Genetic differentiation and species status of the large-bodied leaf-tailed geckos
Uroplatus fimbriatus and U. giganteus

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Abstract. The taxonomy of the Malagasy leaf-tailed geckos Uroplatus fimbriatus and U. giganteus is in need of revision since a molecular study casted doubt on the species status of U. giganteus from northern Madagascar. In this study we separately analyse DNA sequences of a mitochondrial gene (12S rRNA) and of four nuclear genes (CMOS, KIAA1239, RAG1, SACS), to test for concordant differentiation in these independent markers. In addition to the molecular data we provide a comprehensive review of colour variation of U. fimbriatus and U. giganteus populations from the entire distribution area based on photographs. The molecular evidence clearly supports a two-species taxonomy, with U. fimbriatus corresponding to a southern clade and U. giganteus to a northern clade. This conclusion relies on the high mitochondrial divergence among these units, and especially on the full concordance of the mitochondrial signal with differentiation in two of the four nuclear genes, which show no haplotype sharing among the northern and southern clade. This suggests limited or absent gene flow among these units, even in areas where they occur in rather close proximity to one another. The only consistent difference in colour variation between the two species is the whitish iris with rather indistinct brown vertical lines in U. giganteus, versus a yellowish iris with more distinct, continuous lines of typically a more reddish brown tone in U. fimbriatus. In the U. giganteus population of Montagne d’Ambre, a particular colour pattern on the head prevails which is not present in all the specimens studied from Marojejy. The Marojejy population of U. giganteus is also genetically distinct, as is one sample from Ankavanana included in a previous study, requiring in-depth future study.

Key words. Squamata, Gekkonidae, Uroplatus fimbriatus species complex, taxonomy, phylogeny, Madagascar, colour variation, biogeography.

Introduction

The genus Uroplatus is a remarkable group of nocturnal geckos endemic to the forests of Madagascar and currently includes 17 formally recognized species plus several candidate species (Ratsoavina et al. 2017). The largest members of this genus are included in the Uroplatus fimbriatus species group which comprises U. fimbriatus, U. giganteus, U. sikorae, U. sameiti, U. henkelii and one candidate species that is morphologically close to U. henkelii and has been referred to as U. henkelii [Ca11] (Böhme & Ibisch 1990, Ratsoavina et al. 2013). Uroplatus fimbriatus is one of the largest representatives of these geckos and is one of the most famous reptile species from Madagascar. It was already mentioned in the literature of the 17th century (Flacourt 1658) and was formally described by Schneider in 1792 (for a detailed discussion see Glaw et al. 2006 and Ratsoavina et al. 2013) with the imprecise type locality “Madagascar” (Angel 1929). Motivated by the lack of precision of the type locality and some major problems with the holotype, Bauer & Russell (1989) designated as neotype the specimen ZFMK 36503 from Nosy Mangabe, and thereby restricted the type locality to this tiny offshore island in the Bay of Antongil in the North East of Madagascar.
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Figure 1. Map of Madagascar and molecular differentiation between *Uroplatus fimbriatus* (dots) and *U. giganteus* (squares). Locality records of specimens for which locality records have been unambiguously been confirmed with molecular evidence, based on data herein. Colours correspond to those used in the phylogenetic tree (Fig. 1A). Type localities are given with asterisks (*). Underlined are the northernmost (Cap Est without molecular evidence) and southernmost (Kianjavato) locality records for *U. fimbriatus*. Molecular differentiation between *U. fimbriatus* (blue) and *U. giganteus* (orange). (A) Maximum Likelihood tree based on a fragment of the mitochondrial 12S rRNA gene. Values at nodes are bootstrap proportions in percent. (B–E) Haplotype networks of four nuclear genes (RAG1, SACS, KIAA1239, CMOS).
Uroplatus fimbriatus appears to be mainly a species of low-altitude rainforests of eastern Madagascar covering a distribution area roughly from Cap Est in the Masoala Peninsula, southwards at least until Kianjavato (Fig. 1). It is uncertain whether the species occurs further south; reports of this lizard from Vohipeno, Vondrozo and Eminiminy, close to Andohahela National Park in the far South East, are based on unverified historical records (Angel 1942), and intensive recent surveys in the Andohahela and Anosy Massifs and surrounding forests have not yielded any records of U. fimbriatus (Andreone & Randriamahazo 1997, Nussbaum et al. 1999, Ratsaoavina et al. 2013). Uroplatus fimbriatus has a rather unique appearance with lateral dermal fringes along the flanks, arms, legs, and head sides. When not stressed, the body shows dorsally an irregular pattern of grey, brown, black and white, sometimes light green dots, lines, or rather uniform colouration. The iris is characteristically coloured: a yellow background with reddish-brown, largely circular stripes around the pupil.

