# Biodiversity of true lemurs (*Eulemur* spp.):

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# Species delimitation and phylogeography

# in the brown lemur complex

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*Considering the number of species that are close to extinction or even go extinct before they are described, the "species problem" seems absurd.*
*(Matthias Markolf, 2013)*

# Contents

# 1 General Introduction

## 1.1 Biodiversity

Species are the units of fundamental interest in biodiversity studies (Agapow et al. 2004; Balakrishnan 2005). In fact, species are the basic unit of comparison in all biological disciplines, e.g. anatomy, behavior, ecology, evolution, physiology and molecular biology, underlining the importance of biodiversity studies to all fields of biological science (de Queiroz 2005; Sites & Marshall 2004; Wiens & Penkrot 2002; Wiens 2000). In addition, species are the currency used to define areas of conservation priority (Agapow et al. 2004, Balakrishan 2005). A central goal of evolutionary biology is to determine how many species exist, how they evolved, and to devise concepts and methods that allow their delimitation.

The total number of species on the planet might never be determined as most species are probably not yet described. A recent estimate deciphers the total number of the world's species at 1.5 million, with an additional 2-8 million species unknown to science and/or not yet described (Costello 2013). Numerous measures of biodiversity exist for described taxa to adequately set priorities for areas with high biodiversity and to effectively manage and plan global conservation efforts. Among them are concepts of "key biodiversity areas", "biodiversity hotspots", "high-biodiversity wilderness areas", "important plant areas" and many more (see biodiversitya-z.org), which use concrete measures like species richness, genetic composition, species dissimilarity or endemism to define areas of conservation priority. No matter which measure is used, a latitudinal biodiversity gradient exists, and biodiversity is much higher in the tropics (Dowle 2013).

Madagascar, the fourth largest island of the world, is one of the global biodiversity hotspots (Myers et al. 2000). At least 88 mya of isolation from the Indian and African landmasses, together with pronounced climatic regional variation and occasional events of oceanic dispersal, gave rise to today’s endemism of Madagascar‘s fauna and flora (Ganzhorn et al. 2006; Thalmann 2007; Yoder 2013). As a result, Madagascar is in the global focus of biodiversity studies and conservation efforts (Mittermeier et al. 1998;

Ganzhorn et al. 2001). Madagascar's biodiversity and endemism is indeed astonishing and new species are still being described almost every week (Thompson 2011). Around 80 % of animals and ~90% of plants from Madagascar can be found nowhere else in the world. Levels of endemism reach up to 100% for several groups of vertebrates, including amphibians, tenrecs, carnivores and primates (Goodman & Benstead 2003).

One of the most striking examples of Malagasy biodiversity is the infraorder of *Lemuriformes,* a group of basal primates endemic to the island. Following a single colonization of the island in the late Eocene (Yoder et al. 1996; Yoder 2013), the lemur radiation today represents more than 15% of all living primate species and 36% of all primate families (Mittermeier et al. 2010). In 2012, an assessment of the conservation status of all living lemurs by the IUCN-SSC Primate Specialist Group revealed that 90% of lemur species are threatened. This highlights Madagascar‘s international significance for conservation (Myers et al. 2000) and the importance of an objective assessment of the number and distribution of existing taxa in order to effectively conserve its biodiversity.

## 1.2 The species problem

Despite the central importance of species for all fields of evolutionary biology, it seems surprising that there is no general agreement about what a species is, and that the ‘species problem’ is still one of the most discussed topics in evolutionary biology (Sites & Marshall 2003; Knapp 2008; Pavlinov 2013). The discussion goes back to Aristoteles, who used the term "species" ("eidos") to classify organisms and other things of the natural world (Pavlinov 2013; Wilkins 2009). The formulation of "concepts of species", however, of which many exist today, was mainly accelerated by the work of Theodosius Dobzhansky, Julian Huxley, Ronald Fisher and Ernst Mayr during the Modern Evolutionary Synthesis, together with new insights from population genetic theory (Wilkins 2009). Ernst Mayr (1942, 1963) formulated the perhaps most influential definition, the biological species concept (BSC), which considered species as populations of interbreeding organisms that are reproductively isolated from other such groups. This definition has several obvious practical limitations, however. Neither can reproductive isolation be established for fossils, nor can we know whether allopatric populations would interbreed without a geographical barrier (Holliday 2003, Coyne & Orr 2004, Balakrishnan 2005).

Hence, numerous additional species concepts were formulated, but none of them is operational for all taxa (see Claridge et al. 1997, Hey 2001, Mallet 2001, Coyne & Orr

2004). Among them is also the phylogenetic species concept (PSC), which will be of central importance for this study. There are several versions of the PSC, such as "*A phylogenetic species is an irreducible (basal) cluster of organisms, diagnosably distinct from other such clusters, and within which there is a parental pattern of ancestry and descent"* (Cracraft 1983). However, all versions of the PSC stress 'diagnosability' and a 'common descent' as criteria to delineate groups of organisms as species. The PSC has been applied to many major groups of organisms in the last decades and has led to a fundamental increase in the number of species (Agapow et al. 2004, Zachos et al. 2013), often via raising subspecies to species level without collecting additional data. The main reason for this is that diagnosability can be achieved for the smallest unit one can imagine, e.g. populations, demes or family groups, as long as scientist are able to find autapomorphic characters among individuals or groups (Mallet 2001, Zachos 2013). In times of DNA sequencing this allows researchers to use a single base-pair polymorphism as a diagnostic character for the delineation of species under the PSC.

One fundamental reason for the endless debates of species concepts relates to the fact that different levels of the species problem have been discussed and intermixed by many authors over the last decades (de Quieroz 2005, Pavlinov 2013). These levels comprise discussions about the "reality" of species in nature, e.g. are species "real", or at least more real than other biological categories such as genera or families, the aim to find a universal definition of the category of species that can be applied to all living beings, and the discussion about properties, such as diagnosability, reproductive isolation, monophyly or ecological traits that demarcates a group of organisms as a species.

The problem of intermixing different levels of the species problem has been recognized by Mayden (1997) and De Quieroz (1998) (Naomi 2011). They began to differentiate between the conceptual question of how to define the term species as something that is "real" in nature and the question of how to demarcate these units using different criteria such as monophyly, reproductive isolation, ecological or morphological traits. This important distinction led to the formulation of the general metapopulation lineage concept of species (GLC) (De Quieroz 1998, 2005), which argues that all modern species definitions are variations on the same general (metapopulation) lineage concept of species because they equate species either explicitly or implicitly with segments of population level evolutionary lineages.

However, adopting the GLC of species still leaves us with the problem of how to delimit independently evolving lineages. This is a separate problem because contingent properties of lineages such as monophyly, reproductive isolation or ecological adaptions can lead to substantially different conclusions about the independence of those lineages (Frost & Kluge 1994; Wiens & Penkrot 2002). This practical problem is especially pronounced in recent radiations, because different contingent properties will accumulate at different times during the speciation process (de Quieroz 1998). However, the insights of the GLC - away from the conceptual questions whether species are real identities and how we can define them - to the practical one of how to delimit them paved the way for a transition and a new era of "species delimitation" in systematic biology (Sites & Marshall 2003). That this practical approach is beginning to be widely accepted is illustrated by the number of publications about species delimitation since the influential paper of Sites and Marshall in 2003 (Camargo & Sites 2013).

## 1.3 Taxonomic inflation

Describing new species of mammals is an increasingly common event and sometimes referred to as 'taxonomic inflation' (Agapow et al. 2004; Isaac et al. 2004; Tattersall 2007; Zachos 2013). Whereas 4659 mammal species were listed in 1993 (Wilson & Reeder 2005) today we count 5501 (IUCN Red List, 2012) species of mammals on the planet. However, the increase in species numbers is not equally across orders of mammals. The number of ungulates for example recently increased from 250 to 450 species based on one extensive revision of the whole group (Groves & Grubb 2011). Similarly, the number of primates almost doubled in the last 20 years to 479 recognized primate species today (Mittermeier et al. 2013). Within the order Primates, the number of lemur species increased form 36 species recognized by Tattersall in 1982 to almost 100 today. Tattersall (2007, 2012) questioned this development as „taxonomic inflation or cryptic diversity ?“, where 'taxonomic inflation' refers to the increase of the number of species due to the application of different species concepts (Agapow et al. 2004). In fact, the reason for the rapid increase in species numbers is mainly due to the application of the PSC. First, numerous subspecies have been elevated to species level without collecting new data, and second, several of these newly described lemurs, especially nocturnal ones of the genus *Lepilemur* and *Microcebus* have been solely described on diagnostic characters

(single nucleotide polymorphisms) and genetic distances of mitochondrial DNA sequences (e.g. Andriaholinirina et al. 2006, Andriantompohavana et al. 2007; Craul et al. 2007; Louis et al. 2006; Radespiel et al. 2008). Whether species delimitation based on the PSC and mitochondrial DNA alone is indeed a valid method for delimiting species is discussed in the **Chapter 2.**

## 1.4 The true lemurs

Members of the genus of true lemurs *(Eulemur* Simons & Rumpler 1988) range in body mass from 900 g (*E. mongoz*) to 2500 g (*E. fulvus*) (Mittermeier et al. 2010). *Eulemur* species are distributed over most of the island (Fig. 3.1) and occupy almost all biogeographic zones, from the south-western dry spiny forest to the dry deciduous forests of western and the rain forests of eastern Madagascar (Goodman and Ganzhorn 2004). Only the central plateau is not occupied by eulemurs. Eulemurs living in dry deciduous forests are smaller in size than those inhabiting rainforests (Godfrey 1990). Most eulemurs are sexually dichromatic, with males being more variable in pelage coloration across taxa than females (Fig. 1.1, 1.2). Despite the wide range of habitats, eulemurs are predominantly frugivorous, and variation in diet is most pronounced between western and eastern populations (Overdorff and Johnson 2003). Cathemeral activity, defined as significant amounts of traveling or feeding activity within both the light and dark portions of a 24h cycle, is characteristic for all members of this genus, but the degree of nocturnal activity is variable among taxa, populations and seasons (Overdorff & Johnson 2003). In general, members of the genus *Eulemur* seem to be very flexible in their biology and can coexist in most places with sympatric congeners (Johnson 2006, Overdorff & Johnson 2003).

### 1.4.1 Taxonomy

Taxonomically, eulemurs have a long and complicated history, which is nicely illustrated by 13 different synonyms for *E. mongoz* alone (Schwarz 1931). Because a more detailed description of the taxonomic history of the genus *Eulemur* is provided in **Chapter 3**, I only highlight the most important facts necessary for deriving the specific questions for this thesis here. Based on behavioral, anatomical and cytogenetic evidence, Simons & Rumpler (1988) split the genus *Lemur* into two taxa, one containing only *Lemur catta* and

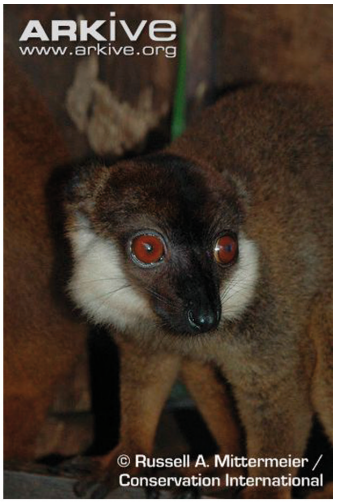

*E. albifrons*


*E. collaris*


*E. fulvus*


*E. rufifrons*

*E. sanfordi*




*E. cinereiceps (m)*

*E. rufus*


*E. cinereiceps (f)*

**Fig. 1.1: *Eulemur* species of the brown lemur complex.** m= male, f= female. Where sex is not indicated females are shown on the left, males on the right of the picture.

the other containing the "true lemurs", i.e. *Eulemur coronatus, E. mongoz, E. rubriventer, E. macaco macaco, E. m. flavifrons E. fulvus albifrons, E. f. albocollaris (*later *E. cinereiceps), E. f. collaris, E. f. fulvus, E. f. mayottensis, E. f. rufus and E. f. sanfordi.* Seven of 12 *Eulemur* taxa were classified as subspecies of the common brown lemur, *Eulemur fulvus,* and grouped in the polytypic "*fulvus* group" also referred to as "brown lemur complex" (BLC) (Wyner et al. 1999).

