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Molecular phylogeny and geographic variation of Malagasy iguanas (*Oplurus* and *Chalarodon*)

Tobias Münchenberg[1], Katharina C. Wollenberg[1], Frank Glaw[2], Miguel Vences[1,*]

Abstract. The iguanid subfamily Oplurinae consists of seven species of small to medium-sized, arboreal, sand-dwelling or rock-dwelling lizards endemic to Madagascar and the Comoros, belonging to the genera *Oplurus* and *Chalarodon*. We here present the first complete molecular species-level phylogeny for Oplurinae based on DNA sequences (865 bp) of the mitochondrial 16S rRNA gene and the nuclear *c-mos* gene. Our study is based on 52 specimens sampled from different populations in Madagascar and includes the geographically isolated population from Grande Comore, hitherto considered as subspecies *Oplurus cuvieri comorensis* or even as a separate species *O. comorensis*. Our results confirm that, within the genus *Oplurus*, the largely arboreal *O. cuvieri* and *O. cyclurus* form the sister clade to the remaining, more rock-dwelling species. Within the latter lineage, *Oplurus quadrimaculatus* is placed most basal, *O. fierinensis* and *O. grandidieri* are closely related sister species with high support, and *O. saxicola* is sister to the *fierinensis/grandidieri* lineage. Within the arboreal *Oplurus*, the Comoran sample shows no genetic differentiation relative to *O. cuvieri* populations from the North West and Sambirano regions of Madagascar, indicating that this population should not be considered as a separate species. In the monotypic genus *Chalarodon*, we discovered deep genetic divergences among populations of *C. madagascariensis* indicating the presence of a previously unrecognized cryptic species and the need for taxonomic revision.

Introduction

The lizard family Iguanidae has a disjunct distribution. Most taxa occur in North and South America, a few taxa on the Fidji Islands in the Pacific, and one subfamily, the Oplurinae (Schulte et al., 1998; sometimes considered as separate family Opluridae), is endemic to Madagascar and the Comoros archipelago (Cadle, 2003). Two oplurine genera are distinguished, the monotypic *Chalarodon*, and *Oplurus* with 6 or 7 species. *Chalarodon madagascariensis* is a common inhabitant of sandy soils that can be recognized by its rather small size and the presence of a distinct dorsal crest (Blanc, 1977). Species of the second oplurine genus, *Oplurus*, can be divided into two ecomorphological groups: (1) a rock-dwelling, terrestrial group consisting of *O. fierinensis*, *O. grandidieri*, *O. quadrimaculatus*, and *O. saxicola*, and (2) a more arboreal group, containing *O. cyclurus* and *O. cuvieri*. These arboreal species differ by their larger and more distinctly spiny scales encircling the tail. The population of *Oplurus cuvieri* from Grande Comore on the Comoro archipelago has initially been described as a variant (Angel, 1942) and subsequently been considered as subspecies *O. cuvieri comorensis* (Savage, 1952). Due to its geographical isolation combined with a more rock-dwelling ecology, differences in morphology (e.g. lack of a black band on the neck), and a possibly larger body size, this form has recently been given full species rank, as *Oplurus comorensis* (Meirte, 2004).

The reconstruction of oplurine phylogeny has been the subject of two major studies so far (fig. 1). Blanc et al. (1983) studied morphological (lepidosis, colour) as well as biochemical (lactic dehydrogenase, serum proteins) characters, while the study of Titus and Frost (1996) combined morphological data initially taken from the literature (e.g. Blanc, 1977; Frost, 1992) with DNA sequences (of the mitochondrial 12S rRNA, $tRNA_{Val}$ and 16S rRNA genes, together about 1130 bp; single specimens per species; missing for *O. fierinensis* and *O. grandidieri*).