In 2006, populations of U. fimbriatus from mid-altitude rainforests of Montagne d’Ambre in northern Madagascar were described as a new species, U. giganteus, based on morphology, colouration, hemipenis structure, and a substantial genetic distance to U. fimbriatus (Glaw et al. 2006). Uroplatus giganteus was diagnosed from U. fimbriatus by having a larger size (snout–vent length 182–200 mm vs. 150–189 mm, total length up to 322 mm vs. 295 mm), a white ground colour of the iris with brownish lines around the vertical pupil, and a particular colouration of the head as follows (Glaw et al. 2006): two distinct, chevron-shaped, blackish markings, both pointing posteriorly, one between the eyes and the other in front of the eyes. One, often poorly delimited, blackish spot is generally present between the nostrils and a further one posterior to the nostrils. Furthermore two black spots are present behind the posterior chevron, and the combined appearance often resembles a “sad smiley” symbol (Fig. 2). No dark pigment was noted in the oral mucosa of either species (Glaw et al. 2006). Further characters that might differ between the two species were observed in genital morphology: the terminal elements of the hemipenes in U. giganteus were found to be almost parallel and not pointed, whereas in U. fimbriatus they were converging and pointed (Glaw et al. 2006).

The original description also relied on the differentiation in a fragment of the mitochondrial 16S rRNA gene, with 4.8% uncorrected pairwise p-distance (UPD) between U. giganteus and one sample of U. fimbriatus from the neotype locality Nosy Mangabe. A subsequent molecular study (Greenbaum et al. 2007) supported the recognition of U. giganteus as distinct species. Ratsaoavina et al. (2013), based on sequences from Raxworthy et al. (2008), additionally found 7% UPD in the 12S rRNA gene between a U. fimbriatus sequence from Betampona, versus U. giganteus from the type locality Montagne d’Ambre and from Analalava, Salafaina and Bezavona. In the COB gene, the highest divergence in the U. fimbriatus complex was reported between U. fimbriatus from Andakibe and U. giganteus from Marojejy, with an UPD of 17.6%.

The multigene phylogenetic analysis of Raxworthy et al. (2008), published two years after the original description of U. giganteus, revealed two well supported clades within U. fimbriatus, corresponding to a northern and a southern clade, and easily referable to U. giganteus (northern clade) and U. fimbriatus (southern clade), respectively. However, because the northern clade also included a sample from a site 50 km north of Nosy Mangabe, the neotype locality of U. fimbriatus, these authors speculated that samples from Nosy Mangabe might also fall into the northern clade once included in the analysis; they argued that due to a purported lack of accurate diagnostic morphological characters, and of comprehensive genetic data from Nosy Mangabe, the description of U. giganteus had been premature. Raxworthy et al. (2008), thus, did not accept U. giganteus as a valid species, but also did not place it formally into the synonymy of U. fimbriatus.

Solving the U. fimbriatus / giganteus taxonomic puzzle requires answering three main questions: (1) Does the neotype locality of U. fimbriatus, Nosy Mangabe, fall into the northern clade of Raxworthy et al. (2008)? In this case U. giganteus would likely constitute a synonym of U. fimbriatus. (2) Is the mitochondrial divergence between the northern and southern clade paralleled by concordant divergence in the nuclear genome, which would support a two-species hypothesis? (3) Is the molecular divergence between both lineages paralleled by consistent, diagnos-
tic morphological or colour differences that, again, would support a two-species hypothesis?

