton, 2013) and high altitude heath and grasslands in central and east Africa, are currently the most speciose chameleon genus (40 species, ~20% of all chameleons). Recognition of this diversity is due, in part, to the application of molecular systematic methods that provided evidence to elevate *Trioceros* from a subgenus of *Chamaeleo* Linnaeus, 1758 (Tilbury and Tolley, 2009b). Molecular data have also provided evidence for the discovery of new species of *Trioceros* (Krause and Böhme, 2010; Stipala et al., 2011, 2012; Tolley and Herrel, 2013a). Notwithstanding some progress in alpha diversity, *Trioceros* remains one of the least understood chameleon genera, with a paucity of basic natural history information. This includes a general lack of good distribution information and limited understanding of variation within and among species that often confounds identifications and challenges taxonomic stability (Tilbury, 2010; Tolley and Herrel, 2013b).

Because *Trioceros* forms a considerable component of chameleon diversity, a better understanding of its systematics and biogeography would be a substantial advancement for African reptile biology. Although recent phylogenies provide a broad overview of species level relationships within *Trioceros* for most of the known taxa (Tilbury and Tolley, 2009b; Tolley et al., 2013), geographic sampling for species – including species that occur across multiple mountain blocks – has thus far been limited (but see Branch et al., 2014). It is well established that montane regions in Africa are important centers of diversity (e.g. Burgess et al., 2007; Plumptre et al., 2007) and current estimates of species richness possibly underestimate the diversity within *Trioceros*.

Here we use molecular methods to assess the diversity within selected species of *Trioceros*, expanding on geographic and taxonomic sampling with regards to previous studies, with a particular focus on multiple populations of species in the EAR. This includes *T. deremensis, T. tempeli,* and *T. werneri* from the Eastern Arc Mountains (EAM) in Tanzania, which are restricted to high altitude forest or grasslands, with multiple populations of each species effectively isolated on distinct mountain blocks currently separated by lowland savannah. Because of their distribution and isolation, there is the potential that some of these species are actually complexes of species. In addition, three species from Ethiopia, *T. affinis*, *T. balebicornutus* and *T. harennae*, were targeted, because *T. affinis* also consists of multiple populations isolated in high-altitude forest patches, while *T. balebicornutus* and *T. harennae* show more restricted distributions. We predict that the nominal species with disjunct populations might be complexes with cryptic diversity, and that their lineage divergences will reflect the dynamic history of forest origin and/or fragmentation in the region since the Oligocene (Couvreur et al., 2008). We tested these propositions by generating new molecular data to estimate a dated phylogeny for ~75% of the nominal species of *Trioceros*, applied different species delimitation approaches, and reconstructed ancestral areas.

## 2. Materials and methods

### 2.1. Phylogenetic reconstructions and divergence time estimates

For reconstructing phylogenies and estimating divergence times of *Trioceros*, a molecular dataset was assembled with DNA sequence data from individuals from West, Central and East Africa (see Fig. 1). The dataset consisted of both published and new sequences (16S rRNA (16S): 18/69 new sequences; NADH dehydrogenase subunit 4 (ND4): 27/58 new sequences; recombination activating gene fragment 1 (RAG1): 16/60 new sequences) for 25 species in the genus *Trioceros* and 6 species of the genus *Kinyongia* as outgroup taxa. An additional 22 individuals of the five target taxa (*T. affinis*, *T. balebicornutus*, *T. deremensis*, *T. harennae*, *T. tempeli*, *T. werneri*) were also included for a final dataset of 73 individuals (Appendix A, Table S1). Two additional markers (all newly sequenced: 12S rRNA (12S) and cytochrome oxidase I (COI)) were sequenced to obtain greater resolution for the target taxa. All tissue samples from the new individuals (thigh muscle and/or liver) were preserved in 96–99% ethanol. Extraction, amplification and sequencing for new material followed standard protocols for amplification and sequencing (Loader et al., 2004; Tilbury and Tolley, 2009b). The following primer pairs were used for amplification: 12S: L1091 and H1478 (Kocher et al., 1989); 16S: 16S-L2510 and 16S-H3080 (Palumbi, 1996); ND4: ND4and $tRNA^{Leu}$ (Raxworthy et al., 2002); COI: RepCOI-F and RepCOI-R (Nagy et al., 2012); RAG1: multiple combinations of primer pairs (see Tolley et al., 2013). PCR products were sequenced using the forward and reverse primers by the Sanger DNA sequencing service of Microsynth AG, Balgach, Switzerland. The complementary sequences were assembled and edited with CodonCode Aligner 4. Sequences were aligned using MUSCLE (Edgar, 2004) in Geneious Pro 5.5.4 (http://www.geneious.com/) with default settings. Alignment ambiguities and gaps (including 12S and 16S stem-loop regions) were excluded from phylogenetic analyses using GBLOCKS version 0.91b (Castresana, 2000). Codon positions for protein coding genes were determined using TranslatorX (Abascal et al., 2010) and sites resulting from heterozygous RAG1 loci were coded using ambiguity codes. In total, 478, 712, 691, 445 and 821 base-pairs