Here we infer oplurine phylogeny by expanding existing molecular phylogenetic data to

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1 - Technical University of Braunschweig, Zoological Institute, Spielmannstr. 8, 38106 Braunschweig, Germany
2 - Zoologische Staatssammlung München, Münchhausenstr. 21, 81247 München, Germany
*Corresponding author; e-mail: m.vences@tu-bs.de

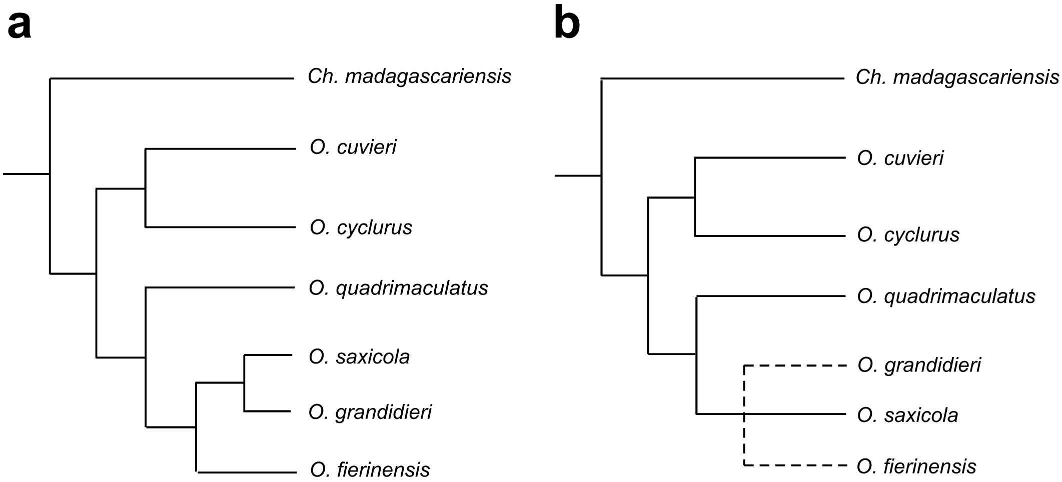

Figure 1. Phylogeny of Oplurinae (*Oplurus* and *Chalarodon*) as inferred by (a) Blanc et al. (1983) based on morphological (lepidosis, colour) as well as biochemical (lactic dehydrogenase, serum proteins) characters, and by (b) Titus and Frost (1996) based on combined and separate parsimony analyses of 34 morphological characters (all species), and molecular genetic data (missing for *O. fierinesis* and *O. grandidieri*; dashed lines).

all oplurine taxa, also including *O. fierinensis*, *O. grandidieri*, and *O. cuvieri comorensis*, using a mitochondrial marker (16S rRNA) combined with a nuclear marker (*c-mos*). For most oplurine species we also include samples from different localities, covering large parts of their distribution ranges, and thereby provide a basis for formulating first phylogeographic hypotheses for these taxa.

Methods

DNA was extracted from 52 muscle tissue samples of *Oplurus* and *Chalarodon* specimens (table 1) using a standard salt-extraction protocol. We amplified a 510 bp-fragment of the mitochondrial 16S rRNA gene using primers 16SAr-L and 16SBr-H (for primer sequences and reaction information see Vences et al., 2003). We furthermore amplified 455 bp of the nuclear *c-mos* gene using the primers CO8 (for) and CO9 (rev) (C08: 5′-GCT TGG TGT TCA ATA GAC TGG-3′, and C09: 5′-TTT GGG AGC ATC CAA AGT CTC-3′; Han et al., 2004). Thermocycling was performed as described in Saint et al. (1998), with an annealing temperature of 48°C. Purified DNA fragments were resolved on automated sequencers, and sequences were submitted to GenBank (Accession numbers EU099653-EU099755; see table 1). The iguanid lizard *Sceloporus grammicus* (GenBank accession numbers L41464 and AF039478; Reeder, 1995; Saint et al., 1998) was used as outgroup. Alignment was carried out using the Clustal algorithm in the software Sequence Navigator (Applied Biosystems) and subsequently refined by eye. The first 100 bp of the 16S rRNA alignment were excluded from further analysis due to different readability of sequences. The *c-mos* alignment contained no indels, while several such insertions and/or deletions were apparent in the 16S rRNA alignment. All indels in the ingroup were concentrated in two hypervariable regions and were usually single and double indels that posed no alignment problems, except for a series of four deletions present in all individuals of *Chalarodon madagascariensis* from Esomony. Initial explorative Neighbor-joining (NJ) analyses of the separate datasets of the two genes were carried out using PAUP*, version 4b10 (Swofford, 2002), under the Kimura-2-parameter substitution model. Explorative phylogenetic analyses after exclusion of all gapped sites did not result in relevant differences in the topology of obtained trees.