In this study, we address these three questions by analysing a comprehensive new molecular dataset of mitochondrial and nuclear DNA sequences of the *U. fimbriatus* complex, and undertake a review of the colour pattern based on all photographic material of the *U. fimbriatus* complex available to us, with a focus on the type localities of *U. fimbriatus* (Nosy Mangabe) and *U. giganteus* (Montagne d’Ambre). Analyses of external morphology or osteology are not undertaken here and will be the subject of future studies.

**Materials and methods**

Rationale for molecular analysis and species delimitation

For molecular genetic analysis, we follow the rationale of previous studies (e.g., Ratsoavina et al. 2011, 2015) in which we specifically searched for concordance between differentiation in mitochondrial and nuclear genes as support for the existence of independent evolutionary lineages with limited gene flow among them. While mtDNA due to its fourfold lower effective population size often shows a strong phylogeographic structure, this can be misleading because (1) it might reflect only a low vagility of females while males might ensure gene flow of nuclear genes among populations, and (2) mtDNA structure can be strongly affected by introgressive hybridization. We therefore analysed nuclear and mitochondrial DNA sequences separately. We chose as mtDNA marker a fragment of 12S rRNA, which allowed the inclusion of previously published sequences (Raxworthy et al. 2008), and complemented it with DNA sequences of four nuclear gene fragments.

DNA sequencing and analysis of sequences

Tissue samples were collected either as tail tips (from specimens subsequently released) or as muscle samples obtained from the femoral region (from preserved voucher specimens). We preserved samples in ethanol (96%) and extracted total DNA following standard salt extraction protocol using proteinase K digestion in a concentration of 10 mg/ml (Bruford et al. 1992). We used polymerase chain reactions (PCRs) to sequence a fragment of mitochondrial 12S ribosomal RNA (12S rRNA) using primers 12SA-L (5’-AAACTGGGATTAGATACCCCATAT-3’) and 12SB-H (5’-GAGGGTGAACGGGCGGTTGT-3’) following protocols of Vences et al. (2003), and fragments of the following four nuclear genes: (1) oocyte maturation factor Mos (CMOS) following protocols of Ratsoavina et al. (2011), (2) the recombination-activating gene 1 (RAG1) using a nested approach as described in Rakotoarison et al. (2015), and (3) leucine-rich repeat and WD repeat-containing protein (KIAA1239) and (4) sacsin (SACS) with primers and a nested PCR approach of Shen et al. (2012).

PCR products were sequenced directly using an automated DNA sequencer (ABI 3130 XL, Applied Biosystems). Quality control of sequences was carried out using CodonCode Aligner (Codon Code Corporation). For sequence alignment, as well as calculation of uncorrected p-distances between sequences, we used MEGA7 (Kumar et al. 2016). Newly determined sequences were deposited in GenBank (accession numbers MG922948-MG922971 and MG925676-MG925780).

We used the Bayesian Information Criterion in jModeltest (Darriba et al. 2012) to determine the best-suited substitution model for the 12S alignment (a HKY+G model), and estimated the mitochondrial phylogeny of the *U. fimbriatus* complex under the Maximum Likelihood (ML) optimality criterion in MEGA7 under this model, assessing node support with 2000 non-parametric bootstrap replicates. Haplotypes of each of the nuclear DNA fragments were inferred using the PHASE algorithm (Stephens et al. 2001) implemented in DnaSP software (Version 5.10.3; Librado & Rozas 2009). For each fragment, we reconstructed a Maximum Likelihood tree with Jukes-Cantor substitution model (chosen to avoid overparameterisation) in MEGA7 (Kumar et al. 2016) and built a haplotype network by entering this tree together with the alignment in the software Haploviever, written by G. B. Ewing (http://www.cibiv.at/~greg/haploviever) which implements the methodological approach of Salzburger et al. (2011).