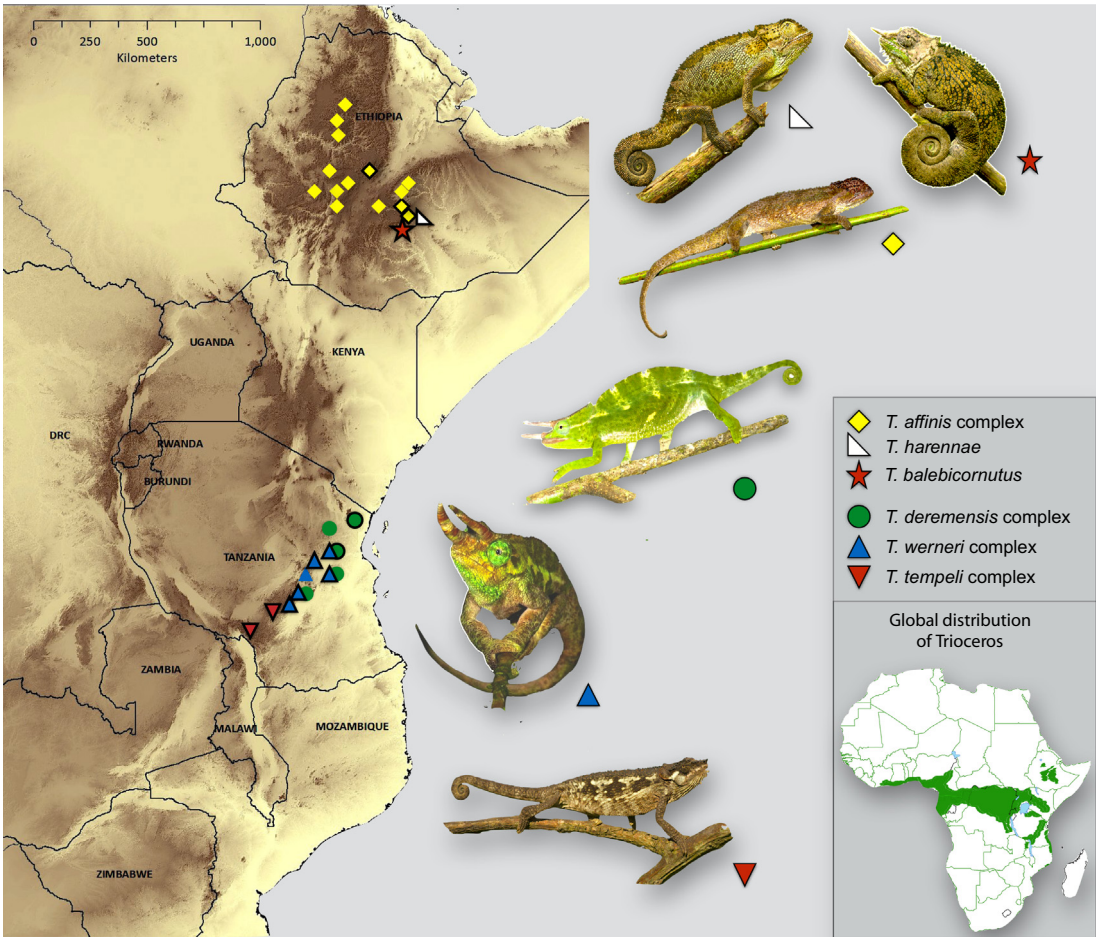


**Fig. 1.** Map of the Eastern Afromontane region with *Trioceros* sample localities. The main map with symbols shows the collection localities of the focal taxa for this study, while the smaller map of Africa shows the general distribution of all *Trioceros* samples included here. Black edged symbols represent samples in our study, whereas symbols with no distinctive edge represent populations not sampled in our study.

were used for the combined analyses of 16S, ND4, COI, 12S and RAG1, respectively (i.e. 2326 base-pairs of mitochondrial and 821 of the nuclear marker). Details of which sequences were incorporated from previous studies (Kosuch et al., 1999; Tilbury et al., 2006; Tilbury and Tolley, 2009b; Tolley et al., 2011, 2013; Townsend et al., 2011), and those generated *de novo* for this study can be found in Table S1 of Appendix A. All new sequences have been deposited in GenBank.

To understand the phylogenetic placement of chameleons from isolated montane forests, a phylogenetic analysis was conducted using both a Bayesian and likelihood framework. The dataset was first partitioned according to marker, and the best-fit model of nucleotide substitution for each marker was identified using the Akaike information criterion (AIC; Akaike, 1973) implemented in jModeltest 2.1.3 (Darriba et al., 2012; Appendix A, Table S2). Two datasets were each analyzed using: three markers (ND4, 16S and RAG1) which were available for all individuals, followed by the addition of two more markers (12S and COI) which were only available for the new material.

The datasets were analyzed with maximum likelihood (ML), and Bayesian inference (BI) using the CIPRES Science Gateway V. 3.3. (Miller et al., 2010). The ML analysis were conducted in RAxML version 7.0.4 (Stamatakis, 2006) using the rapid hill climbing algorithm and the GTRGAMMA substitution model (Stamatakis et al., 2007). The BI was carried out with MrBayes version 3.2.1 (Ronquist et al., 2012) implementing parallel runs of four simultaneous Markov chains for 10 million generations, sampling every 1000 generations and using the default parameters. The partitioning scheme used for the analyses involved applying separate models to each of the codon positions of COI and ND4, while RAG1 was analyzed without partitioning the codon positions (for details see Appendix A, Tables S2 and S3). For both ML and BI analyses, model parameters were independently optimized for each partition. The first one million generations were discarded as burn-in, based on stationarity of the log-likelihood tree scores, and whether the effective sample size of all parameters were >200, using Tracer v.1.5 (Rambaut and Drummond, 2007). Node support was evaluated by non-parametric bootstrapping (Felsenstein, 1985) with 1000 replicates performed with RAxML (ML), and by posterior probabilities (BI).

A dated phylogeny was constructed for the *Trioceros* in a Bayesian framework using the program BEAST v. 1.8.0 (Drummond et al., 2012) with a Markov chain Monte Carlo (MCMC) simulation for 100 million generations, sampling trees every 5000 generations. Two separate runs were carried out and the last 10,000 trees from each run combined after establishing in Tracer v. 1.5 that the runs had stabilized at similar likelihood values (correct "mixing" of chains). The dataset was partitioned per marker and substitution models were unlinked, applying the appropriate model (as selected by jModeltest 2.1.3) to each partition. Clock models were also unlinked, applying an uncorrelated lognormal relaxed clock prior to each partition. In the absence of reliable clock rate data for

our gene fragments, we used the default settings for the mean clock rate with a lognormal distribution, allowing for auto-optimization as the runs progressed. Tree models on the other hand were linked and a birth–death tree prior was applied. For node calibrations, a secondary calibration point on the node of the most recent common ancestor of *Trioceros* (Tolley et al., 2013) was used, applying a normal distribution with a mean age of 36.13 Million years ago (Mya; s.d. = 3.0 Mya), to obtain a curve that closely mirrored the 95% HPD confidence intervals of Tolley et al. (2013) for that particular node. TreeAnnotator v.1.7.5 (Drummond et al., 2012) was used to choose the maximum clade credibility tree with the "mean node heights" option from the 20,000 output trees from the two BEAST runs. Output parameters were examined in Tracer v. 1.5 to determine whether the effective sample size of the parameters was >200.