To explore if the two analysed gene fragments have a congruent phylogenetic signal we performed partition-homogeneity tests (= ILD tests) as implemented in PAUP*. Because one partition (the *c-mos* fragment) had identical sequences for many of the included specimens, performing this test on the full data set under the most extensive search settings proved not feasible for computational reasons. We therefore carried out (1) a test based on the full data set, but with simple addition sequence, a maximum number of trees to be saved ("Maxtrees" setting in PAUP*) of 100 000, and nearest-neighbor interchange branch swapping, and (2) a test on a subset of 34 taxa including all species and all lineages of divergent haplotypes, with random addition sequence with ten replicates, no Maxtrees limit, and tree-bisection reconnection branch swapping. Both tests did not provide evidence for phylogenetic incongruence of the two gene fragments ($P = 0.82$ and 0.34, respectively). Hence, for final analysis, sequences of the two genes were concatenated.

Table 1. Voucher specimens used in molecular genetic analysis, locality information and GenBank Accession numbers. Acronyms are as follows: FGMV, field numbers of F. Glaw and M. Vences; FGZC, Frank Glaw Zoological Collection; TM, sample numbers of Tobias Münchenberg; UADBA, Université d'Antananarivo, Département de Biologie Animale; ZMA, Zoological Museum Amsterdam; ZSM, Zoologische Staatsammlung München. FGZC and FGMV specimens are to be catalogued in the UADBA and ZSM collections.

Species	Sample	Voucher number	Locality	Genbank Accession 16S	Genbank Accession *c-mos*
Chalarodon madagascariensis					
	TM64	UADBA/FGZC 954	Antsalova	EU099705	EU099654
	TM71	ZSM/FGZC 2330	near Esomony	EU099710	EU099659
	TM72	UADBA/FGZC 2363	near Esomony	EU099711	EU099660
	TM73	UADBA/FGZC 2549	near Esomony	EU099704	EU099653
	TM74	ZSM/FGZC 2550	near Esomony	EU099712	EU099661
	TM75	UADBA/FGZC 335	Betioky	EU099713	EU099662
	TM76	UADBA/FGZC 336	Betioky	EU099714	EU099663
	TM85	UADBA 21059	Analalava/Isalo	EU099706	EU099655
	TM86	FGMV 2002.1551	Toliara	EU099707	EU099656
	TM87	FGMV 2002.2017	Toliara	EU099708	EU099657
	TM88	UADBA 21038	Toliara	EU099709	EU099658
Oplurus cuvieri					
	TM61	UADBA/FGZC 708	Tsingy de Bemaraha	–	EU099676
	TM62	ZSM 86/2006	Tsingy de Bemaraha	EU099730	EU099677
	TM63	UADBA/FGZC 655	Antsalova	EU099729	–
	TM105	FGMV 2002.2248	Maevatanana	EU099728	–
	TM110	UADBA/MV 2001.363	Ankarafantsika	EU099715	EU099664
	TM112	UADBA/MV 2001.324	Ankarafantsika	EU099734	EU099681
	TM113	ZSM 461/2001	Itremo	EU099716	–
	TM114	Specimen not collected	Antsirasira	EU099717	EU099665
	TM115	Specimen not collected	Antsirasira	EU099718	EU099666
	TM116	Specimen not collected	Antsirasira	EU099719	EU099667
	TM137	ZSM 320/2002	Grande Comore	EU099753	EU099701
Oplurus cyclurus					
	TM67	ZSM/FGZC 2570	near Marohotra	EU099733	EU099680
	TM89	ZSM 939/2003	Analalava/Isalo	EU099720	EU099668
	TM90	FGMV 2002.1474	Analalava/Isalo	EU099721	EU099669
	TM91	FGMV 2002.1475	Analalava/Isalo	EU099722	EU099670
	TM92	ZSM 940/2003	Analalava	EU099732	EU099679
	TM93	FGMV 2002.1479	Analalava/Isalo	EU099723	EU099671
	TM94	FGMV 2002.1517	Analalava/Isalo	EU099724	EU099672
	TM96	FGMV 2002.2001	Toliara	EU099725	EU099673
	TM97	FGMV 2002.2002	Toliara	EU099726	EU099674
	TM98	ZSM 952/2003	Toliara	EU099727	EU099675
	TM99	FGMV 2002.2092	Ifaty	EU099748	EU099696
	TM100	FGMV 2002.2093	Ifaty	EU099749	EU099697
	TM101	ZSM 960/2003	Ifaty	EU099750	EU099698
	TM111	ZSM 462/2001	Ankarafantsika	EU099731	EU099678
Oplurus fierinensis					
	TM95	FGMV 2002.1583	near Toliara	EU099747	EU099695
	TM138	UADBA/FGMV 2000.557	Andatabo	EU099754	EU099702
Oplurus grandidieri					
	TM139	ZSM 30/2004	Zazafotsy	EU099755	EU099703