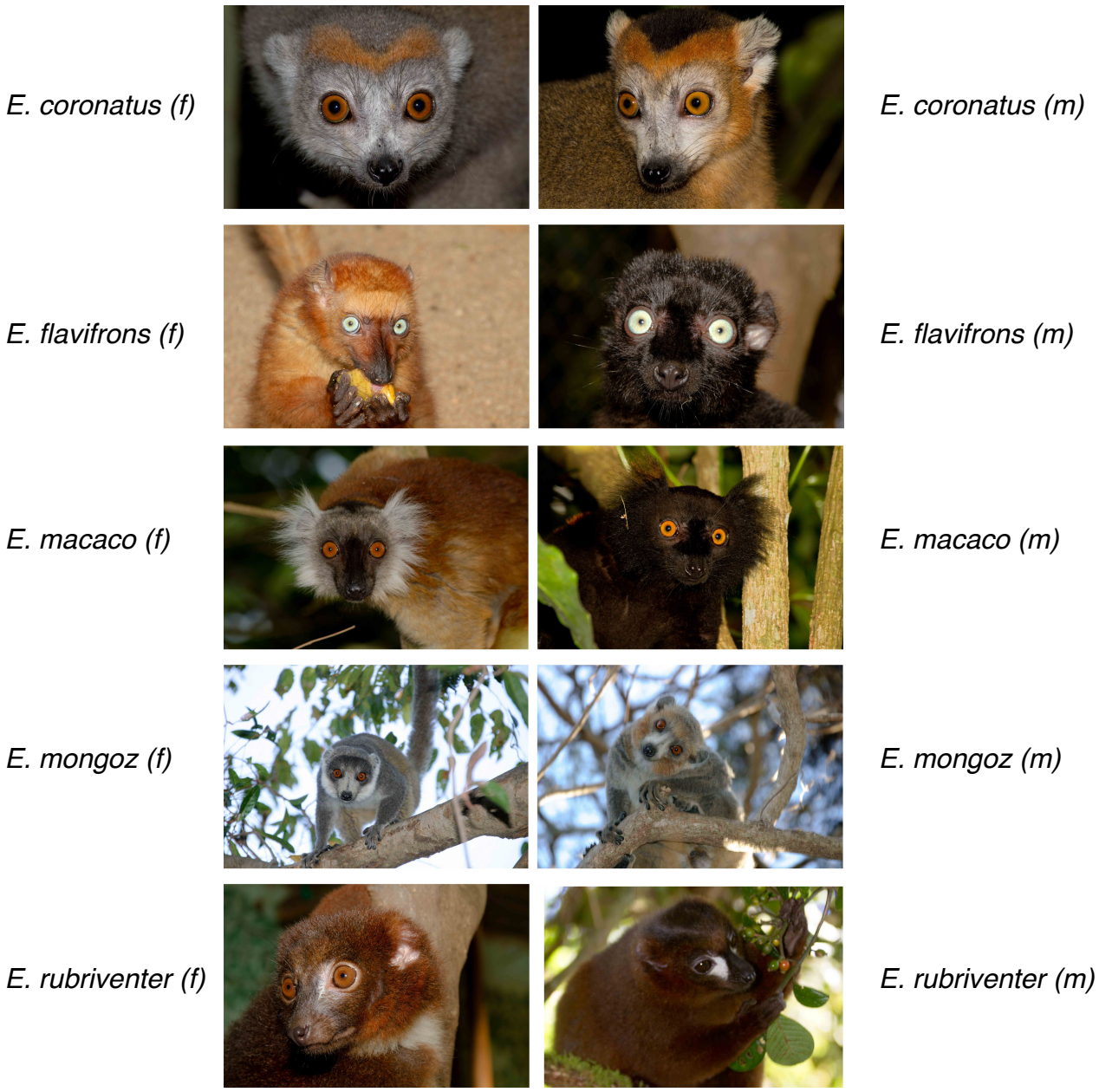



**Fig. 1.2: Other species of the genus *Eulemur*.** m= males, f= females.

Several authors tried to resolve the relationships among eulemurs based on different methods. Macedonia & Shedd (1991) investigated phaeomelanin hair- banding patterns after the principle of metachromism (Hershkovitz 1968) in females and found the following sequences from ancestral to derived states among the members of the genus: coronatus-mongoz-rubriventer-flavifrons-macaco and collaris-fulvus-sanfordi-rufus-albifrons. Macedonia & Stanger (1994) used acoustic data to derive a phylogeny for the eulemurs, however they lumped all subspecies of the BLC for practical purposes. Morphological analyses (Groves & Eaglen 1988; Groves & Trueman 1995; Schwarz 1931;

Tattersall & Schwartz 1991; Viguier 2002) resulted in various polytomies and in particular highlighted extensive homoplasy found among members of the BLC. Several phylogenetic reconstructions of molecular sequence data (Delpero et al. 2006; Pozzi et al. 2006; Pastorini et al. 2003; Wyner et al. 1999; Wyner et al. 2000) resulted in different phylogenies and discordances among major nodes and were based solely on mitochondrial DNA. Other phylogenies based on several nuclear genetic loci or SINE integrations suffer from incomplete taxon sampling (Horvath et al. 2008; McLain et al. 2012, Perelman et al. 2011; Roos et al. 2004). Although phylogenetic relationships are still unresolved, Johnson (2006) clearly stated that the taxonomy of the BLC is not fully resolved and populations have not yet been shown to have speciated (Tattersall 2007, 2012). Nonetheless, Groves (2001a) elevated all subspecies of BLC to species rank without new evidence.

We know today that individuals of *E. f. mayottensis* from the island of Mayotte represent introduced individuals of *E. fulvus* (Mittermeier et al. 2010) and that *E. albocollaris* is a synonym for *E. cinereiceps* (Johnson et al. 2007). A further split of populations of *E. rufus* into *E. rufifrons* south of the Tsiribihina river and *E. rufus* north of it (Groves 2006) leaves us with seven species that were formerly treated as subspecies within the polytypic BLC. However, this decision was entirely based on a different interpretation of existing data under the PSC. Groves (2001a, pp. 74-75) argued that subspecies of the BSC qualify as species under the PSC, because they *"are sharply distinct externally"*...and..."*appear consistently different in craniodental characters (Tattersall & Schwartz 1991)".* Moreover, two of them, *E. collaris* and *E. cinereiceps* (formerly *E. f. albocollaris*) have unique mitochondrial DNA sequences, there would be "*no evidence in overlap of phenotypic character states among members of the group"* and *"little or no evidence that they form a genetic continuum in the wild"*. Tattersall & Schwartz

(1991), however, clearly stated that the degree of homoplasy in craniodental characters among members of the *fulvus* group does not allow to infer any relationships among members of the group. Thus, apparently homoplastic characters have been used to delimit species under the PSC. Moreover hybridization, *sensu* "a genetic continuum" is not only likely between members of the "*fulvus* group", but has also been suggested for *E. rufifrons* and *E. fulvus* at Betsakafandrika (Lehmann & Wright 2000) and *E. albifrons* with either *E. fulvus along the Mananara*- Zahamena corridor or with *E. sanfordi* north of the Bemarivo (Mittermeier et al. 2006).

Thus, there is substantial evidence that the members of the BLC form a genetic continuum in the wild. Consequently, the only argument that holds is that these species are distinct externally, whereas “sharply” is only true for facial patterns and male coloration. However, explicit tests of overlap in phenotypic character states with an appropriate geographical sampling at the intraspecific level are still lacking. While all taxa in question may be said to represent potential new species, because of remarkable phenotypic differences of males, none of them could yet be shown to have speciated (Tattersall 2007, 2013). Whether members of the brown lemur complex have indeed speciated is therefore investigated in **Chapter 3**, using multiple lines of evidence.

### 1.4.2 Phylogeography

As mentioned earlier, Madagascar's exceptional biodiversity and its many endemic lineages, in particular at higher taxonomic levels such as families or genera, qualify the island as one of the global biodiversity hotspots (Myers 2000). Our current understanding of the origin of those numerous endemic lineages at higher taxonomic levels is that Madagascar was surrounded by an oceanic barrier for at least 88 million years and that most lineages arrived in Madagascar via oceanic dispersal from Indian or African landmasses during the Cenozoic (Yoder 2013). Extensive research during the last decades has shown that many species are indeed endemic at a local scale and diversified extensively upon arrival in Madagascar or after separation of the island from the mainland (Goodman & Benstead 2003, Vences et al. 2009, Wilmé et al. 2006). For example ~242 species of amphibians and ~100 species of lemurs (IUCN Red List, 2012) must have diversified in the last 88 million years on the island. How did this diversification happen?

Several diversification mechanisms have been proposed to explain the tremendous amount of micro-endemism in Madagascar and were recently reviewed in Vences et al.

(2009). These authors listed several different diversification hypotheses for the evolution of micro-endemism in Madagascar and proposed predictions to test these hypotheses in single radiations or comparative approaches across lineages. Among them the 'ecogeographic constraint hypothesis', the 'western refugia hypotheses', the 'mountain refugia hypothesis', the 'riverine barrier hypothesis' and the 'watershed hypothesis', which are all explained in detail in **Chapter 4**. Moreover, Vences et al. (2009) proposed Madagascar as a biogeographic model region, as it allows testing speciation mechanism in a geographically small, but diverse region within the borders of one country. Furthermore, they highlighted the necessity to test the above-mentioned diversification mechanisms using statistical phylogeographic methods with *a priori* defined predictions.

Phylogeography is a fairly recent discipline that combines population genetics and phylogenetic theory (Avise et al. 1987; Hickerson et al. 2010) as well as ecological niche modeling and geographic information system (GIS) modeling approaches (Chan et al. 2011). Whereas variation in mitochondrial DNA sequences was in the focus of studies in the early years of phylogeography, recent advances in sequencing technology provide the possibility to use multilocus sequencing data to analyze the evolutionary history of populations and species. However, analyzing genealogies of multiple independent nuclear loci revealed that individual gene trees can substantially differ from the species tree, because each individual locus may have its own evolutionary history (Brito & Edwards 2008; Brumfield et al. 2003; Carstens & Knowles 2007). Consequently, discordance among gene trees and species trees resulted in the development of methods that simultaneously estimate individual gene trees and the species tree (Knowles & Kubatko 2011). This is a major improvement as we are normally more interest in the phylogeny and divergence of the species instead of single genes, in particular if we want to draw conclusions about the evolutionary history of species in space and time. Beside the estimation of divergence times and phylogenies of species, multiple independent genetic loci can also be analyzed with population genetic methods to infer mixed ancestry, past migration rates or effective populations sizes of species in a Bayesian framework. This allows to test the fit of the data to specific predictions derived from different diversification hypotheses such as the ones mentioned above.