### 2.2. Species delimitation

To establish species boundaries within the genus *Trioceros*, particularly for the four target species, a General Mixed Yule-Coalescent (GMYC) model (Pons et al., 2006) was implemented in the R v. 3.0.2 (R core team, 2013) package "splits" v. 1.0–19 (Ezard et al., 2009). Both single and multiple rate GMYC models were implemented for the dated phylogeny obtained from BEAST, as well as the Bayesian phylogeny converted to an ultrametric tree using r8s v. 1.71 (Sanderson, 2003). In addition, a Bayesian implementation of the GMYC model ("bGMYC" package v. 1.0.2 for R, Ried and Carstens, 2012) was applied to a random sample of 100 of the last 500 trees from the two BEAST runs, setting the Markov Chain Monte Carlo simulation at 50,000 generations with a burn-in at 40,000, sampling every 100th generation. The default priors for the Yule and coalescent rate change parameters were used, whereas the upper bound of the threshold parameter was set to 67 (number of tips in our trees). The scripts for both the GMYC and the bGMYC can be found in Appendix A, Scripts S1. Additionally, the net evolutionary divergence between *Trioceros* species, or postulated species complexes, were estimated for each marker separately in MEGA v. 6.0.5 (Tamura et al., 2013).

Bayes Factor species Delimitation (BFD; Grummer et al., 2014) was used to compare four alternative scenarios for the *T. werneri* species complex: (1) six candidate species (matching the GMYC outcome, see Results); (2) five candidate species, where the two individuals from Uluguru were treated as one species; (3) four candidate species, as in scenario 2 but with all individuals from Udzungwa treated as one species, and (4) a single species for *T. werneri*. All four scenarios included the 15 *T. werneri* individuals, plus *T. goetzei*, *T. tempeli*, *T. fuelleborni* and *T. laterispinis* as outgroup taxa. Two alternative scenarios were also tested for *T. affinis*: scenario (1) two candidate species (one from Addis Ababa region and the other with individuals from the Bale Mountains) and (2) all individuals considered one species. Likewise, two scenarios were tested for *T. deremensis* and *T. tempeli*: (1) two candidate species (one from East Usambara and the other from Nguru for *T. deremensis*, and Udzungwa and the other from Southern Highlands (Njombe) for *T. tempeli*) versus (2) one species for both *T. deremensis* and *T. tempeli*. These scenarios were tested using the coalescent species tree algorithm in *BEAST (Heled and Drummond, 2010) adding a script for estimating the marginal likelihood (MLE) by path-sampling (PS; Lartillot and Philippe, 2006) and stepping-stone sampling (SS; Xie et al., 2011). PS and SS can be used as a means of comparing the MLEs of the runs, also taking into account the importance of proper priors (Baele et al., 2012, 2013). Each of the coalescent species tree analyses were run twice, using the dataset consisting of 16S, ND4 and RAG1. Molecular clock rates, tree priors and MCMC settings were as for the initial BEAST analysis, while nucleotide substitution models were simplified to HKY for all partitions to avoid over-parametrisation. Bayes Factors (2*ln*Bf) were estimated from the MLE to compare species group scenarios and to choose the most likely scenario (Kass and Raftery, 1995; 2*ln*Bf = 0–2 "not worth more than a bare mention", 2*ln*Bf = 2–6 "positive" support, 2*ln*Bf = 6–10 "strong" support and 2*ln*Bf > 10 "decisive" support in distinguishing between competing hypotheses).

### 2.3. Biogeographic history

To estimate the ancestral areas of *Trioceros* species, each species was coded as belonging to one of the following 8 areas in Africa: A = Eastern Arc Mountains, B = northern Tanzania/western Kenya, C = Ethiopia, D = northern Albertine Rift, E = Congo basin, F = southern Tanzania/Malawi, G = southern Malawi/Mozambique, H = central/West Africa. These areas were chosen based on a combination of geological features (e.g. major mountain chains and large basins) and current distributions of the focal taxa. This allowed for a dataset with a fairly large-scale geographic resolution that was suitable to the data resolution. Three reconstruction approaches were used: Bayesian binary MCMC (BBM; (Yu et al., 2013), S-DIVA (Yu et al., 2010) based on the original formulation of Ronquist, 1997), and Dispersal-Extinction-Cladogenesis (DEC; Ree et al., 2005; Ree and Smith, 2008). BBM and S-DIVA can be run in the program RASP version 2.1b (Nylander et al., 2008; Yu et al., 2013), the former implementing source code modified from MrBayes 3.2.1 (Ronquist and Huelsenbeck, 2003) and the latter source code from DIVA 1.2 (Ronquist, 2001). Both methods allow for inferences to be made regarding the most likely ancestral area, as well as identification of nodes where vicariance, dispersal or extinction events are likely. In the case of DEC, the analysis infers ancestral areas at nodes, as well as allowing the detection of two anagenetic (internodal) events: dispersal (or range expansion) and extinction (or range contraction). As fro cladogenetic events, DEC detects alloperipatric speciation (similar to classical vicariance, but with the daughter areas of uneven size), lineage duplication (or within-area diversification) and peripatric speciation (for a review see Ronquist and Sanmartin, 2011).

Both the Bayesian and S-DIVA algorithms were run in the program RASP version 2.1b, with the consensus trees from the MrBayes and the BEAST analyses as input trees for the S-DIVA analyses and the last 1000 trees from the aforementioned programs as input for the BBM algorithm. For both these analyses the maximum number of ancestral areas was set to 3. Bayesian binary MCMC was set to 100,000 cycles, 10 chains, estimated state frequencies, gamma among-site rate variation and a widespread root distribution, whereas default settings were used for the S-DIVA analysis. The DEC analysis was carried out using the program Lagrange v. 20130526 (Ree and Smith, 2008) on the time calibrated tree obtained from BEAST, using the web-based configurator (http://www.reelab.net/lagrange/configurator/index). Four scenarios were examined, in which the dispersal probability between areas was altered (Appendix A, Table S4). For the scenarios, the probability of dispersal between areas was lowered with increasing geographic distance (e.g. Loader et al., in press), and these probabilities were also adjusted according to time-dependent dispersal constraints based on periods of aridification and geological activity (e.g. Couvreur et al., 2008), which would have presumably reduced the probability of dispersal (Appendix A, Table S4).