Table 1. (Continued).

Species	Sample	Voucher number	Locality	Genbank Accession 16S	Genbank Accession *c-mos*
Oplurus quadrimaculatus					
	TM65	ZSM/FGZC 2511	Andohahela	EU099739	EU099686
	TM77	UADBA 24768	near Ambositra	EU099742	EU099689
	TM83	UADBA/FGZC 320	Tranoroa	EU099741	EU099688
	TM102	ZMA 19513	Andringitra	EU099751	EU099699
	TM104	FGMV 2002.2103	Andringitra	EU099752	EU099700
	TM106	FGMV 2002.62	Antoetra	EU099736	EU099683
	TM107	FGMV 2002.63	Antoetra	EU099737	EU099684
	TM108	ZSM 220/2002	Tolagnaro	EU099738	EU099685
	TM109	ZSM 617/2000	Toliara	EU099735	EU099682
Oplurus saxicola					
	TM70	UADBA/FGZC 2551	near Esomony	EU099744	EU099692
	TM78	ZSM 33/2004	near Tsihombe	EU099743	EU099691
	TM79	UADBA/FGZC 54	near Tsihombe	EU099745	EU099693
	TM80	ZSM 34/2004	near Tsihombe	EU099746	EU099694
	TM82	ZSM 170/2004	west of Tsihombe	EU099740	EU099687

We constructed phylogenies using Maximum Parsimony (MP), Maximum Likelihood (ML) and Bayesian inference. A heuristic MP search with 500 bootstrap replicates was performed with PAUP*. Previous to ML and Bayesian analyses we estimated the best suited substitution model using the Akaike information criterion as implemented in Modeltest (Posada and Buckley, 2004; Posada and Crandall, 1998). The analysis selected as best fitting the data a GTR + G + I model with a proportion of invariable sites of 0.5793 and a gamma shape parameter of 0.4935. The ML phylogeny was established in PhyML (Guindon and Gascuel, 2003) with 2000 bootstrap replicates. The Bayesian phylogeny was constructed using MrBayes V.3.1.2 (Huelsenbeck and Ronquist, 2001) by running four chains for 5×10^7 generations. We verified that the posterior likelihood values reached stability by visual inspection and stationarity of the PRSF (Potential Scale Reduction Factor). Every 1000th tree was sampled and the first 10 000 generations were discarded.

To represent the obtained strict consensus tree from the MP analysis with branch lengths, we saved this consensus tree in PAUP*, then loaded it again from file and printed it as phylogram with branch lengths. Here we present this MP phylogeny, with MP and ML bootstrap values and Bayesian posterior probability scores (fig. 2).

