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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.

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