Because the phylogenetic analysis (see Section 3) indicated several distinct clades within *T. werneri* that correspond with mountain ranges, a separate DEC analysis was carried for this species, coding the areas according to mountain range (Appendix A, Table S5). As in the full analysis, combinations of time/distance dispersal constraints were enforced, resulting in four different scenarios (Appendix A, Table S5).

## 3. Results

### 3.1. Species delimitation and divergence

The Bayesian and ML analyses yielded essentially the same topologies with similar support, regardless of whether three or five markers were used (Appendix A, Figs. S2–S4). The topology is also in agreement with previously published higher-level phylogenies that include *Trioceros* (e.g. Tilbury and Tolley, 2009b; Tolley et al., 2013). In general, most west and central African species are sister to those from eastern Africa, although the placement of species from Ethiopia (*T. affinis*, *T. balebicornutus* and *T. harennae*) is not resolved, while the clade consisting of these three species is well-supported (MrBayes and BEAST; see Fig. 2 and Appendix A, Fig. S2). The results of the GMYC species delimitation did not differ substantially based on whether the BEAST or MrBayes phylogeny was used (see Table 1), and the bGMYC, GMYC and BFD results are comparable (see Fig. 2, Appendix A and Appendix B). *Trioceros affinis*, which is widespread in the Ethiopian highlands, formed a well-supported sister group to *T. balebicornutus* and *T. harennae* (both from the Bale Mountains). Within *T. balebicornutus* and *T. harennae* there was little divergence, consistent with uninterrupted gene flow between sampled populations.

*Trioceros affinis* showed some degree of geographic structure across the Rift Valley. Representatives of the western (Addis Ababa) and eastern (Bale Mountains) populations are considerably different (ca. 5% sequence divergence ND4; Appendix C). This is supported by the results of both the GMYC and to some extent the bGMYC analyses (Fig. 2, Appendix B) that indicate a branching rate shift consistent with species-level variation. For the BFD analysis, the highest marginal likelihood values, both by path-sampling and stepping-stone sampling, suggest that *T. affinis* comprises two species-level units (Appendix A, Table S6-a). In the case of *T. deremensis* the GMYC analyses do not support species-level variation between the East Usambara and Nguru populations, while the bGMYC analyses neither support nor reject this split. The preferred hypothesis using BFD includes two species-level units for *T. deremensis* (Appendix A, Table S6-b). For *T. tempeli* the results of both the GMYC and bGMYC analyses identify, albeit with low support, potential species-level divergence between the Udzungwa and Njombe (Southern Highland) populations. BFD neither supported nor rejected two species of *T. tempeli,* as BFD using PS favoured two and SS one species, yet neither hypothesis received strong support over the other (PS: 2*ln*BF = 0.52; SS: 2*ln*BF = 0.58; Appendix A, Table S6-c). Potentially the presence of only two gene partitions for one sample (Njombe) might account for this ambiguous result.

In the case of *T. werneri*, the phylogeny and the associated GMYC and bGMYC analyses identifies six monophyletic units – one for each of the Nguru and Ukaguru samples (both well-supported; $p > 0.95$) and two for each of the Uluguru and Udzungwa samples, the latter two each being sister-group pairs (support values for both GMYC and bGMYC $0.5 > p > 0.95$). Estimated divergence between the two Udzungwa clades is only ca. 1.8 Mya and examination of sequence differences (uncorrected net *p*-distances) are 1.7% for ND4 and 0.7% for 16S (Appendix C), which is more similar to values found between populations within chameleon species, rather than between species (e.g. Tilbury and Tolley, 2009b; Tolley et al., 2011). The two candidate species from the Uluguru Mountains are more distinct and estimated to have diverged ca. 3.6 Mya. Sequence differences are, however, at the lower end (3.2% for ND4 and 1.1% for 16S) for minima typically encountered at the species level for chameleons (e.g. Tolley et al., 2006, 2011). Regardless, species boundaries inferred by sequence divergences should be viewed with caution, as the values are not unanimously accepted as evidence for delimiting species, but are only a guideline. In the case of the BFD analyses for *T. werneri*, the hypothesis including six species units received stronger support than that of four or one (PS: 2*ln*Bf = 21.76 and 2*ln*Bf = 113.78, respectively; SS: 2*ln*Bf = 18.96 and 2*ln*Bf = 111.38, respectively). There is some support (2*ln*Bf = 1.58) for a five-species solution for *T. werneri*, with two Udzungwa and one Uluguru unit (Appendix A, Table S6-d).

Estimated times of species divergences across *Trioceros* show late Miocene/early Pliocene divergence among populations in fragmented mountain regions. *Trioceros werneri* from the Uluguru Mountains are sister to the remainder of the *T. werneri* clades, having diverged approximately 9.5 Mya, followed by *T. werneri* from Nguru (ca. 8.4 Mya) and Ukaguru/Udzungwa (ca. 6 Mya). The *T. deremensis* samples from Nguru and East Usambara are estimated to have diverged approximately 1.3 Mya, and populations of *T. tempeli* from Udzungwa and Njome, *T. tempeli* diverged approximately 4 Mya. There appears to be at least two divergent lineages (divergence dated at approximately 4 Mya) within *T. affinis*. The two clades, both in the Ethiopian highlands, are separated by the Great Rift Valley. More recent splits are estimated within *T. balebicornutus* (ca. 0.36 Mya) and *T. harennae* (ca. 0.33 Mya) (Appendix A, Table S7).