The 'brown lemur complex' is a particularly interesting group to test different diversification hypotheses for the evolution of micro-endemism in Madagascar. As a consequence of their broad distribution covering almost all biogeographic regions,

eulemurs have been fundamentally involved in the development of all major biogeographic hypotheses for Madagascar. For example, according to the “watershed hypothesis” ("Centers of endemsim hypothesis", see Fig. 4.1) (Wilmé et al. 2006), the island's three major mountains with altitudes above 2000 m and their associated river catchments played a fundamental role for the diversification of many taxa during the late Quarternary. During drier and cooler periods induced by glacial cycles, suitable habitat for forest-dependent species was restricted to refugia along river catchments. Therefore, rivers with watersheds at high elevational ranges served as 'retreat-dispersal watersheds', which allowed for dispersal among neighboring retreat-dispersal watersheds or even between eastern and western populations, whereas rivers with watersheds at low altitude were zones of isolation and provoked the evolution of micro-endemic taxa. The idea of retreat-dispersal watersheds stems mainly from the fact that *E. fulvus* and *E. rufifrons* today have disjunct populations, occurring in the east as well as in western parts of the island. However, explicit tests for these taxa and concordance with the watershed hypothesis have been based solely on present distributions of these taxa. As ancestral distributions of these taxa during the time of speciation are not known and can hardly be inferred precisely, I will test the concordance of the 'watershed' and other diversification hypothesis proposed for the evolution of Madagascar's micro-endemic biota with the evolution of the *Eulemur* clade in space and time.

Against this background, the following specific questions will be addressed in this dissertation:

**Chapter 2:**

Are approaches based on genetic distance or diagnosability of mitochondrial DNA a valid method for species delimitation in lemurs, and how does sampling influence these approaches?

**Chapter 3:**

How many *Eulemur* species can be delineated, and are members of the brown lemur complex valid species or subspecies?

**Chapter 4:**

Phylogeography of the genus Eulemur

Is *Eulemur* evolution in space and time concordant with major biogeographic hypotheses proposed to explain the present distribution of taxa across Madagascar?

# 2 On species delimitation: Yet another lemur species or just genetic variation?

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# Abstract

**Background**

Although most taxonomists agree that species are independently evolving metapopulation lineages that should be delimited with several kinds of data, the taxonomic practice in Malagasy primates (Lemuriformes) looks quite different. Several recently described lemur species are based solely on evidence of genetic distance and diagnostic characters of mitochondrial DNA sequences sampled from a few individuals per location. Here we explore the validity of this procedure for species delimitation in lemurs using published sequence data.

**Results**

We show that genetic distance estimates and *Population Aggregation Analysis* (PAA) are inappropriate for species delimitation in this group of primates. Intra- and interspecific genetic distances overlapped in 14 of 17 cases independent of the genetic marker used. A simulation of a fictive taxonomic study indicated that for the mitochondrial D-loop the minimum required number of individuals sampled per location is 10 in order to avoid false positives via PAA.

**Conclusions**

Genetic distances estimates and PAA alone should not be used for species delimitation in lemurs. Instead, several nuclear and sex-specific loci should be considered and combined with other data sets from morphology, ecology or behavior. Independent of the data source, sampling should be done in a way to ensure a quantitative comparison of intra- and interspecific variation of the taxa in question. The results of our study also indicate that several of the recently described lemur species should be reevaluated with additional data and that the number of good species among the currently known taxa is probably lower than currently assumed.


## 2.1 Introduction

Species are the fundamental units of evolutionary biology as they define the entities that are studied and compared in every field of biology (de Quieroz 1998). Moreover, they are the currency for biodiversity classification of geographic regions, and are therefore

used to define regions of conservation priority, so-called biological hotspots (Agapow et al. 2004; Balakrishnan 2005) Despite the central importance of species, there is no general agreement about what a species is, and the 'species problem' is one of the most discussed topics in evolutionary biology (Coyne & Orr 2004; Knapp et al. 2005; Sites & Marshall 2003).

An overview of species concepts is beyond the scope of this article, but it should be emphasized that the discussion has shifted away from the philosophical and conceptual questions towards a more pragmatic approach in recent years (Hausdorf 2011; Mayden et al. 1997). De Quieroz (1998) argued that all modern species definitions are variations on the same general lineage concept of species, because these definitions equate species either explicitly or implicitly with segments of population level evolutionary lineages (Hey 2006; Sites & Marshall 2004; de Quieroz 1998; Wiens & Penkrot 2002).

Adopting a concept of species as population level lineages will not solve the problems related to species delimitation in practice, but there would no longer be a discussion of the species concept (de Quieroz 1998). In doing so, the concept of species and the question how we recognize a species in practice are encapsulated (de Quieroz 2005), which means that no single property is necessary to be considered crucial, as is reproductive isolation for the Biological Species Concept (BSC) or a phylogenetically distinct cluster for the Phylogenetic Species Concept (PSC), because every single criterion is likely to fail or to yield ambiguous results (Frost & Kluge 1994; Sites & Marshall 2003; Wiens & Penkrot 2002). As emphasized by Ernst Mayr (1996), species should therefore be delimited with different datasets (criteria) (Balakrishnan 2005; Dayrat 2005; Sanders et al. 2006; Sites & Marshall 2004; de Quieroz 2005). In practice, morphological and molecular approaches are mutually informative (Avise 2004) and often feasible.

The recent taxonomic practice in the primates of Madagascar (Lemuriformes) looks quite different for the most part. Tattersall (2007) recently questioned whether the dramatic increase of recognized lemur species in recent years is due to previously unnoticed cryptic diversity or to taxonomic inflation. In 1982, he counted 36 lemur species, whereas in 2007 already 83 species were recognized. This is an increase of 1.88 lemur species per year over 25 years, which is partly due to the fact that small, nocturnal animals were actually being captured for the first time, that research effort has increased, that remote forests have been visited and that new molecular techniques have become available. In 2011, the count is currently at 101 species (Mittermeier et al. 2010), which means that the rate of new species descriptions more than doubled (to 4.5 species per year) in the last 5 years

alone. Are we still unraveling cryptic taxonomic diversity or has the use of particular methods or criteria kindled taxonomic inflation? Because Tattersall's question seemed to have been largely ignored, we re-visit this problem, using quantitative genetic methods to scrutinize methods and concepts used to describe new lemur taxa.

It is particularly striking that several recent taxonomic studies of lemurs are based almost exclusively on evidence from mitochondrial DNA (but see Groeneveld 2009, 2010; Rasoloarison et al. 2000; Weisrock et al. 2010; Yoder et al. 2000; Zimmermann et al. 1998). Even where morphometric data were available, they were not analyzed statistically (Andriantompohavana et al. 2007; Louis et al. 2006a; Louis et al. 2006b). Specifically, a relatively small number of individuals per location were typically sampled in formerly uninvestigated areas. Mitochondrial DNA was then sequenced and compared with previously published data. If the sampled individuals clustered together in a phylogenetic tree and interspecific genetic distances between the new and other taxa were in the range of previous published interspecific distances within the genus under study, and if additional diagnostic sites could be determined via Population Aggregation Analysis (PAA) (Davis & Nixon 1992), a new species was proposed and eventually described.

Genetic distances are valid tools for taxonomy because sequences of different organizational levels (e.g. within species, within genera, within families) exhibit different amounts of divergence, which do not overlap and create a gap (Hebert et al. 2003). This gap can be used as an objective threshold for a species boundary. One indispensable prerequisite for this procedure is to calculate genetic distances at both levels of organization (within and between species) in order to identify the gap. This was often not the case in lemurs (e.g. see (Andriantompohavana et al. 2007; Louis et al. 2006a; Louis et al. 2006b). For example, comparisons of intraspecific levels of divergence for populations of *Microcebus* (Olivieri et al. 2007) and *Lepilemur* (Craul et al. 2007) were based on as few as 3 individuals (*M. bongolavenesis),* but it is not known whether this is sufficient for a representative characterization of the existing intraspecific variation. Similarly, (Louis et al. 2008) divergence estimates of the D-loop of 3.7 % between *M. margotmarshae* and *M. mamiratra* were used in identifying the former as a new species. This approach needs to be reconciled with the observation of Fredsted et al. (2004), who found genetic divergences of up to 8.2 % among potentially interbreeding individuals of *Microcebus murinus* within an area of 3 $km^2$ of continuous forest. In light of these overlapping levels of genetic variance within and between taxa, the question arises on which criteria species

delimitations should be based and which sample sizes are likely to be sufficient to identify true differences.

The problem of an appropriate sample size is also relevant for PAA, a method frequently used to support inferences about the existence of new taxa in combination with the PSC (e.g. Craul et al. 2007; Louis et al. 2006a, Louis et al. 2006b; Louis et al. 2008; Olivieri et al. 2007). PAA compares homologous sequences drawn from different populations. A position (base in DNA sequence) that is fixed (i.e. fully conserved) in one population, but has a different state (base) compared to another population is treated as diagnostic site or character. Although it is known that PAA is prone to small sample sizes Walsh 2000; Wiens & Servedio 2000), we also asked how PAA would be influenced by sample size, using a simulation with data from a real population of *Microcebus*, a genus with particularly many recently described new species.

The aims of this study were, therefore, to use the publicly available information about genetic variation from different lemur taxa to identify typical levels of intra- and interspecific genetic variation at loci commonly used in species delimitation and to determine minimal reliable sample sizes for these types of analyses. It is explicitly not our intention to single out particular studies for criticism. We know from personal experience that field work in Madagascar can be extremely difficult, that some species live at low densities and or high up in the canopy, making access to a desirable number of samples very difficult. We also realize (but do personally not endorse) the view that sacrificing potentially rare animals for proper description and deposition in an accessible museum is ethically challenging for some; a fact that may also contribute to false positives and an inflation of species numbers. Finally, it can also be argued that assigning species status to a potentially endangered taxon is a politically justified strategy in order to achieve maximal preemptive conservation effects because extinction cannot be reversed. This approach will also favor splitting over lumping and contribute to an increase in species numbers. All these aspects and problems at the interface of sound scientific procedures, practical difficulties of fieldwork and conservation politics can benefit from sound empirical criteria, which we hope to contribute with these analyses.

## 2.2 Methods

### Genetic distances

We searched the NCBI database for published lemur sequences and downloaded those in the application Geneious Pro (version 4.8.5). Sequences were grouped by genus and sub-grouped by sequenced loci. Taxonomic identity of each sequence was either based on the publication or on locality, if taxonomy was likely to have changed over years. Sequences were aligned using the ClustalW plugin in Geneious and afterwards checked by eye. Distances were estimated using the software MEGA (Tamura et al. 2007). We calculated p-distances, as it is the mostly used method in previous lemur publications and report distances as percentage genetic distances. Gaps or different length of sequences were not used for calculations as we chose the pair-wise deletion option in MEGA.

We calculated genetic distances within species (intraspecific) and between species (interspecific). Values were exported to Excel to process and to visualize distances. Afterwards we plotted the mean and the range to the lowest and highest value of intra- and interspecific distances per marker and taxon.

### Simulation

To simulate the impact of sample size to the results of PAA on the number of species, we used one of the best-studied mouse lemur population at Kirindy Forest. The published dataset consists of 202 different gray mouse lemur individuals (*Microcebus murinus*), which showed 22 haplotypes for the mitochondrial D-loop (Fredsted et al. 2004). All sequences were aligned and cut to equal length (529bp) The gray mouse lemur population at Kirindy showed significant genetic structure between 3 local study sites (CS5, CS7 and N5), which are 2-3 km apart (see Fredsted et al. (2004) for details of the study area). This substructure was used for the simulation as different sampling areas for a fictive taxonomic study. We divided the population into two sampling areas (CS5 and N5 vs CS7), including approximately the same number of individuals in each population.

Afterwards 2, 4, 6, …20 sequences were drawn randomly from each population 10,000 times for the entire dataset and for males and females separately. After each step the number of diagnostic characters were determined and the mean was plotted against the number of sequences drawn from each population. Simulations were done using PERL (PERL script can be received by request from the authors).