Analyses of colour variation

To test whether there are chromatic differences between individuals or populations assigned to genealogical groups (identified through phylogeographic analyses), especially between the northern (*U. giganteus*) and southern (*U. fimbriatus*) mitochondrial clade, we compared colour patterns of the body, eye and buccal mucosa based on photographs of specimens from the entire distribution range of the two species, in order to follow an integrative taxonomic approach (Pašič et al. 2010). All photographs had been taken of live geckos in the field during day and/or night in the following localities: Ambodiriana, Ambodi (Anaravana, Cap Est, Fanolamkely, Hiaraka, Tampolo), Marolambo, Marojejy, Montagne d’Ambre, Nosy Boraha, and Nosy Mangabe. These localities comprised intact or slightly to moderately disturbed rainforest and ranged in elevation from about 0–1000 m (Supplementary Materials: Table S1).

**Results**

Molecular analysis and species delimitation

The ML tree based on mitochondrial sequences (12S rRNA) separated all included samples in a northern and a southern clade, supported by bootstrap support values of 79% and 65%. Despite the relatively low support of the
U. fimbriatus individuals from the Masoala Peninsula showed three light white patches of variable shape well delimited by dark lines on the head and shoulders (Fig. 4C). The ventral surface is whitish. The colouration of the buccal mucosa in all U. fimbriatus populations is unpigmented. In some individuals the anterior tip of the tongue is light to dark red, but it is uncertain whether this is a stable individual character and if this colouration is caused by pigmentation or simply perfusion of superficial blood vessels (Fig. 9D).

Photographs from within the range of the northern lineage, and thus assigned to U. giganteus, were available for 13 individuals from two localities: Montagne d’Ambre (3; Fig. 7) and Marojejy (10; Fig. 8) Many of these correspond to specimens assigned to this species also based on molecular data.

The specimens from Montagne d’Ambre exhibited the following dorsal colouration (an overview is given in Fig. 7): The dorsal surface of unstressed specimens is mainly covered with brown, grey, and black, often reminiscent of the bark of trees (Fig. 7A and B). The colouration of stressed individuals is much more contrasting: the brownish and greyish spots mostly have changed to yellow and a black reticulation or marbling covers most dorsal parts of back, tail, head and limbs (Fig. 7C). Most of the photographed individuals agree in having one large beige symmetrical patch in the neck, one at middorsum, and one in the sacral region. These patches can vary from poorly defined (e.g., Fig. 7D) to very distinct (GLaw et al. 2006; Fig. 7B) and their borders can be well delimited by black or poorly recognizable. The light patches are often filled with brownish pattern and their shape is variable. The head colouration of U. giganteus from Montagne d’Ambre is rather characteristic: there are two distinct, chevron-shaped, blackish markings, both pointing posteriorly, one between the eyes and the other in front of the eyes. One, often poorly delimited, blackish spot is generally present between the nostrils and a further one posterior to the nostrils. The area between these spots and the two chevrons is usually beige, brown or yellow. Two black spots are present behind the posterior chevron, and the combined appearance often resembles a “sad smiley” symbol. The ventral surface is whitish. No dark colour was noted in the open mouth, and the anterior tip of the tongue is light red in the photographed individual (Fig. 9C).

Specimens of U. giganteus from Marojejy differ clearly in terms of dorsal colouration from the Montagne d’Ambre individuals (an overview is given in Fig. 8): generally the Marojejy population seems to be much more variable in dorsal colouration and pattern. Some individuals resemble the general pattern as observed in Montagne d’Ambre (Fig. 8E) whereas others have an irregular pattern of indistinct dark vertical, sometimes ruptured lines on greyish-brown ground colouration (e.g., Figs 8A, 8D, 8F). One individual showed several light white patches of variable shape well delimited by dark lines on the head, shoulders, dorsum, feet, and tail (Fig. 8D), very similar to the pattern observed in some U. fimbriatus (Fig. 4C). The head colouration of U. giganteus from Marojejy is rather heterogeneous and chevron-shaped, blackish markings are absent or