### 3.2. Biogeographic history

Because approximately 25% of *Trioceros* species were unavailable for this study, and therefore not included in the phylogeny, inferences regarding the biogeographic history of the genus are tentative. Based on the species included in the ancestral area reconstruction using Bayesian binary MCMC analyses, no ancestral area for the basal split within *Trioceros* could be inferred with any degree of certainty (area H: ~30%). Using S-DIVA, highest support was received for the ancestral area being either a combination of the Congo basin and central/West Africa (areas E and H: 50%), or for central/West Africa only (area H: 50%). The DEC analyses also favored a widespread ancestral area for *Trioceros* spanning the EAM, the northern Albertine Rift (NAR) and the Congo basin (areas A, D and E, respectively) but the relative probability for this is low (0.37). Overall, the results are equivocal because of low probabilities, the large multiple-area combinations inferred to be ancestral ranges, and conflicting results from the three analyses (Fig. 3; Appendix A, Table S8 & Figs. S5–S8). However, all of the analyses indicated that the ancestral area for *Trioceros* was unlikely to have been within the southernmost geographic regions (e.g. Southern Highlands of Tanzania/Malawi or Mozambique). Given that the ancestral area reconstructions are ambiguous, no inferences regarding dispersal/vicariance events can be made for the most recent common ancestor (mrca) of *Trioceros*. However, the two most deeply nested clades in our phylogenies – one of which includes *T. werneri* – show current distributions either within northern Tanzania/western Kenya (B) and the northern Albertine Rift (D), or within the Eastern Arc Mountains (A) and southern Tanzania/Malawi (F) and appear to have diversified mainly during the late Miocene after divergence from their mrca during the Oligocene–Miocene boundary (Fig. 3). This evidence suggests that some event during that time separated the two clades, promoting *in situ* diversification. However, until we are able to reconstruct a phylogeny that includes comprehensive taxon sampling, interpretations are speculative. The DEC analyses focusing on only *T. werneri* with areas divided into EAM mountain blocks suggest a widespread ancestral area, across all four mountain blocks (Udzungwa, Uluguru, Ukaguru and Nguru, see Fig. 3). The results indicate with high probability (0.98–1.0) that the four clades of *T. werneri* currently found in each mountain block are a result of alloperipatric speciation (or vicariance; Appendix A, Table S9).

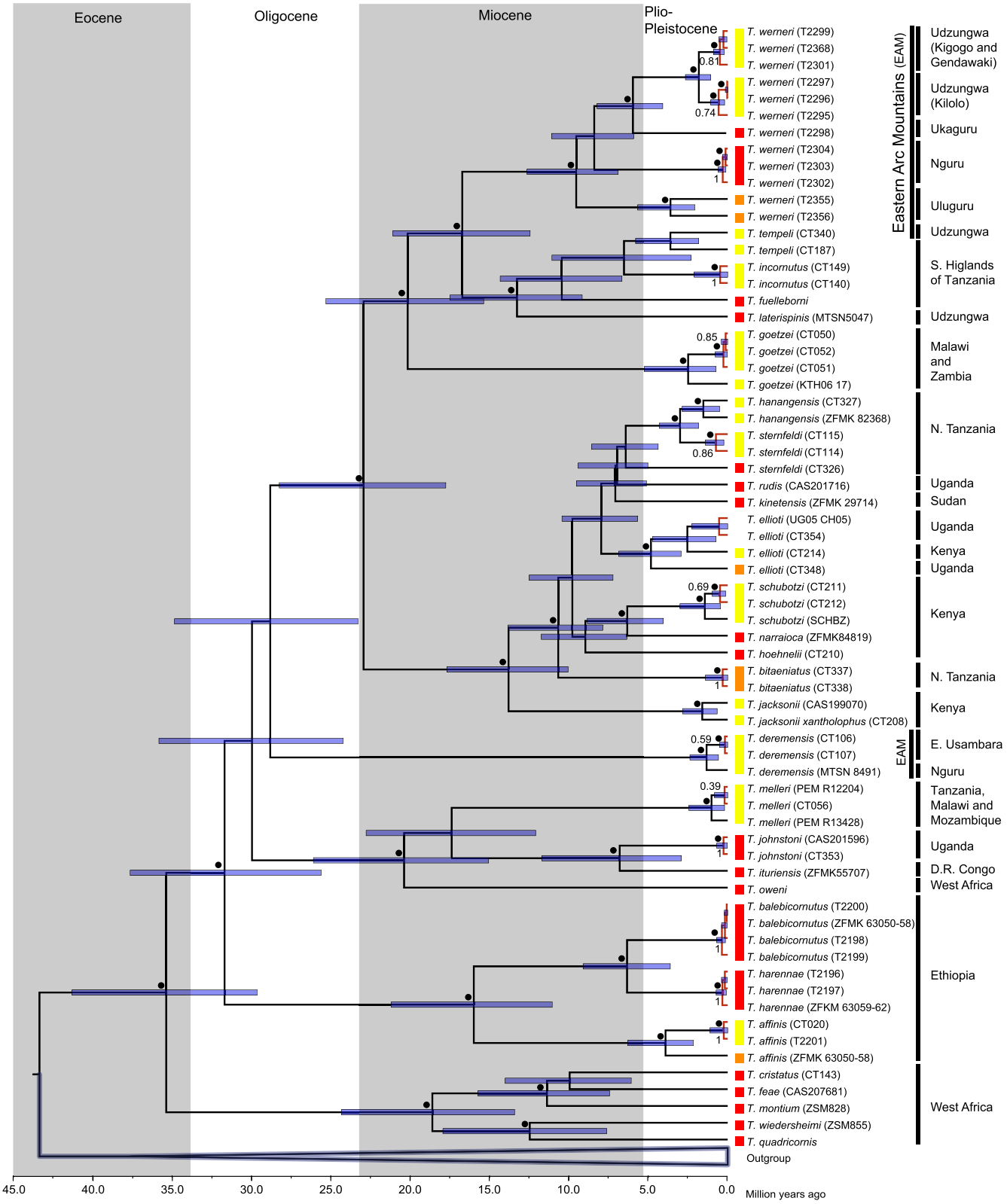


**Fig. 2.** Time-calibrated phylogeny of *Trioceros*. Blue bars at nodes represent the 95% confidence intervals of the dates, while black circles above/below branches represent Bayesian posterior probability values >0.95. Terminal branches colored red represent maximum likelihood clusters (i.e. individuals belonging to the same species) as delimited by the single-threshold GMYC algorithm, with support values indicated by the numbers next to the clusters. Vertical bars to the left of the names represent "species" delimited by the bGMYC algorithm, color-coded according to the probability values obtained (yellow = $0.5 < p \leqslant 0.9$, orange = $0.9 < p \leqslant 0.95$, red = $0.95 < p \leqslant 1$). Vertical bars to the right of the names indicate the distribution areas, in most cases given by the country name, in the case of central and West African countries given as "West Africa" and for specimens from the Eastern Arc Mountains given as "EAM", or in the case of *T. werneri* and *T. deremensis* given in more detail as the mountain block (and locality for Udzungwa). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