## 2.3 Results

### Genetic distances

Intra- and interspecific genetic distances are plotted pair-wise for each taxon and marker in Fig 2.1. Only the genetic distances of *Lepilemur* for the tRNA marker, the *Microcebus* distances for the PAST fragment (Pastorini et al. 2000) and the cytochrome B distances for *Mirza* show no overlap. All other pair-wise plots show more or less overlap of intra- and interspecific genetic distances. In several cases the smallest interspecific value even exceeds the lower level of intraspecific variation. None of the different markers show a superior performance over different genera. *Lepilemur* and *Microcebus* exhibit the highest intra- and interspecific variation for all markers.

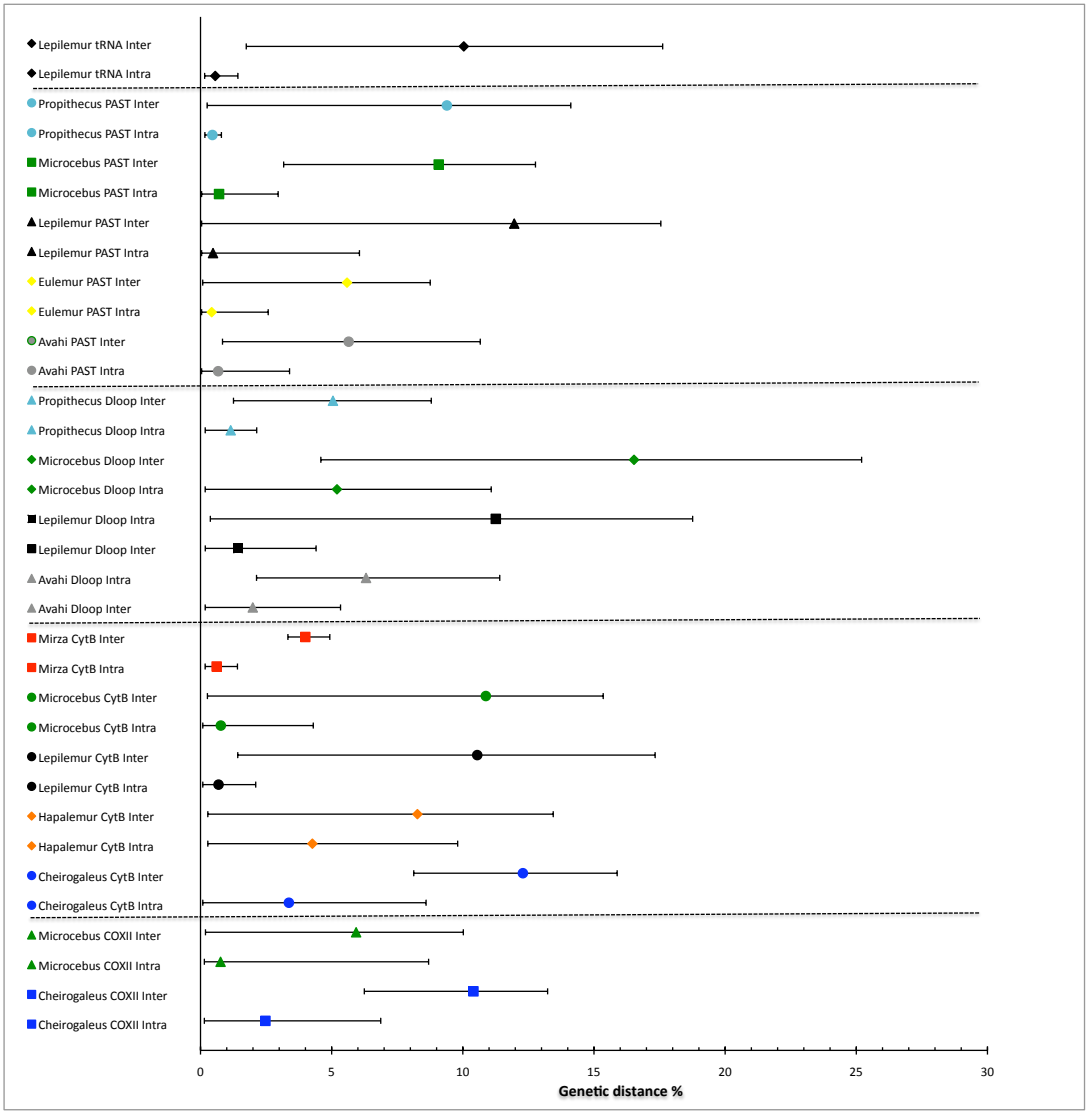


**Fig. 2.1: Genetic distance plot.** x- axis= Genetic distance in %; y- axis= lemur genera and analyzed marker with unique identifier. Interspecific distances per taxa are plotted above intraspecific. Plots are grouped by marker.

**PAA Simulation**

The simulation of diagnostic characters (Fig. 2.2) revealed that two individuals drawn from a population lead to 11-12 diagnostic sites that would argue for a separation into two species. The curve describing the relationship between sample size and the number of diagnostic sites drops relatively fast. However, 10 individuals randomly drawn from each population can still occasionally lead to the identification of a diagnostic character as the curve has not reached 0 yet. What is also evident is that sampling only females is much more likely to produce diagnostic sites than sampling only males. Random sampling of 8 females per population still results in one diagnostic character, on average, arguing for separation into two species according to the PSC.

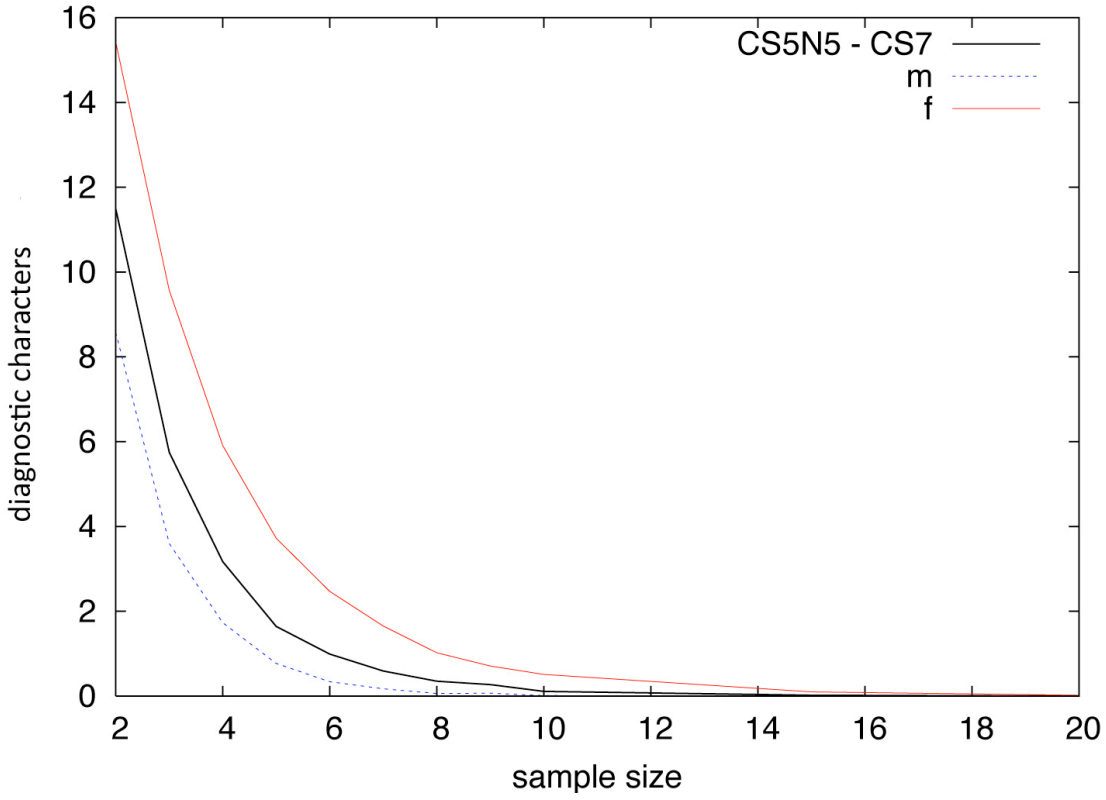


**Fig. 2.2: Plot of mean diagnostic characters per sample size.** X-axis= samples drawn from each population, Y-axis = diagnostic characters (a site in a DNA sequence of a population that has a fixed but different state as in another population), CS7= Population 1, CS5= Population 2, males= blue, females= red; 2,4,6,...,20 individuals were randomly drawn 10 000 times.

## 2.4 Discussion

**Genetic distance**

The comparison of intra- and interspecific distances across several lemur genera and markers revealed that none of the commonly used markers are generally suitable for distance-based species delimitation in this taxon. One possible error in our estimation could be the wrong assignment of an individual to a certain species, because of changing taxonomy. However, we checked affiliation several times in all cases and used the most recent publication referring to the sequence in question.

The overlap of intra- and interspecific distances in most cases is best explained by paraphyly and polyphyly of the mitochondrial DNA (Funk & Omland 2003) of the relevant taxa. For example, the overlap of *Avahi* distance estimates for the D-loop and PAST fragment is due to paraphyly of *Avahi peyriasi* (Andriantompohavana et al. 2007; Lei et al. 2008). Three types of *Avahi peyriasi* are distinguished. The fact that all of them actually occur at one site (Ranomafana) indicates that the taxonomy of the south-eastern *Avahi* taxa (*A. peyrierasi, A. betsileo, A. ramanantsoavanai, A, meridionalis*) is highly questionable and should be revised.

The same problem applies to *Eulemur fulvus*, which was also paraphyletic for the PAST fragment (Pastorini et al. 2000). *Hapalemur aloatrensis* is not distinguishable from *Hapalemur griseus* on a molecular basis. This, and the paraphyly of *Hapalemur griseus* subspecies, leads to the observed overlap in cytochrome B (Fausser et al. 2002; Rababrivola 2007). Interspecific distances of *Lepilemur* (D-loop; PAST) are as small as the lower limit of intraspecific distances. Zinner et al. (2007) already questioned the existence of *L. mittermeieri* and *L. tymerlachsonorum*. Where intraspecific divergence reaches high levels, e.g. 8% in *Microcebus* for COX II, we can expect that more species are going to be described if this locus is being used. Indeed, these 8% are caused by individuals from Bemanasy, which seem to form an independently evolving lineage (Weisrock et al. 2010).

Another factor influencing the overlap of intra- and interspecific distances might be the geographical distribution of different taxa. Whereas some taxa like *M. murinus* are widespread (but see Weisrock et al. 2010), others, such as *M. tavaratra* occur only in very restricted areas (Yoder 2000).

Whatever the explanation for the overlap of intra and interspecific distances in different taxa, the present analysis indicates that a constant “threshold species

delimitation", as it is used in barcoding approaches, cannot be recommended (Cognato 2006).

**PAA simulation**

For the present simulation, we used as diagnostic characters only those sites that are fixed and different between populations. Sites that are variable within populations, but different between populations are sometimes also referred to as being diagnostic attributes (Kelly et al. 2007; Sarkar et al. 2009), and would lead to an even higher number of diagnostic characters.