Results and discussion

The concatenated data set included 865 base pairs (410 for 16S rRNA and 455 for *c-mos*) for 52 specimens of all oplurine taxa. Of these 865 characters, 705 characters were constant (318 for 16S rRNA, 387 for *c-mos*) and 92 were parsimony informative (69 for 16S rRNA, 23 for *c-mos*). Results of the Maximum Parsimony analysis are shown in figure 2. The ML and Bayesian trees (both not shown) agreed with the MP tree (fig. 2), with the exception of identifying *O. cuvieri* as paraphyletic with respect to *O. cyclurus*. However, despite the paraphyletic arrangement of *O. cuvieri* in ML and Bayesian analyses we consider monophyly of this taxon as very probable based on general sequence similarity (reflected by 92% bootstrap support in a Neighbor-joining analysis; results not shown). In general, support values for many nodes were relatively low, especially in ML and Bayesian analyses. This and the paraphyletic arrangement of some otherwise well-defined clades (such as *O. cuvieri* in our case) appears to be a common theme in studies where relatively short DNA sequences of a large number of taxa are analysed, with many similar (conspecific) haplotypes included (e.g., Boumans et al., 2007). Although it probably reflects a general problem of likelihood methods with such data sets, we emphasize that only those phylogenetic relationships here supported by relevant bootstrap values and posterior probabilities should be considered as well assessed while others are pending further studies with longer DNA sequences.