**Table 1**
Summary of results obtained from GMYC species delimitations of *Trioceros* phylogenetic reconstructions by MCMC in MrBayes (+r8s) and BEAST.

| | M | T | NC | NE | $L_0$ | $L_{GMYC}$ | LR |
|---|---|---|---|---|---|---|---|
| BEAST | s | −0.742 | 15 (14–17) | 45 (36–47) | 22.99 | 38.85 | 31.72* |
| | m | n.a. | 18 (15–18) | 43 (32–43) | 22.99 | 39.21 | 32.43* |
| MrBayes + r8s | s | −0.028 | 13 (8–17) | 47 (35–54) | 249.52 | 261.75 | 24.47* |
| | m | n.a. | 19 (11–20) | 40 (13–48) | 249.52 | 263.57 | 28.11* |

M = method (s = single, m = multiple); T = threshold time from the branch tips where the coalescent-speciation transition occurred; NC = number of clusters (GMYC "species" with more than one individual) with confidence interval in brackets; NE = number of Maximum Likelihood entities (GMYC "species") discriminated with confidence intervals in brackets; $L_0$ = likelihood of null model; $L_{GMYC}$ = likelihood of GMYC model; LR = likelihood ratio.
* $p < 0.001$ for LR.

## 4. Discussion

### 4.1. Taxonomy and species relationships

The estimated phylogeny suggests some novel taxonomic findings within *Trioceros*. The sampling of *T. werneri* from across its range shows that this nominal species consists of multiple species-level taxa, forming a species complex. Each of the main mountain blocks of its distribution contains a well-supported lineage, with the earliest divergence among them occurring in the late Miocene. The species delimitation analyses all suggest that *T. werneri* may consist of six candidate species, despite being found in only four main mountain chains, although some support values are low. The Ukaguru and Nguru mountains each contain a distinct clade, diverging ca. 6–8 Mya, while the Udzungwa and Uluguru mountains each contain two clades that were also identified as potential species by the species delimitation algorithms. Given that the clades from the Udzungwa and Uluguru mountains are not highly divergent, particularly for the Udzungwa populations, it would be precautionary to rather treat them as distinct populations of a single species until additional samples can be obtained. Analyses of molecular data have already helped to identify 'cryptic' species of other vertebrates inhabiting EAM mountain blocks (e.g. Bowie and Fjeldså, 2005; Bowie et al., 2009). Currently there is a poor understanding of morphological variation across all *T. werneri* populations, and this precludes any assessment of whether these 'cryptic' species are phenotypically diagnosable. However, characters including relative length of the horns, shape and size of dorsal crests, relative size of flank tubercles might prove to be useful in diagnosing these units (M. Menegon, pers. obs.). Future studies will address intra- and inter-specific morphological variation in *T. werneri*.

*Trioceros deremensis* and *T. tempeli* both show more recent divergences (ca. 1.3–4 Mya) between clades from mountain ranges in the north (East Usambara and Nguru Mountains, respectively) and south (Udzungwa and Southern Highlands, respectively) compared to those in *T. werneri*. Overall, the results show clear geographic structuring of populations in *Trioceros* (*T. deremensis*, *T. tempeli* and *T. werneri*) from the Eastern Arc Mountains, also evident in other montane species previously examined including reptiles (Tolley et al., 2011), amphibians (e.g. Loader et al., 2011, in press), plants (e.g. Dimitrov et al., 2012), insects (e.g. Voje et al., 2009; Mlambo et al., 2014) and rodents (Huhndorf et al., 2007; Demos et al., 2014). Moreover, the two specimens of *T. werneri* from the Uluguru Mountains were found in forest and in open grassland, within a few kilometers of each other, yet our results allude to near species-level molecular divergence. However, the geographic distance between them is not sufficient to explain an isolating mechanism. Rather, locally adapted populations may be isolated due to habitat preferences. Although ecological speciation in chameleons has been demonstrated only in the Southern African *Bradypodion* to date (daSilva et al., 2014a,b; Potgieter, 2013; Tolley et al., 2008), the results found here suggest a similar process might be occurring within the *T. werneri* from Uluguru. In general, the high numbers of species from various groups restricted to one EAR mountain, support the idea that the EAR is an exceptionally biodiverse region as a consequence of its geo-climatic history (e.g. Fjeldsa and Lovett, 1997; Loader et al., in press).

*Trioceros affinis* is found on both sides of the Great Rift Valley, which is thought to be a strong biogeographic barrier for highland species (Evans et al., 2011; Kebede et al., 2007; Kingdon, 1989; Wüster et al., 2007). Although our sampling of *T. affinis* was sparse, divergence between the lineages sampled is not particularly deep when compared to other *Trioceros* species. *Trioceros affinis* are not strongly associated with forest or highland habitats (Largen and Spawls, 2010) and this might explain the lack of strong geographic structuring. The remaining two focal taxa, *T. balebicornutus* and *T. harennae* did not show any evidence of cryptic species, although this is not unexpected because they are not particularly widespread or fragmented in their distribution. Both are found only in the Bale Mountains of Ethiopia and do not extend into the highlands north of the Great Rift as does *T. affinis* (though see Necas, 2004 for potential variation in *T. harennae*).

The congruence of species delimitation estimates from GMYC, bGMYC and BFD approaches in *Trioceros* is interesting to note, because there has been controversy surrounding the GMYC species delimitation method (e.g. Carstens et al., 2013). For example, Miralles and Vences (2013) found that GMYC over-estimated the number of species when compared with other evidence (e.g. morphology, pairwise DNA differences), returning false positives for candidate species in chameleons, while other studies have found GMYC to be useful in cases where the taxa present few to no discernable morphological features that would allow species boundaries to be established (e.g. Martinez-Aquino et al., 2013). In cases where only mtDNA is used, factors such as the mean population size of the species affect the species delimitation outcome (Esselstyn et al., 2012; Fujisawa and Barraclough, 2013). Although we are unable to assess all lines of evidence for *Trioceros* (e.g. morphology), support from BFD, bGMYC and pairwise sequence divergences indicates the GMYC approach for *Trioceros* to be consistent, especially for *T. affinis*, *T. deremensis* and *T. werneri*. Potentially, the inferred candidate species for *T. werneri* from Uluguru and Udzungwa might represent over-estimations, but due to our limited sampling we cannot make any conclusive statements regarding this. Future work will need to clarify these aspects, and application of additional methodologies would be useful to determine the robustness of these results.