Our simulation showed that sampling fewer than 10 individuals can falsely lead to diagnostic characters and to an argument for identifying a new species under the PSC. The number of published diagnostics characters for several recently newly described lemur species for the mitochondrial D-loop are far below 10 (e.g. Andriantompohavana et al. 2007). Because this analysis was focused on the highly variable mitochondrial D-loop, this value should not be used as a general guideline for taxonomic sampling. For less polymorphic markers, such as cytochrome b for example, the curve would probably need fewer individuals to reach zero. However, to establish a general sampling threshold the same analysis ought to be repeated for several different markers and populations. Walsh (2000) estimated necessary sampling values of > 50 individuals in order to perform well with PAA. Wiens & Servedio (2000) even argued that hundreds and thousands of individuals would be necessary to identify diagnostic characters that are valid for the species boundary. This is unpractical and impossible for most taxonomic studies, however. Hence, other species delimitation methods should be favored and are discussed below.

Finally, the simulation revealed a clear difference between males and females. Because of its uniparental inheritance and male-biased dispersal in *Microcebus,* mitochondrial DNA exhibits necessarily higher divergence between populations (Fredsted et al. 2005). That does not mean that there is no genetic exchange via males, however. Gene flow is an important feature of species, especially in introgressed species. Therefore, genetic markers with high levels of gene flow in the dispersing sex should be more effective for species delimitation (Petit & Excoffier 2009).

**How to delimit species?**

We have argued that sole analysis of uniparentally inherited genomes, like mtDNA, is not sufficient to delimit species, as it does not realistically reflect the population history

(Funk & Omland 2003). On the other hand, sequencing other parts of the genome revealed that gene trees can differ substantially between different loci (Carstens & Knowles 2007; Edwards & Beerli 2000; Nichols 2001; Riddle et al. 2008) because each locus has its own evolutionary history (Thalmann et al. 2007). These differences between loci can challenge the delimitation of species via nuclear DNA, but can also be used to draw inferences about population size and subdivision, gene flow and hybridization (Nichols 2001), all of which play a role in generating new taxa and biodiversity. The use of multiple loci including nuclear and sex-specific markers in studying the evolutionary history of populations has already been applied in several other organsims (Carstens & Knowles 2007; Fischer 2004; Fischer et al. 2006; Hey & Nielsen 2004; Thalmann et al. 2007) apart from lemurs (for exceptions see Groeneveld 2009, 2010; Weisrock et al. 2010), and is highly recommended to obtain a realistic picture of the population history (Brumfield et al. 2003) and to adequately describe phylogenies at and below the species level (Hewitt 2001). Recent advances in sequencing technology provide the possibility for multilocus analyses, even of non-model species (for lemurs see Horvath et al. 2008). The use of multilocus sequence data requires different statistical procedures, which become more and more sophisticated. Likelihood and Bayesian summary statistics are now commonly used in phylogeographic and phylogenetic inference and replace older methods that rely on single gene trees (Beaumont & Rannala 2004; Brito & Edwards 2008).

Using Bayesian structure analysis (Pritchard et al. 2000) and the Genealogical Sorting Index (gsi) (Cummings et al. 2008) Weisrock et al. (2010) confirmed the high number of *Microcebus* species using several nuclear markers, although species were not reciprocally monophyletic. In contrast, using also several nuclear markers in combination with morphological data, Groeneveld et al. (2009, 2010) reduced the number of *Cheirogaleus* species from 7 to 4, indicating the suitability to delimit species with several types of information (Alstrom et al. 2008; Balakrishnan 2005; Dayrat 2005; de Quieroz 2005; Payne & Sorenson 2007; Rach et al. 2008; Sanders et al. 2006; Seppä et al. 2011; Sites & Marshall 2004). For example, morphologically distinct mouse lemurs (Rasoloarison et al. 2000) could be confirmed as separate species with genetic data (Yoder et al. 2000). Similarly, Zimmermann et al. (2000) and Nietsch & Kopp (2000) have emphasized the suitability of vocalizations for species delimitation in non-human primates, and this type of data has been used to clarify the taxonomy of tarsiers, for example (Groves & Shekelle 2010). Whatever these data might be, genetic samples, morphological measurements or other types of data should be sampled in a way that intraspecific variation can be

assessed and compared to interspecific variation before new species are described.

Why lemur taxonomists have not used the above-mentioned criteria to delimit species is only speculative, but one reason might have been that collecting high quality samples for DNA analyses from many individuals is anything but easy. Furthermore, the methods to extract nuclear DNA from low quality samples such as fecal or museum samples and sequencing those at low costs as well as nuclear primers were only recently developed (Horvath et al. 2008). Finally, from a conservation perspective, the urgent need to protect several highly threatened areas in Madagascar may have favored splitting species over lumping as well.

**Conclusions**

We conclude that PAA and genetic distances are inappropriate singular methods to delimit lemur species. Furthermore, we encourage the use of several nuclear and sex- specific genetic loci as well as the combination of different datasets for species delimitation. Populations that are considered to be different species should be sampled in a way that intraspecific variation can be compared with interspecific variation. Recently described lemur species should be critically re-evaluated, and we predict a taxonomic deflation for several genera.

# 3 True lemurs...true species? - Species delimitation using multiple data sources in the brown lemur complex

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**Abstract**

Species are the fundamental units in evolutionary biology. However, defining them as evolutionary independent lineages requires integration of several independent sources of information in order to develop robust hypotheses for taxonomic classification. Here, we exemplarily propose an integrative framework for species delimitation in the "*brown lemur complex*" (BLC) of Madagascar, which consists of seven allopatric populations of the genus *Eulemur* (Primates: Lemuridae), which were sampled extensively across northern, eastern and western Madagascar to collect fecal samples for DNA extraction as well as color photographs and vocal recordings. Our data base was extended by including museum specimens with reliable identification and locality information. Between-group analyses of principal components revealed significant heterogeneity in skull shape, pelage color variation and loud calls across all seven populations. Furthermore, post-hoc statistical tests between pairs of populations revealed considerable discordance among different data sets for different dyads. Despite a high degree of incomplete lineage sorting among nuclear loci, significant exclusive ancestry was found for all populations, except for *E. cinereiceps,* based on one mitochondrial and three nuclear genetic loci. Using several independent lines of evidence, our results confirm the species status of the members of the BLC under the general lineage concept of species. More generally, the present analyses demonstrates the importance and value of integrating different kinds of data in delimiting recently evolved radiations.

## 3.1 Introduction

Species are the fundamental units in biology (Fujita et al. 2012; de Queiroz 2005; Naomi 2011). In fact, species are the fundamental units of comparisons in all fields of biology, including anatomy, behavior, ecology, molecular biology or physiology, underlining the importance of taxonomic studies for all biological disciplines (de Queiroz 2005; Sites & Marshall 2004; Tobias et al. 2010; Wiens & Penkrot 2002). Furthermore, species are also the currency for biodiversity classification and define regions of conservation priority, so-called biological hotspots (Agapow et al. 2004; Balakrishnan 2005). Despite their fundamental importance and widespread application, identifying, defining and delimiting species is still one of the most disputed and controversial tasks in evolutionary biology (Pavlinov 2013).

Dozens of species concepts have been formulated, but none of them seems to be operational for every individual taxon (see Claridge et al. 1997; Coyne & Orr 2004; Mallet 2001; Mayden et al. 1997; Pavlinov 2013). De Queiroz therefore proposed a definition of species that is in agreement with all modern species concepts. Under this so-called general (metapopulation) lineage concept (GLC), the conceptualization of the notion of species and the operational criteria necessary to delimit them became separated (de Queiroz 1998; de Queiroz 2005). Instead of using a single operational criterion, such as monophyly or interbreeding, seeing species as separately evolving metapopulation lineages through time offers and highlights the importance of using multiple lines of evidence for their delimitation (Bacon et al. 2012). In fact, different criteria can lead to important biases in estimates of biodiversity, especially in macroevolutionary and conservation studies depending on species lists (Agapow et al. 2004; Isaac & Purvis 2004), and are expected to give incongruent results for the boundaries of recently evolved radiations (Bacon et al. 2012; Leaché et al. 2009). However, evaluating multiple lines of evidence not only increases our capacity to detect recently diverged populations, but also can provide stronger evidence of lineage separation when different operational criteria are in concordance (Dayrat 2005; de Queiroz 2007).

The fauna of Madagascar has enjoyed a constant increase in species numbers in recent years. Descriptions of newly discovered species from all vertebrate groups were based on various criteria for species delimitation, however (Andriaholinirina et al. 2006; Craul et al. 2007; Gehring et al. 2011; Goodman et al. 2011; Groeneveld et al. 2009; Louis et al. 2006; Radespiel et al. 2008; Wollenberg et al. 2008; Weisrock et al. 2010). In this context, an almost threefold increase in the number of endemic primate species (Lemuriformes) over the last three decades has been questioned by several authors (Groeneveld 2008; Markolf et al. 2011; Tattersall 2007). For example, newly described lemur species have been delimited solely based on minor variation in mitochondrial DNA (summarized in Markolf et al. 2011). Moreover, sampling per "species" was often limited to one locality encompassed by a pair of Madagascar's larger rivers. Thus, we have limited information on intraspecific genetic variation across a species' geographic range, so that the documented extent of mtDNA divergence might just be a result of local population structure. Other taxa have been subject to taxonomic revision without new data and were raised to species level (Groves 2001a) solely based on the application of the phylogenetic species concept (PSC) in favor of the the biological species concept (BSC). These

taxonomic revisions, especially in the genus *Eulemur*, were based on little evidence (Tattersall 2007), as we outline in the following.

Based on behavioral, anatomical and cytogenetic evidence, Simons and Rumpler (Simons & Rumpler 1988) erected and defined the genus *Eulemur* by splitting the former genus *Lemur* into two taxa, one containing only *Lemur catta* and the other containing the "true lemurs“, *Eulemur coronatus, E. mongoz, E. rubriventer, E. macaco, E. fulvus fulvus, E. f. albifrons, E. f. collaris, E. f. albocollaris, E. f. rufus* and *E. f. sanfordi.* A further subspecies, *E. f. cinereiceps,* was resurrected by Groves (2001a) based on a drawing by Milne-Edwards from 1890. More recent investigations revealed that this taxon is identical to *E. albocollaris* and thus the older name *E. cinereiceps* was adapted for this taxon (Johnson et al. 2007).

Although hybridization occurs between wild *E. f. rufus* and *E. mongoz* (Pastorini et al. 2009), lineage separation of *E. coronatus, E. macaco, E. mongoz* and *E. rubriventer* from each other and from the *E. fulvus* group is considered to be significant by most authors (Johnson 2006, Tattersall 2007) due to frequent sympatry, smaller social units and greater phenotypic differences. The remaining *Eulemur* taxa were treated as subspecies of the common brown lemur (*Eulemur fulvus*) and grouped into the polytypic BLC (Tattersall 1982), also referred to as the "brown lemur complex“ (BLC) (Wyner et al. 1999). Species status for *E. f. albocollaris (cinereiceps)* and *E. f. collaris* was later proposed by Wyner et al. (1999), although both taxa hybridize with *E. f. rufifrons* (Jekielek 2004; Wyner et al. 2002). In fact, hybrids of *E. cinereiceps* and *E. collaris* are not able to produce fertile offspring, but both taxa can produce fertile offspring with other members of the BLC. Although a number of studies tried to resolve the phylogeny among *Eulemur* taxa using morphology (Groves & Eaglen 1988; Groves and Trueman 1995; Tattersall & Schwartz 1991), loud calls (Macedonia & Stanger 1994), hair banding patterns (Macedonia & Shedd 1991), chromosomal banding patterns (Djelati et al. 1997; Rumpler et al. 1989) or molecular genetics (Delpero et al. 2006; Horvath et al. 2008; Pastorini 2000; Pastorini et al. 2003; Pozzi et al. 2006; Wyner et al. 2000; Yoder & Yang 2004), phylogenetic relationships among *Eulemur* taxa, especially among the members of the BLC remain unresolved. Nevertheless, Groves (2001a) elevated all members of the BLC to species status without new evidence or new data.