Analysis of colour patterns

Photographs from within the range of the southern lineage, and thus assigned to U. fimbriatus, were available for 32 individuals from 11 localities: Ambodiriana, Ambodivoangy (close to Makira), Kianjavato, Mahaso, Masoala Peninsula (Anaravana, Cap Est, Hiaraka [photos examined but not included in the figures], Tampolo), Marolambo, Nosy Boraha, and Nosy Mangabe. Many of these corresponded to the specimens identified via molecular data (i.e., from all localities except Mahaso, Marolambo, Nosy Boraha, and several sites in Masoala). The specimens exhibited the following dorsal colouration (an exemplified overview is given in Figs. 3–6). Dorsal ground colour varies between light beige to dark brown to black mainly covered with brown, grey and black patterns, often reminiscent of the bark of trees. Some specimens show a more distinct pattern with up to three well-delimited light patches on the neck, middorsum and one in the sacral region (e.g., Figs 3B and 4D). Other individuals show only a poorly recognizable pattern of indistinct dark vertical, sometimes ruptured lines on greyish ground colouration (e.g., Figs 3G, 6C). Some individuals show an irregular pattern of fine-grained beige-greenish to brown dots (e.g., Figs 3D, 5D, 5E). One individual from the Masoala Peninsula showed three light white

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Figure 3. Representative specimens and colour variation of \textit{U. fimbriatus} of the Nosy Mangabe population. Picture B shows a subadult specimen whilst all others show adult specimens. Photos D and G. by A. HARTIG; photo E by H.-P. BERGHOFS; other photos by the authors.
not as characteristic as in the Montage d’Ambre population (Fig. 9E). The ventral surface is whitish (Fig. 2). No dark colour of the buccal mucosa was noted; the anterior tip of the tongue is light red in one photographed individual and in another individual the middle part of tongue has a light yellowish colouration (Fig. 9C).

The most important and largely consistent difference between *U. fimbriatus* and *U. giganteus* is the colour of the
Figure 5. Colour variation of *U. fimbriatus* form north-eastern populations. (A–B) adult specimen from Ambodivoangy, Makira; (C) adult female form Ambodiriana; (D) subadult from Ambodivoangy, Makira; (E) specimen from Mahasoa; (F) specimen from the island Nosy Boraha; (G) subadult from Ambodiriana; (H) defense behavior in an adult male from Ambodiriana. All photos by the authors.
iris, as shown in Figure 9A. In *U. giganteus* it is whitish to beige, with brownish lines running vertically along the vertical pupil. These lines typically are chocolate brown without a distinct reddish-brown tone, thin and poorly defined, i.e., often interrupted and connected with each other in an irregular network pattern. In *U. fimbriatus*, the iris has a more beige-yellowish to olive greenish ground colour, and the vertical lines typically have a reddish-brown tone. Furthermore, these lines are more distinct, i.e., at least 2–3 of them are relatively wide and continuous; in some specimens, the pattern of wide and continuous vertical lines is so dense that it covers most of the iris surface.

Additional photographs of *U. fimbriatus* and *U. giganteus* which in general comply with the pattern described below can be found in the publications of Love (2001a, b), Svatek & van Duin (2002) and Schönecker (2008) as well as on the internet.