### 4.2. Biogeographic history

The dynamics of EAR geomorphology have been invoked as causal factors in the divergence and allopatric speciation of some taxa (e.g. Goodier et al., 2011; Nicolas et al., 2008; Schwarzer et al., 2012), but their impact is difficult to evaluate for *Trioceros* given the ambiguities of the ancestral area reconstruction. Furthermore, linking cladogenetic events to geomorphological changes is

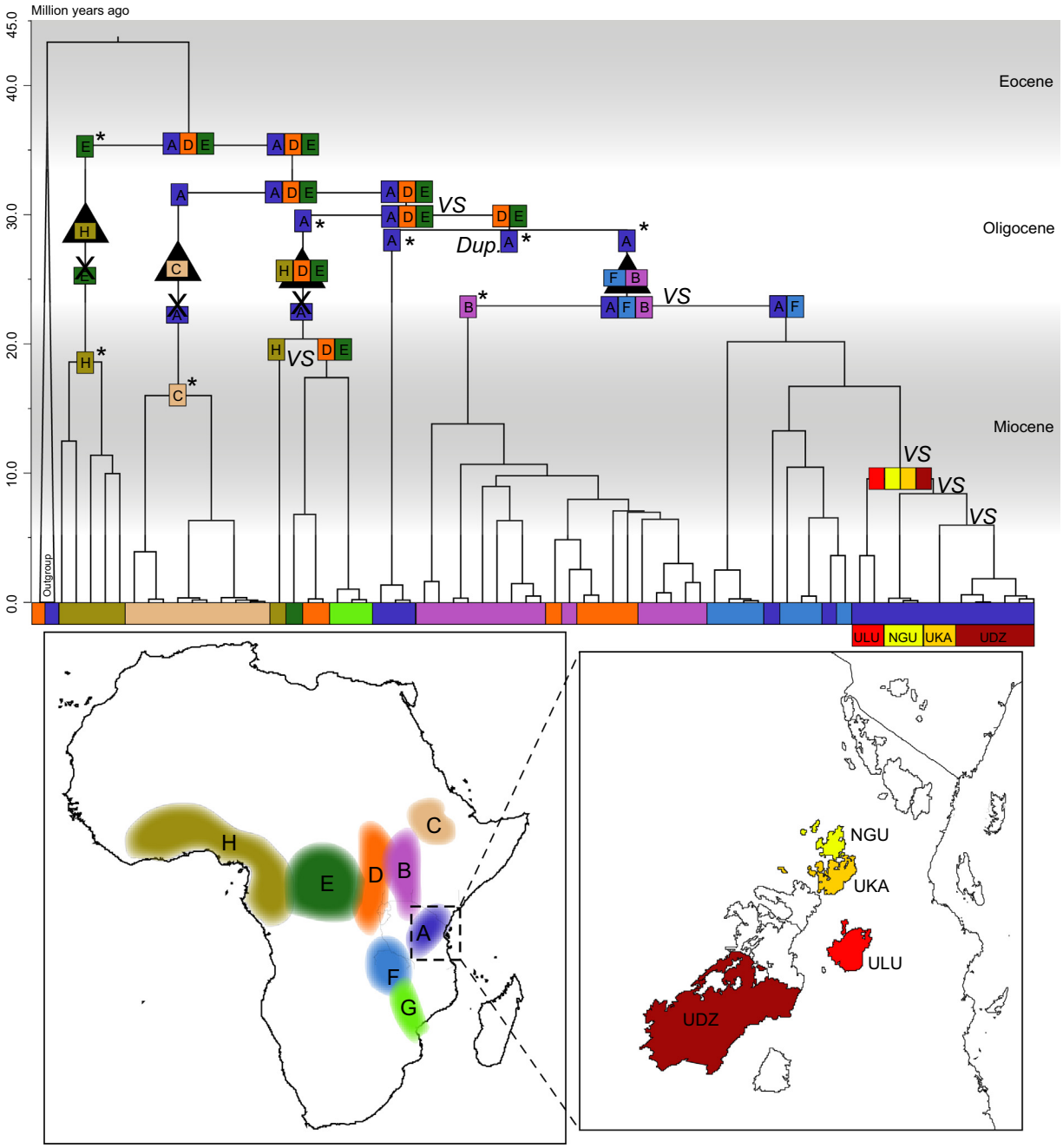


**Fig. 3.** Time-calibrated tree of *Trioceros* and map showing coded areas for ancestral range estimation. Bottom left map indicates large scale geographic areas, color- and letter-coded (A = Eastern Arc Mountains, B = northern Tanzania/western Kenya, C = Ethiopia, D = northern Albertine Rift, E = Congo basin, F = southern Tanzania/Malawi, G = southern Malawi/Mozambique, H = central/West Africa); map on the bottom right shows the Eastern Arc Mountain blocks used in the fine scale analyses for *T. werneri* (NGU = Nguru, UDZ = Udzungwa, UKA = Ukaguru, ULU = Uluguru). Colored squares at each node represent ancestral areas recovered by the DEC analyses. Squares at nodes represent the areas of that particular mrca, with left/right splitting of descendant lineages into areas shown at the ends of horizontal lines passing through nodes. Based on the pattern of area splitting, the major inferred cladogenetic event is also shown, represented by the italicized capital letters (*Dup.* = lineage duplication, *VS* = vicariant speciation, or alloperipatric speciation). Anagenetic events are represented on the branches: black triangles represent dispersal (or range expansion, to the areas indicated inside the triangle) and crosses represent extinction (or range contraction, in the areas shown under the cross). Asterisks (*) indicate areas that received a combined relative probability > 0.7. Map of the EAM modified from Platts et al. (2011). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

problematic when there are frequent, spatiotemporally overlapping geological events, as is the case for many parts of the Eastern Afromontane region. Block faulting (e.g. Uluguru Mountains) may have been initiated several hundred million years ago, whereas other areas (e.g. Usambara, North and South Pare) are thought to have uplifted in the Neogene (Griffiths, 1993). The Eastern Arc Mountains have been impacted by recent orogenic events and volcanism, with the present topographic surface most likely formed at the start of the Pliocene (Griffiths, 1993). These geological changes, coupled with large fluctuations in climate – both global (Cane and Molnar, 2001) and localized (e.g. Hamilton, 1982) – would have impacted the extent of forest and savannah habitats in the EAR and consequently the distribution of species. Linking geographic events to speciation processes in this case is therefore problematic.