Groves (2001a, pp. 74-75) justified his decision to split *E. fulvus* into 7 species as follows: *"What one can insist on is full species status for what are currently regarded as subspecies of E. fulvus. These species are not only sharply distinct externally, but they*

*also appear to differ consistently in craniodental characters (Tattersall & Schwartz 1991). Two of them, collaris and albocollaris (cinereiceps), have unique DNA sequences and are already acknowledged as diagnosably distinct entities (Wyner et al. 1999). There is no evidence of overlap in phenotypic character states among members of the group, so they qualify as species under the PSC; there is little or no evidence that they form a genetic continuum in the wild, so they also qualify under the BSC."*

However, Tattersall & Schwartz (1991, p. 17) stated: *"...so little of that 'craniodental' variation can be made pertinent to relationships within the group. Clearly we are dealing with a high degree of homoplasy."* Thus, apparently homoplastic characters have been used to delimit species under the PSC. Moreover hybridization *sensu* "a genetic continuum" is not only likely between members of the BLC, but has also been suggested for *E. rufifrons* and *E. fulvus* at Betsakafandrika (Lehmann & Wright 2000) and *E. albifrons* with either *E. fulvus along the Mananara*-Zahamena corridor or with *E. sanfordi* north of the Bemarivo (Mittermeier et al. 2006). Thus, it appears that there is more evidence that species of the BLC form a genetic continuum in the wild than not, and explicit tests of overlap in phenotypic character states are still lacking. While all taxa may be said to represent potential new species, because of remarkable phenotypic differences of males, none of them can yet be shown to have speciated (Tattersall 2007).

Considering the poorly justified decision to split the subspecies of the BLC into seven different species, the main aim of this study was to test this taxonomic hypothesis with new data, and to critically appraise the conceptual and empirical approaches used in delineating these and other lemur species using an approach for species delimitation that covers intraspecific variation of hypothesized lineages for multiple independent data sets. With the present paper we aim to contribute to the topic of species delimitation in recently diverged populations in general, while clarifying the taxonomy of the BLC using several lines of evidence. The usefulness of each type of data for delimiting populations of the BLC can be characterized as follows:

*Genetic data*

Several studies have investigated the phylogenetic relationships of the members of the *Lemuridae* (Delpero et al. 2006; Pozzi et al. 2006) without completely resolving the relationships within the BLC. Moreover, these studies used either only mitochondrial DNA (Pastorini et al. 2003) or included not all taxa or only one specimen from captivity (Horvath et al. 2008; Perelman et al. 2011; Yoder & Yang 2004;) in their analyses, which limits their

usefulness for delimitation of natural taxa. Therefore, we analyzed one mitochondrial and three nuclear introns to infer species boundaries of natural populations, using phylo- and population genetic methods.

*Morphology*

Several authors, including Groves & Eaglen (1988), Tattersall & Schwartz (1991) and Groves & Trueman (1995), investigated cranidodental features of the *Lemuridae* without resolving relationships between members of the BLC. Later, Viguier (2002) claimed that skull disparity is more controlled by geography than by phylogeny, confirming the homoplasy found in previous studies. Because sample size for taxa of the BLC was quite small in the latter study, we revisit the morphology of lemur skulls, using a geometric morphometric approach.

*Acoustic data*

Vocalizations in non-human primates are predominantly innate and may thus provide an additional trait for species delimitation. Loud or long distance calls represent the most distinctive calls in the vocal repertoire and are common in most primates (Wich & Nunn 2002). They typically have a species-specific acoustic structure and have therefore been used to infer phylogenetic relationships (Konrad & Geissmann 2006; Mendez-Cardenas et al. 2008; Merker et al. 2009; Nietsch & Kopp 1998; Pozzi et al. 2009; Thinh et al. 2011; Zimmermann et al. 2000). Macedonia & Stanger (1994) investigated the phylogeny of the *Lemuridae* based on loud calls which often, but not always, consist of an introducing series of short explosive elements (chucks), followed by a long lasting scream (croak). These authors found considerable variation in what they called "disturbance advertisement calls" between members of the BLC, but they lumped all of them together for practical purposes so that variation among members of the BLC remains unknown.

*Pelage coloration*

Based on genetic data and pelage coloration of a single type specimen of *E. f. rufus*, this taxon was split into two species: *E. rufus* occurring north of the Tsiribihina river and *E. rufifrons* south of it (Groves 2006). There are indeed phenotypic differences in pelage coloration among the members of the BLC, but a quantitative comparison of variation within and between populations has not been conducted so far.

Using new data from the field in combination with museum specimens, we examined variation in all four traits among the members of the BLC in order to assess the validity of all species assignments as well as to evaluate the usefulness and consistency of these four data sets in delineating species.

## 3.2 Methods

We collected data from 34 different field sites in Madagascar (Fig. 3.1). Sampling localities were *a priori* chosen based on published distribution data of *Eulemur* species. We sampled at least 3 different populations per target taxon to cover intraspecific variation, except for *E. cinereiceps*. Additional data were collected in 5 national history museums (Appendix Tab. 1-3) to further increase sample size for genetic (mtDNA) analyses, and to obtain measurements on skull morphometry and fur coloration. Only museum specimens that could unequivocally be assigned to a taxon based on their phenotype, genetic characteristics or confirmed locality were included in the analyses.

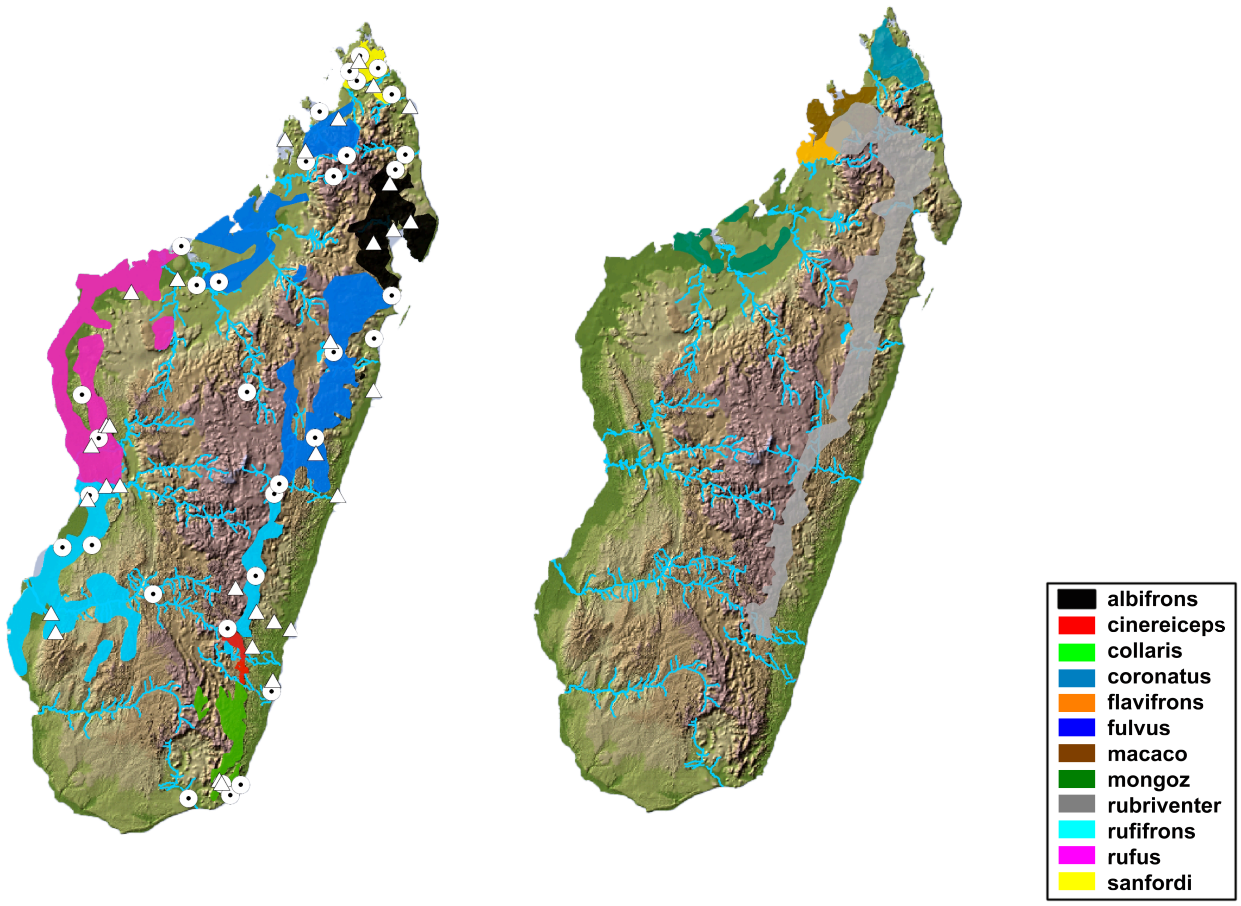



**Fig. 3.1: Maps of Madagascar showing the distribution of the members of the genus *Eulemur*** left= members of the *fulvus* group with our sampling localities, right= remaining members of the genus (right). Triangles= Museum samples, circles= field samples. A color legend is shown at the right.

### Acoustic data

In total, we analyzed 1170 loud calls from 24 *Eulemur* populations. Loud-calls were elicited by presenting species-specific loud calls given during group encounters via a loudspeaker (Davidactive, Visonik) and a Marantz digital solid state recorder (PMD 660; sampling rate: 44.1 kHz, 16 bit amplitude resolution) hidden in the vegetation. Vocalizations were recorded with a Marantz and a Sennheiser directional microphone (K6 power module and ME66 recording head with MZW66 pro windscreen; Sennheiser, Wedemark, Germany). Vocalizations were digitized using AVISOFT-SASLab pro 5.0.07 (R. Specht, Berlin, Germany). We visually inspected and sampled only calls of good quality and low background noise at a sampling frequency of 44.1 kHz. As loud calls often, but not always, consist of an introducing series of short explosive elements (chucks), followed by

a long lasting scream (croak), croaks and chucks were processed and analyzed separately. A spectrogram of a typical loud call is given in Appendix Fig. 1.

Single calls were submitted to a fast Fourier transformation (1024-pt FFT; time step: 5 ms; frequency range: 22.05 kHz; frequency resolution: 21 Hz) with AVISOFT-SASLab pro. Frequency-time spectra were analyzed with LMA 9.2, a custom software tool to extract different sets of variables from acoustic signals (Schrader & Hammerschmidt 1997). We focused on acoustic variables that characterize the general call structure and are comparable with acoustic variables that were measured in other studies characterizing the structure of mammalian vocalizations (Fichtel & Hammerschmidt 2002; Fichtel *et al.* 2005; Fichtel in press; Gros-Louis *et al.* 2008; Manser 2001). Also, we briefly describe the acoustic variables that were used for the analysis. We measured the mean duration, the mean frequency range, the mean central frequency (DFA2) and the first and second dominant frequency bands, as well as the percentage of time of the call in which the 3rd dominant frequency could be identified (Schrader & Hammerschmidt 1997). Acoustic variables entered in the analysis were revealed by Pearson's correlation analysis. We excluded variables exhibiting a correlation coefficient higher than 0.8; the remaining variables were retained and entered into the analysis.