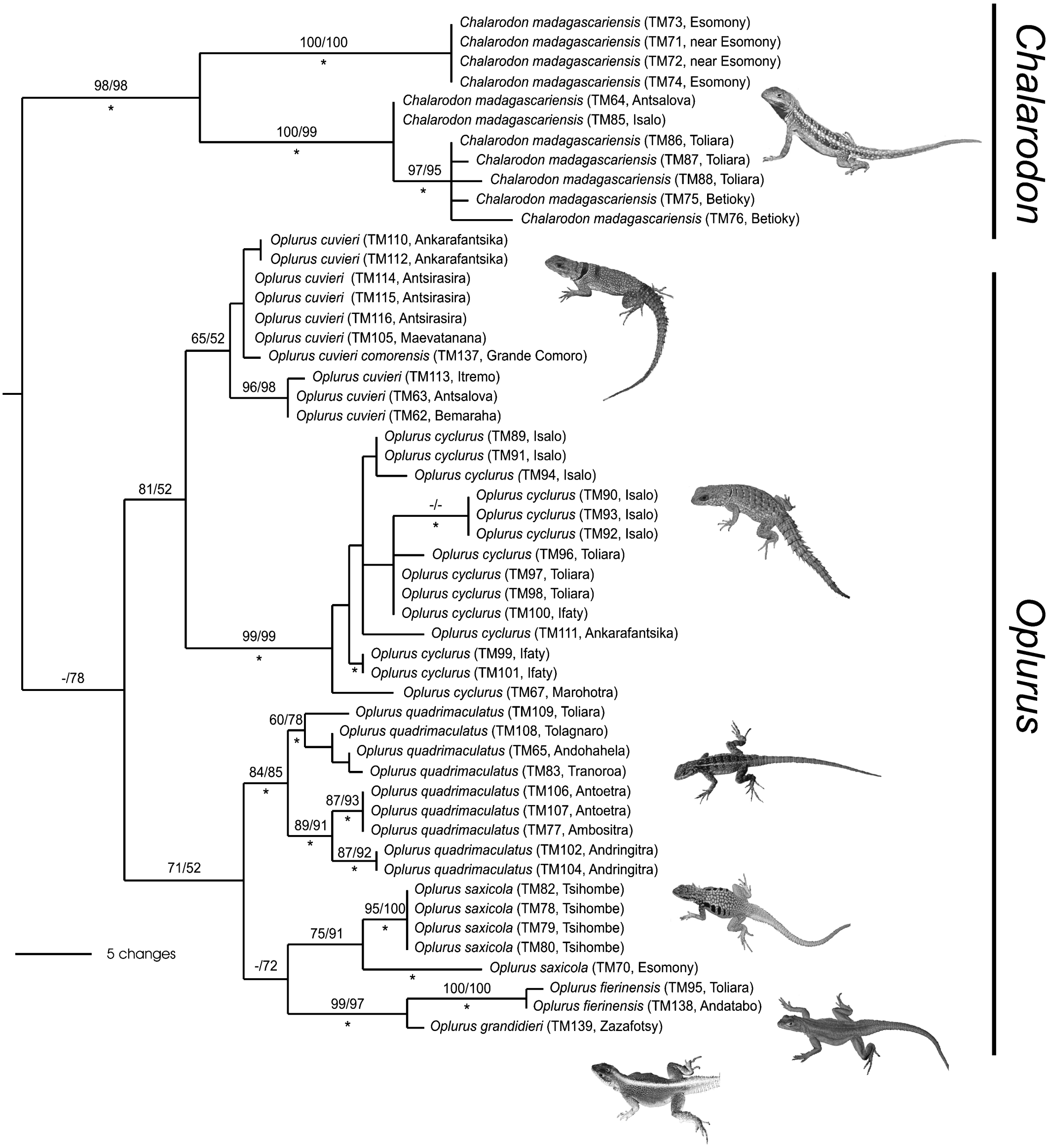

Figure 2. Maximum parsimony tree of Oplurinae based on combined 16S and *c-mos* sequences. The tree is a strict consensus of 3168 equally most parsimonious trees (265 steps), with a consistency index of 0.751 and a retention index of 0.924. Numbers above branches: bootstrap values of MP (500 replicates)/ML (2000 replicates). Dashes indicate bootstrap values < 50% in the respective analysis. Bayesian posterior probabilities (> 0.95) are marked with asterisks below branches.

All analyses supported two major evolutionary lineages corresponding to the genera *Chalarodon* and *Oplurus*. The MP and ML analyses (but not the Bayesian tree) supported two clades within *Oplurus*, corresponding to the more arboreal species (*cuvieri*, *cyclurus*), and to the rock-dwelling species (*fierinensis*, *grandidieri*, *quadrimaculatus*, *saxicola*). This result fully conforms to previous molecular and morphological studies. In the MP tree, all species were monophyletic, largely supporting current classification.

First explorative NJ trees based on the 16S rRNA sequences (not shown) almost fully agreed in topology with the combined analysis. The *c-mos* gene had a much lower variation, and consequently a lower phylogenetic resolution (not shown): the separation between *Chalarodon* and *Oplurus* and the split between the two major *Oplurus* lineages were recovered, but species in the rock-dwelling clade were not resolved.

In the following we discuss a number of aspects of our analysis that were recovered unambiguously by our analyses. Considering the partly low ML and Bayesian support values, we largely limit our interpretations to those aspects where support was high in all cases.

Cryptic species within *Chalarodon madagascariensis*. — The monotypic *Chalarodon* turned out to be composed of two strongly supported and highly divergent subclades, which can be strictly allocated to sample sites (fig. 3): populations of the first clade occur in the plains of South Madagascar (sample sites Toliara and Betioky; see Boumans et al. [2007] for definition of informal biogeographic regions), and in the West and at the border of the Central region (Isalo and Antsalova). Members of the second clade occur in the slightly mountainous eastern parts of the South region (sample site Esomony). The deep genetic divergences between these two lineages were concordantly found in both the 16S rRNA and *c-mos* sequences. In fact, in the *c-mos* data set, the two lineages are more strongly divergent (1.1-1.8% uncorrected pairwise p-distances) than are sister species of *Oplurus* (between 0-0.5%), and even more so than representatives of the arboreal vs. the rock-dwelling clades of *Oplurus* (1.3-1.5%). The 16S rRNA divergences between the two *Chalarodon* lineages are very high as well, amounting to 6.2%. This value is distinctly higher than between other closely related but distinct and well-established species of Malagasy reptiles, such as the different chameleon species of the *Calumma brevicorne* complex (1.0-5.0%), the skinks *Trachylepis elegans* and *T. gravenhorstii* (2.9-5.0%), the pseudoxyrhophiine snakes *Madagascarophis colubrinus* and *M. meridionalis* (2.4-5.0%), and the giant snakes *Acrantophis madagascariensis* and *A. dumerilii* (1.0-1.8%) (Vences and Glaw, 2004; Boumans et al., 2007; Nagy et al., 2007). This suggests the presence of two previously unrecognized cryptic species within what is currently considered as *Chalarodon madagascariensis* and emphasizes the need for a detailed taxonomic revision of the genus *Chalarodon*.