Within the Eastern Arc, *T. dermensis*, *T. tempeli*, and *T. werneri*, show diversification events among areas (mostly mountain blocks)

that are between 1.3 and 9, but generally approximately 5 Mya. These results are similar to the patterns shown among other vertebrate groups, which show substantial genetic differences, even among populations on different mountains that are geographically relatively close (e.g. Blackburn and Measey, 2009; Gravlund, 2002; Loader et al., 2011, in press; Tolley et al., 2011). Our results provide further support for the idea that although recent climatic events might have connected forest between many parts of the Eastern Arc (Hamilton, 1982), montane forest species were less frequently able to disperse across such barriers in recent times and remained mainly isolated. Whether the spatial isolation and divergence of these populations was caused by any particular environmental event is difficult to evaluate because both uplift of the Eastern Arc Mountains (Griffiths, 1993) and substantial climatic changes seemed to have occurred between 1.3 and 9 Mya.

Within the *T. werneri* species complex, the dated phylogeny indicates that the population from the Uluguru Mountains diverged from the most recent common ancestor (mrca) of *T. werneri* earlier than populations from the other mountain blocks studied here (Udzungwa, Ukaguru and Nguru), approximately 9.5 Mya (Appendix A Table S6), suggesting a relatively old mrca for the *T. werneri* species complex. At present, the Uluguru Mountains are separated from the other EAM blocks by a comparatively large expanse of savannah, presumably forming a formidable barrier for these high altitude forest-living chameleons, which has perhaps remained in place since the late Miocene. This pattern is repeated for a number of other small, non-volant forest dependent taxa, whereby the Uluguru lineages diverged earlier than related lineages on nearby mountain ranges (e.g. Lindqvist and Albert, 2001; Loader et al., 2011, in press; Stanley and Olson, 2005), including other chameleons (*Kinyongia* and *Rhampholeon*). For example, for *Kinyongia*, the Uluguru lineage within the *K. oxyrhina* species complex diverged from the more northern lineages (e.g. Nguru) approximately 10 Mya (Tolley et al., 2011). *Rhampholeon uluguruensis*, also from Uluguru, appears to have diverged from its closest living relative in the same time frame (Matthee et al., 2004), lending support to the concept that isolation of the montane forests promoted allopatric speciation in forest and/or high altitude dependent taxa here (e.g. Fisseha et al., 2013; Tolley et al., 2011, 2013). Our ancestral area reconstructions for lineages within *T. werneri* suggests successive vicariant events splitting populations across the mountain fragments of the EAM, which further fits the scenario proposed for *Rhampholeon*.

The divergence between *T. affinis* and *T. balebicornutus* + *T. harennae* (ca. 16 Mya) occurred during a prolonged period in the Miocene when the Ethiopian dome was fractured by the Great Rift Valley (Chorowicz, 2005; Corti, 2009; Ebinger et al., 2000; Woldegabriel et al., 1990; Wolfenden et al., 2004). Potentially the speciation event between these clades might correspond with this prolonged splitting event but given the trans-Rift distribution of this clade, the precise events that correlate with (and might have caused) their current distribution are unclear – with one species currently having a trans-Rift distribution (*T. affinis*) and the others currently isolated in the East (*T. balebicornutus* + *T. harennae*). The east–west separation within the trans-Rift species *T. affinis* might have been initiated by the final phase of east–west rifting, which is thought to have ended ca. 3 Mya (Corti, 2009) but this requires further examination beyond the preliminary analyses outlined here. However, the relatively recent (i.e. towards the end of the rifting) divergence of *T. affinis* across the Rift found here may not be surprising, given the relatively widespread distribution of *T. affinis* and its tolerance of non-forest habitats (Largen and Spawls, 2010, p. 256). Whether this recent split within *T. affinis* across the Rift Valley reflects a more common pattern is not yet clear but other similar studies support the final phase of rifting as corresponding to splitting trans-Rift species into east and west clades of plants and vertebrates (Evans et al., 2011; Kebede et al., 2007; Kingdon, 1989; Wüster et al., 2007). Future species-level studies for each of our focal taxa separately with denser sampling and phylogeographic analyses may provide more in-depth information about the species and the areas they currently inhabit.

## 5. Conclusions

The results obtained in this study indicate that numerous *Trioceros* species are awaiting description from across the EAR. The results are generally congruent across delimiting approaches (GYMC, bGMYC and BFD) and are also reflected in substantial genetic distances between populations. In particular, *T. werneri* appears to be a complex of four to six species, some of which are isolated on single mountain ranges. Although there is some structuring in the other EAR species (*T. deremensis*, *T. tempeli*), the present sampling makes it difficult to determine whether they represent species complexes. The Ethiopian species, *T. balebicornutus* and *T. harennae*, show only population level divergence, although *T. affinis* showed species level structure despite the sparse sampling. The evidence for a number of candidate species in *Trioceros* further highlights the importance of the EAR as a region rich in endemic reptiles. Species delimitation approaches are a valuable tool in making first estimates of species diversity across an area, directing future taxonomic research. The strongest candidates for new species are found on single mountain blocks (either within the Eastern Arc Mountains or the highlands associated with the Great Rift in Ethiopia). Speciation processes and ancestral area reconstructions are difficult to identify in *Trioceros* but the inferred relationships among units within *T. werneri* are consistent with a process of successive vicariant events across the central and southern Eastern Arc region. Potentially, because of their inferred age, these vicariant events might have been caused by the separation of ancestral areas due to geological, climatic, or a combination of these events. Trans-Rift populations of the Ethiopian species *T. affinis* diverged during the final phase of rifting the Great Rift Valley, which is potentially a causal mechanism.

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## Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2014.07.023.

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