Due to high variation in the number of calls available for each individual, we used the mean for each individual for further statistical analysis. Between-group analysis of principal components (bgPCA) was used to infer and visualize separation between taxa. BgPCA allows to separate and maximize within-group and between-group variation. This is similar but superior to discriminant function analysis (DFA), because DFA needs more cases than variables to reliably discriminate between groups (Mitteroecker & Bookstein 2011). Significance of group separation was afterwards tested using a randomization test with 999 randomizations. BgPCA and randomization were conducted with the Ade4 package in R (r-project.org). To identify significant differences between pairs of, we conducted a permutational MANOVA (PERMANOVA) with the program PAST (Hammer et al. 2001) on the first four principal components of the bgPCA. Significance levels were corrected using the false discovery rate (FDR) (Benjamini & Hochberg 1995) in R.

**Morphometric data**

High resolution (18 Megapixels, RAW format) digital photographs of the ventral view of skulls were taken with a Canon 7d digital camera, a Sigma lens (70-200mm) and with help of a photographic stand. To avoid distortion, which is higher at the fringe of the lens,

photos were taken with a distance of 90 cm between the work space of the photographic stand and the sensor of the camera and with a focal length of 200mm. Skulls were placed in the centre of the image together with a ruler. Use of modeling paste and a water level assured orientation in the horizontal plane. The program tpsDIG (Rohlf 2004) was used to place 17 homologous landmarks on the ventral view of the skull. Landmarks (Appendix Fig. 2) were afterwards subjected to generalized procrustes superimposition in R, using the function procGPA of the shapes package (Dryden 2013). Generalized procrustes superimposition scales, centers and rotates raw coordinates to reduce size differences between objects. BgPCA and a subsequent randomization test on the superimposed coordinates were applied to decompose intra- and interspecific variation and to test for differences between species. The function testmeanshapes of the shapes package in R was used to test for pairwise difference between taxa with subsequent FDR correction of p-values.

**Fur color data**

Following the method of Bergman & Beehner (2008), raw digital photographs of the dorsal view of museum skins were taken with the same equipment as mentioned above. Pictures were intentionally underexposed to avoid clipping of color channels (Stevens et al. 2007). Focal length was reduced to 70mm, and a color chart (MiniColorChecker, Munsell) was included in each photo to control for differences in ambient light conditions. To determine color variation, each image was opened with the raw converter in Photoshop CS5 and all parameters were set to zero, except for the temperature, which was set to 5100K for all photos. Using the PictoColor plugin (www.pictocolor.com), we applied a new color profile to each photo based on the 24 colors of the color checker chart.

We measured three areas of each skin by taking the Red, Green and Blue value (RGB) of an area of 50 x 50 pixels with the help of the rectangular marking tool (Appendix Fig. 3.). One area was a combined measure of two squares of 50 x 50 pixels of the dorso-lateral torso of each specimen. The second area was located on the meso-dorsal stripe that some taxa possess and the third on the centre of the head. Grids and reference lines were used to control for homologous positions of the rectangles in each specimen. Mean RGB values were noted down in an Excel sheet for each area for further statistical analysis. BgPCA with subsequent permutational MANOVA on the first two principal components was conducted to test for pairwise difference between taxa.

**Genetic data**

More than 500 individual fecal samples were collected from eulemurs in the field from 2008-2011. Feces were stored on silica gel and/or 90% ethanol. After completion of fieldwork, feces were stored at 4°C until DNA extraction. Genomic DNA from the fecal samples was extracted using the QIAamp DNA Stool kit DNA (Qiagen) with a slightly modified protocol as follows. Samples were run for 24 hours at room temperature on a lab rotator in ASL buffer and only a 1/2 InhibitEx tablet was used for 600 µl supernatant of ASL-Buffer. Additionally, centrifugation steps of Qiagen spin columns were done at 8000 rpm instead of 13000 rpm as suggested in the Qiagen protocol. The same sample was sometimes extracted two or three times, which still resulted in sufficient amount of genomic DNA for PCR.

DNA extraction and subsequent PCR for the museum samples was done at a different institution (Abt. Historische Anthropologie, Universität Göttingen) under strict conditions for contamination prevention following Hummel (2003), such as separation of pre- and post-PCR laboratories and the use of disposable protective clothing, glasses, and disposable gloves. Further, all experiments took place with disposable laboratory ware, such as pipette tips and cups, while workbenches and other laboratory equipment were cleaned with detergents (AlconoxTM Detergent, Aldrich, Germany), bi-distillated water and ethanol before use for each sample. Automatic DNA extraction of these samples was done with the QIAGEN EZ1 robotic station and the QIAGEN EZ1 DNA Tissue Kit.

Whereas the whole (1140bp) cytochrome B gene was analyzed for fecal samples, only a shorter fragment of 223 bp was analyzed for the museum samples consisting of tissue remnants on skulls or pieces of the skin. Primers, PCR mixtures and annealing temperatures are listed in Appendix Table 4 and 5. We used Roche High Fidelity Taq Polymerase for amplification of DNA extracted from feces and the Qiagen Multiplex PCR plus Kit for the extractions of ancient DNA from museums.

*Nuclear DNA*

Three nuclear introns were sequenced, using 454 amplicon sequencing on a Roche GS Junior 454 Sequencing platform, which allows to directly score both alleles in a diploid individual without extensive cloning procedures. However, prior to sequencing, amplicon libraries have to be constructed and each amplicon requires its own combination of MID tags to assign individuals to the correct sequence after pooling all amplicons for emulsionPCR and subsequent sequencing. A two-step PCR procedure was used to

construct amplicon libraries of the three introns nramp (natural resistance macrophage protein), vwf (van willebrand factor) and eno (enolase). Initially, target-specific primers (Appendix Tab. 4) were designed with help of published sequences from Horvath et al. (2008) and Perelman et al. (2011). These primers were equipped with a universal tail (M13) for the first PCR. After control on an agarose gel, PCR products were purified using magnetic beads (Beckmann and Coulter), and purified products were diluted with Molecular Biology Grade Water to approximately equimolar (5-20 ng/µl) concentrations for the next PCR. Primers for the second PCR included the GS Junior Titanium fusion primer sequences, 1-10 different MIDs for both forward and reverse primers and the template-specific sequence, which in our case were the universal tails of the previous PCR. This approach allowed us to use only 10 different forward and reverse fusion primers to individually tag 10x10=100 individuals for all three introns. The second PCR was run with the same conditions as the first. For the rest of the procedure we followed the GS junior Amplicon Library Preparation Method Manual, the GS Junior emPCR Amplification Method Manual Lib-A and the GS Junior Sequencing Method Manual from Roche.

*Genotyping of individuals*

After initial quality filtering and processing (i.e. adaptor removal) by the Roche/454 GS Junior software, further preprocessing was carried out by custom Perl scripts. First, sufficiently long reads were selected that perfectly matched a pair of barcode (MID) tags. Target-specific primers were removed that need to be found at the 5' and (as reverse complement) at the 3’ end. All reads from the same gene locus were moved to a separate file. Then each sequence file was compressed by (a) removing (duplicate) reads with a perfectly identical copy in the same individual, and (b) noting the number of read copies in the FASTA comment, together with the individual identifier (corresponding to the MID tag pair).

After preprocessing, the unique sequences were aligned in SeaView (Gouy et al. 2010, using the muscle alignment option and subsequent manual inspection for each intron separately. Sequences were sorted by individual in Geneious 4.5 (Biomatters). As 454 sequencing is prone to sequencing errors, specifically chimeras and insertion/deletion errors due to homopolymers (Gilles et al. 2011), we used the following protocol to infer the correct genotypes from all variants:

- All sequences with <10-fold coverage were discarded from the dataset.

- Insertions/deletions that occurred only in one non-duplicate sequence in the whole dataset of a gene locus were discarded from the dataset, because they were likely to be a consequence of homopolymers.
- Variants of each individual were sorted for coverage and checked for chimeras. If one of the sequences was likely to be a chimera of the sequences with highest coverage, they were discarded.
- The two sequences with highest coverage were finally taken as the true alleles for diploid individuals, if more than one sequence was left in the end.

*Phylogenetic analyses*

Final alignments for each locus were produced with SeaView and manually inspected by eye. The best fitting substitution models were calculated for each locus with jModeltest2 (Darriba et al. 2012) and chosen based on Akaike's Information Criterion (AIC). Haplotypes were collapsed using FaBox (Villesen 2007) and translated into a genotype matrix for population genetics analyses. Input files for different software packages were also created with help of the web server GALAXY (Goecks et al. 2010) and Microsoft Excel.

For the combined analysis of the cytb of museum and field samples, a simple Neighbor Joining Tree was calculated using the pairwise deletion option in SeaView with 10.000 bootstraps. Phylogenetic trees for the cytb without museum samples and the three nuclear loci were estimated separately using MrBayes 3.2.1. (Ronquist et al. 2010). In all analyses, we used two runs with four Monte Carlo Markov Chains (MCMC), the default temperature of 0.2, 10.000.000 generations and a sampling frequency of 1000. After a burn-in of 25% we retained 15.002 trees. Substitution model parameters were adjusted as before according to the results from jModeltest. The program Tracer and the uncorrected potential scale reduction factor (PSRF, should approach one) in MrBayes were used to check for the adequacy of the burn-in and sufficient convergence of the Markov chains.

We calculated the genealogical sorting index (gsi) (Cummings et al. 2008) to quantify exclusive ancestry of lineages. The gsi ranges form zero to one, where zero indicates complete lack of divergence and one indicates monophyly. As the significance of the gsi statistic is measured through randomizations of group labels across the tips in a rooted gene tree, hypothesized lineages are tested against the null hypothesis of no divergence. Therefore, significance of the gsi statistic indicates exclusive ancestry of

lineages, whereas the value of the gsi measures the degree of lineage divergence. The gsi was calculated separately and combined for all loci using the Bayesian phylogenetic trees.

As phylogenetic trees are often not appropriate to illustrate relationships due to reticulate evolution or incomplete lineage sorting, we calculated statistical parsimony haplotype networks for the three nuclear loci using NETWORK 4.611 (www.fluxus-engineering.com) (Polzin & Daneshmand 2003).

*Population structure*

We used two population genetic methods to test for population structure with the nuclear genotype matrix. STRUCTURE version 2.2 (Pritchard et al. 2000, 2007) was used for Bayesian clustering of individuals into populations. To infer the correct number of K (clusters), 20 independent runs of 1.000.000 generations and a burn-in of 250.000 generations was used in an admixture model with correlated allele frequencies from K=1-20. The number of K was inferred over all runs with STRUCTURE HARVESTER (Earl & von Holdt 2011) after the ln likelihood of the data and after the method of Evanno et al. (2005). CLUMPP (Jakobsson & Rosenberg 2007) was used to permute over all runs for a given K, and assignment probabilities were plotted in R.

Discriminant Analysis of Principal Components (DAPC) (Jombart et al. 2010) of the adegenet package in R was used to infer the probability of individuals belonging to predefined phenotypic species. DAPC is a multivariate method to infer the genetic structure of populations. The advantage of this method is that it does not assume Hardy Weinberg Equilibrium and linkage disequilibrium as STRUCTURE and other population genetic clustering methods, which is likely to be violated in most natural populations (Jombart et al. 2010). The alpha score was used to choose the number of retained principal components and subsequent discriminant functions in order to avoid over-fitting of the data by retaining to many principal components as suggested by the manual.

## 3.3 Results

### Acoustic data

Results for the bgPCA of chucks and croaks are depicted in Fig. 3.2. The overall randomization test of between-group differences was significant ($p < 0.001$) for both call types. However, pairwise comparisons (Appendix Tab. 6) between taxa of the PERMANOVA ($p < 0.001$) revealed only significant differences between two dyads *(E.*

*collaris* - *E. fulvus* and *E. collaris*- *E. rufifrons)* for croaks. In contrast, chucks were significantly different between more species pairs. Whereas *E. collaris* was significantly different from all other taxa, *E. albifrons* and *E. cinereiceps* showed the fewest significant differences in pairwise comparisons. In general, the decomposition of the total variance in between-group and within-group variation revealed that only 33% of the total variation in chucks was explained by variation between taxa. Between-group variation was even lower (25%) for croaks. This pattern is well reflected by extensive overlap of groups in the scatter plots for both call types and shows that most variation in both call types is explained by intra-specific variation.