Relationships among rock-dwelling species of *Oplurus*. — Within the ground-dwelling species group, the monophyly of *O. quadrimaculatus* receives support in the combined analysis. Its placement sister to a subclade containing the remaining three species (*O. saxicola*, (*O. fierinensis*, *O. grandidieri*)) is only weakly supported but is in agreement with the studies of Blanc (1977) and of Titus and Frost (1996) where *O. quadrimaculatus* was placed as sister taxon to the other three taxa, the latter being referred to as the "saxicolous group" (fig. 1). The saxicolous group is morphologically and biochemically (allozymes) highly uniform (for details see Titus and Frost, 1996), though its phylogeny could neither be solved through morphological (Blanc et al., 1983; fig. 1a) nor molecular genetic analyses due to missing data (Titus and Frost, 1996; fig. 1b). Blanc (1977) placed *O. grandidieri* and *O. saxicola* as sister species (fig. 1a). In contrast, our analysis reveals a highly supported sister relationship of *O. grandidieri* and *O. fierinensis*, with average genetic distances between these two well established *Oplurus* species being rather low (pairwise uncorrected p-distances 2.0% for 16S rRNA, 0% for *c-mos*).

Differentiation within *Oplurus quadrimaculatus* and *O. saxicola*. — *Oplurus quadrimaculatus* is separated into two well-supported clades, one containing populations from the highlands in the north of its distribution range (Antoetra, Ambositra and Andringitra), and the other one containing the more southern populations (Toliara, Tolagnaro, Andohahela, Tranoroa). In *O. saxicola*, the population from