Figure 6. Colour variation of *U. fimbriatus* from central-eastern populations. (A) adult specimen from Marolambo; (B) specimen from Kianjavato; (C) male and female from Marolambo. All photos by the authors.
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**Discussion**

Taken together, the molecular evidence supports a two-species taxonomy, with *U. fimbriatus* corresponding to the southern and *U. giganteus* to the northern mitochondrial clade. This conclusion relies on the high mitochondrial divergence among these units, and especially on the full concordance of the mitochondrial signal with that found in

Figure 7. Colour variation of *U. giganteus* from the Montagne d’Ambre population. Photo A by A. Hartig; other photos by the authors.
Figure 8. Colour variation of *U. giganteus* from the Marojejy mountain massif in north-eastern Madagascar. Note the white spots on the head, shoulders, arms and flanks of the specimen D. Photos by the authors except D by H. P. BERGHE.
Figure 9. Variation in iris and buccal mucosa pigmentation of *U. fimbriatus* and *U. giganteus*. Row (A) Iris colouration in *U. giganteus* (from left to right): Marojejy and Montagne d’Ambre (last). Row (B) Iris colouration in *U. fimbriatus* (from left to right): Masoala, Nosy Mangabe, Nosy Mangabe, Amboriana, Marolambo, and Ambodiriana. Row (C) Colouration of the buccal mucosa in *U. giganteus* (from left to right): Marojejy and Montagne d’Ambre (last). Row (D) Colouration of the buccal mucosa in *U. fimbriatus* (from left to right): Amboriana, Ambodiriana, Marolambo, Nosy Mangabe, Masoala. Row (E) Detailed photographs of the head colouration of *U. giganteus* from Marojejy.
two out of four nuclear genes (i.e., no RAG1 and KIAA1239 haplotype sharing among specimens of either mtDNA clade). This suggests limited or absent gene flow among these units, even in areas where they occur in rather close proximity to one another, and thus provides a rationale for species delimitation under the genealogical concordance criterion (Avise & Ball 1990).

However, this taxonomic separation is less obvious than in many other Uroplatus taxa. As previously known from Uroplatus (e.g., Rats oavina et al. 2012), the samples analysed showed a high mitochondrial variability. Distance values of inter- and intra-species comparisons overlapped due to the high differences among locations within each clade. The geographic location of the basal split between the northern (U. giganteus) and southern (U. fimbriatus) clade along the northern boundaries of the Masoala Peninsula is apparently paralleled by a similar split into a northern and southern clade within Uroplatus lineatus (Fig. 3 in Raxworthy et al. 2008) and in accordance with an identified area of high species turnover in reptiles by Brown et al. (2016). This also holds true for other geckos, such as the Phelsuma pusilla complex: in these small-bodied day geckos, Gehring et al. (2012) identified at least three independent mitochondrial clades in Madagascar's northeastern coastal lowlands.

No obvious geographical signal was apparent in the mitochondrial or nuclear gene trees of U. fimbriatus, suggesting a relatively recent expansion or ongoing gene flow. In contrast, U. giganteus showed a clear differentiation of samples from the Marojejy Massif to those from the northernmost localities Forêt d’Ambre and Montagne d’Ambre, consistently among mitochondrial and nuclear genes. Whether this might suggest the existence of taxonomically separate units requires additional study. This genetic differentiation may help to explain the pattern of geographic variation in colouration within U. giganteus.

It is known that natural selection for crypsis plays an important role in the evolution of colour variation. As in other lizard groups, body colour pattern has been shown to have an adaptive basis (e.g., other lizard groups, body colour pattern has been shown important role in the evolution of colour variation. As in differentiation may help to explain the pattern of geographic separate units requires additional study. This genetic differ...
individuals to species based on colour patterns, especially of the iris. Nevertheless, due to the uncertain taxonomic status of the Marojejy population of *U. giganteus* further research into the taxonomy, populations and geographical range is needed to be able to conserve and manage these unique geckos more effectively.

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Supplementary material

Supplementary Table S1. Locality name, GPS coordinates, and field number of U. fimbriatus and U. giganteus individuals sequenced or mentioned in this study.

Supplementary Figure S1. Unrooted Maximum-Likelihood tree based on sequences of the RAG1 gene.

Supplementary Figure S2. Unrooted Maximum-Likelihood tree based on sequences of the KIAA1239 gene.

Supplementary Figure S3. Unrooted Maximum-Likelihood tree based on sequences of the SACS gene.

Supplementary Figure S4. Unrooted Maximum-Likelihood tree based on sequences of the CMOS gene.