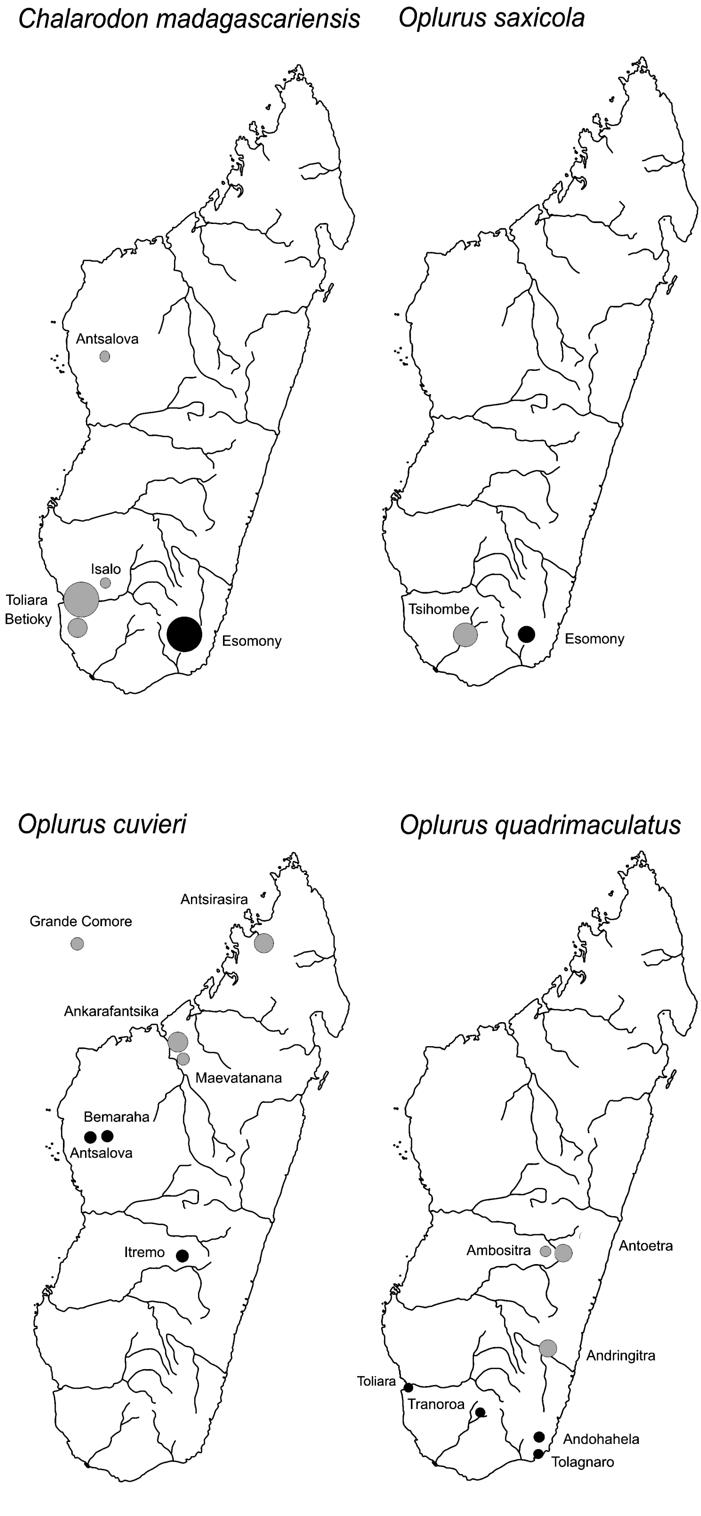

Figure 3. Distribution of major haplotype clades in four species of oplurine iguanas. Black and grey circles symbolize haplotypes belonging to either of two major haplotype clades identified in a species (fig. 2). Size of the circles is representative of the number of sequences determined from one locality.

Esomony (where also the divergent *Chalarodon* clade occurs) is genetically distinct (*c-mos*: 0.4%; 16S rRNA: 2.4-2.8%) from the Tsihombe population. More samples from throughout the range of *O. saxicola* are needed to evaluate this distinction.

Differentiation in the arboreal species and status of *Oplurus cuvieri* from Grande Comore. — Within the arboreal species group, five populations of *Oplurus cyclurus* sampled from throughout its distribution area reveal no geographically concordant genetic sub-structure. For instance, two different haplotype lineages (16S rRNA divergence up to 2.0%) are observed at one locality (Isalo). This indicates probable recurrent gene flow among the populations of this species.

Within *Oplurus cuvieri*, one subclade appears to be distributed in the West and Center (Bemaraha, Antsalova, Itremo) and a second subclade in the North West and Sambirano regions (Maevatanana, Ankarafantsika and Antsirasira). The sample from Grande Comore belongs into the North West subclade, and shows a 16S rRNA divergence of only 0.25% to the other samples from this subclade. This differentiation is remarkably smaller than that between the two Malagasy subclades of *O. cuvieri* (average 16S rRNA distance: 1.48%).

Due to its phylogenetic nestedness within *O. cuvieri* and its very low genetic divergence, we conclude that a species status of the Grande Comore population is not warranted, and its subspecies status is in need of re-evaluation. Several vertebrates of the Comoro islands are known to be introduced by humans, such as the lemurs *Eulemur fulvus* and *E. mongoz* (see Pastorini et al., 2003) and the snake *Leioheterodon madagascariensis* (see Meirte, 1993). However, because the Grande Comore population of *Oplurus cuvieri* appears to be morphologically and ecologically differentiated (Meirte, 1992, 2004), its introduction is unlikely. Furthermore, its distribution seems to be restricted to a few coastal lava habitats in the north of the island which are far away from the harbour of the island capital Moroni. Grande Comore is the youngest of the volcanic Comoro islands, with an approximate age of subaerial emergence between 0.13-0.5 million years (estimates from dating of oldest lavas and calculations of plate motion; Nougier et al., 1986; Louette et al., 2004). Any colonization of this island must therefore be relatively recent as well and *Oplurus* fossils from Aldabra island (Arnold, 1976) demonstrate the general ability of these iguanas for overseas dispersal. We therefore hypothesize that the ancestors of the Grande Comore population of *O. cuvieri* arrived in the Pleistocene via overseas dispersal from the North West or Sambirano regions in Madagascar, in accordance with the probable dispersal route of several other reptile species (Raselimanana and Vences, 2003), and that its morphological differentiation can be explained by fast adaptation to local (insular) environmental condition which is a phenomenon well known in lizards (e.g., Thorpe and Malhotra, 1998). Despite its lack of strong genetic differentiation, the probable natural occurrence of *Oplurus cuvieri* on Grande Comore, and the hypothesized adaptations it has undergone, underline the relevance of conservation of this island population. Factors potentially constituting a severe threat are the tiny distribution range of these Comoran iguanas, and possible competition with the recently introduced agamid lizard *Agama agama* on Grande Comore (Meirte, 2004).

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