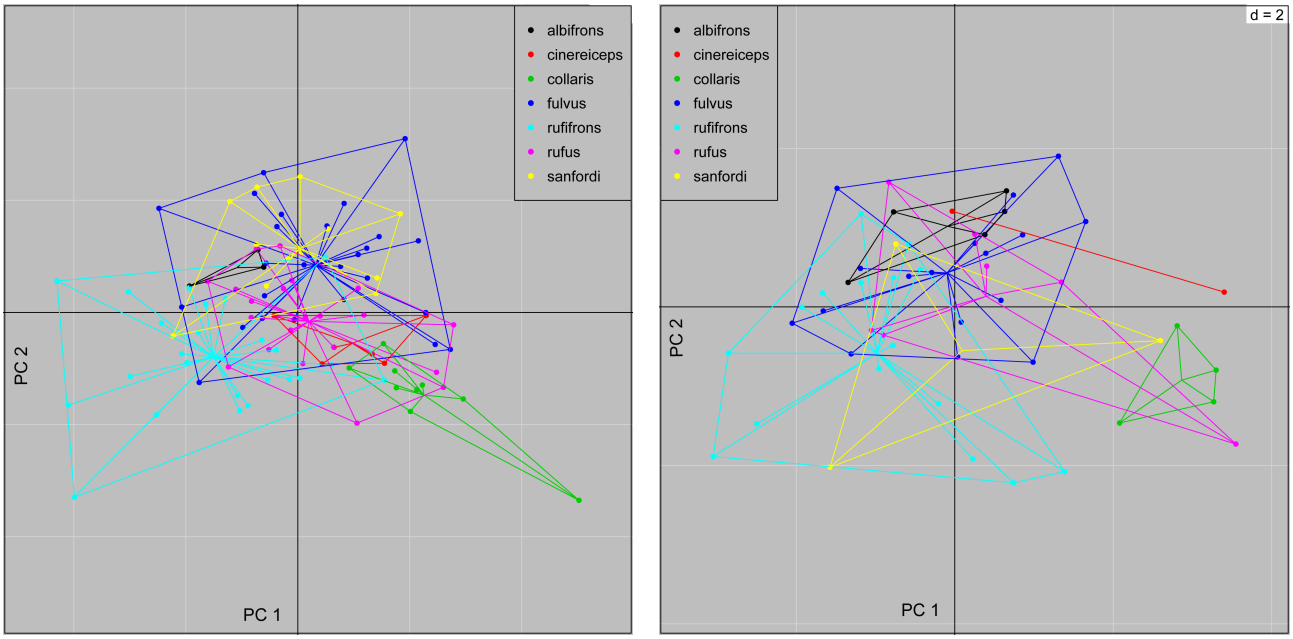



**Fig. 3.2: Scatter plot of bgPCA for chucks (left) and croaks (right).** Points represent individuals along the first and second principal component. A color legend for the different species is given inside the plot. p= < 0.001 (999 randomizations)

**Morphometric data**

Figure 3.3 shows the scatterplot of the bgPCA of procrustes shape coordinates of the members of the BLC. For comparative reasons we included also the three more distantly related taxa *E. coronatus*, *E. mongoz* and *E. rubriventer* for the morphological shape analysis (Appendix Fig. 4). Variance decomposition revealed that variation is much higher within (87%) than between (13%) groups. Nevertheless, the overall randomization test of between-group differences was significant (p<0.001). Results of pairwise comparisons are presented in Appendix Tab. 7. *Eulemur cinereiceps* was only significantly

different from the three smaller bodied *E. coronatus, E. mongoz* and *E. rubriventer,* but not from any of the members of the BLC. *Eulemur sanfordi* did also not differ significantly in shape from *E. albifrons, E.collaris*, *E. fulvus* and *E. rufus.* However, p-values between the geographically adjacent taxa *E. albifrons* and *E. fulvus* approached significance with p=0.068 and p=0.05, respectively. *Eulemur rufus* could not be distinguished from *E. fulvus* and *E. rufifrons* based on shape analyses. Finally, *E. coronatus, E. mongoz* and *E. rubriventer* were significantly different from each other and differed from all members of the BLC (see Appendix Fig. 4).

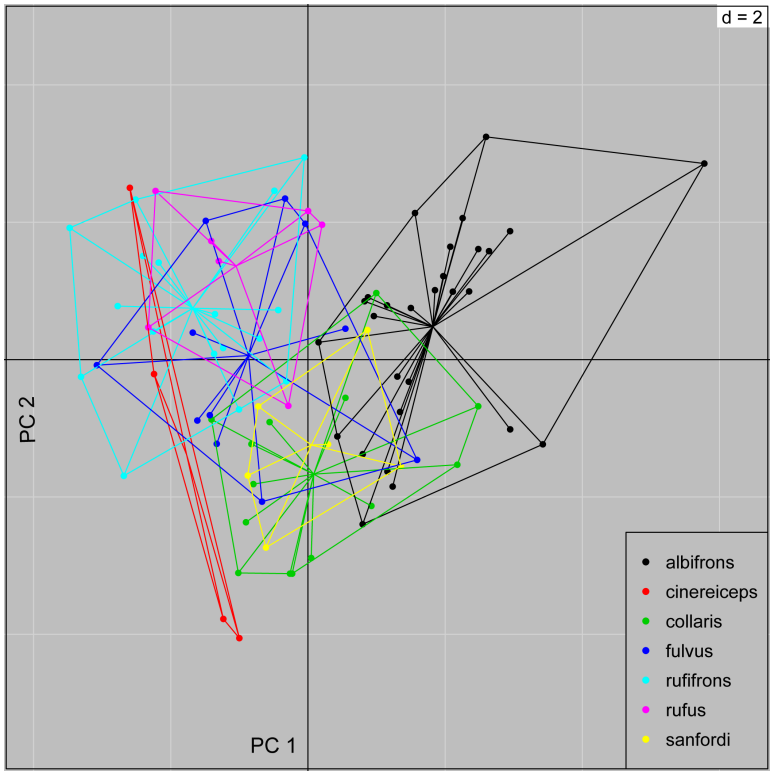



**Fig 3.3:** Scatterplot of bgPCA of morphological shape analysis. Points represent individuals along the first and second principal component. A color legend for the different species is given inside the plot. p= < 0.001 (999 randomizations)

**Pelage coloration**

Variance decomposition of the pelage coloration data revealed that in males 64% and in females 50% of the variation is explained by differences between groups. The overall test of difference between groups was significant (p<0.001). As expected from widespread sexual dichromatism, differences were more pronounced in males (Fig. 3.4).

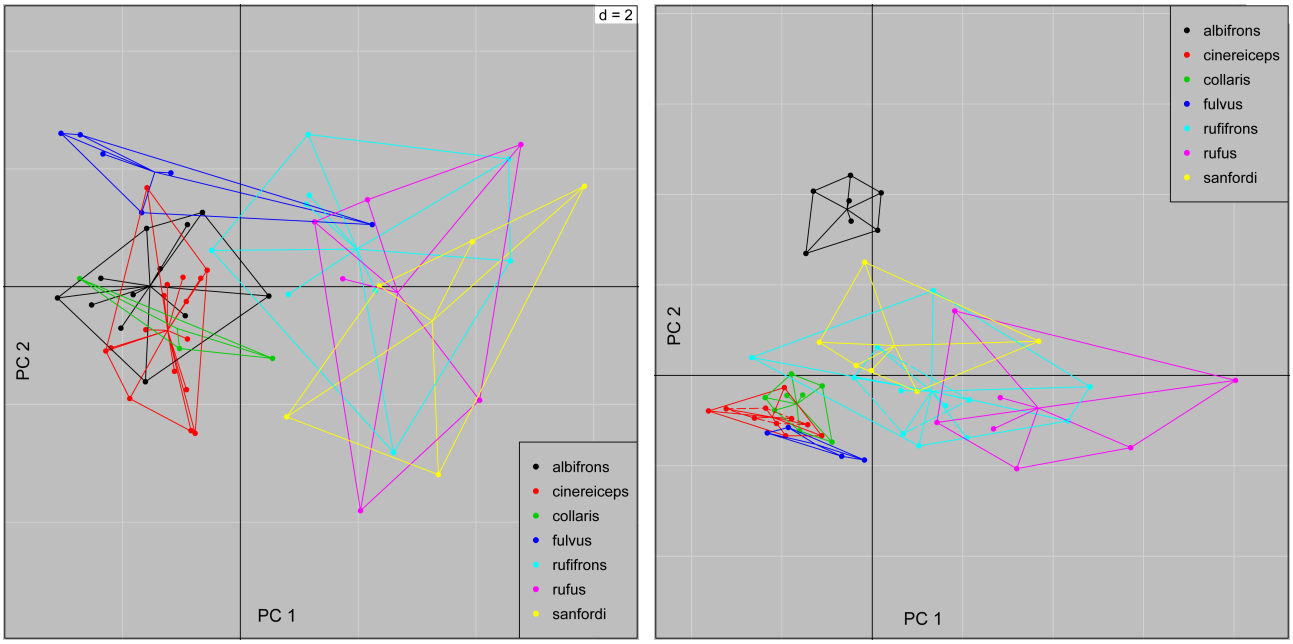



**Fig. 3.4: Scatterplot of bgPCA of female (left) and male (right) pelage coloration.** Points represent individuals along the first and second principal component. A color legend for the different species is given inside the plot. p= < 0.001 (999 randomizations)

Subsequent pairwise comparisons significantly differentiated males of *E. albifrons* from all other taxa (Appendix Tab. 8) Female *E. albifrons*, however, were not different from *E. cinereiceps* and *E. collaris*, but from the geographically adjacent *E. fulvus* and *E. sanfordi.* In contrast to Groves (2006), who postulated female color differences between *E. rufus* and *E. rufifrons,* the present analysis revealed massive overlap and no significant differences between females, but between males. *Eulemur cinereiceps* was also significantly different from its neighbors, i.e. *E. collaris* and *E. rufifrons.*

## Genetic data

*Sequence data*

In total, sequence data were generated from 123 field samples. Due to high variation in the amount of genomic DNA from feces, we were unable to sequence all four loci for all individuals. Missing data are indicated in Appendix Tab. 1. The complete cytochrome B of 1140 basepairs(bp) had 57 individual haplotypes and 318 polymorphic sites. The smaller fragment of 223 bp was sequenced for additional 32 museum specimens and had 42 polymorphic sites. The number of alleles/haplotypes for the three

nuclear loci were 56 for the vwf locus, 49 for the eno locus and 26 for the nramp locus, respectively (Tab. 3.1). The vwf locus had a total length of 288 bp with 56 polymorphic sites and contained two indels of one bp, one indel of 2-3 bp and one indel with seven bp. The eno-locus was 231 bp in length, contained two indels of one and two bp, one indel of three bp and had 28 polymorphic sites. The nramp- locus was 290 bp in length had 25 polymorphic sites and contained one indel. Table 3.1 shows the minimum, maximum and mean coverage for the individual genotyping of the three nuclear loci. Overall, there was high mean coverage of individual alleles for all loci. The AIC of JModeltest found the best fit of the cytb loci with a HKY+I+G model. The eno and vwf loci best fitted a TPM2uf+I (analyzed with GTR+I in Bayesian analysis) and a HKY+G model was favored for the nramp locus.

**Tab. 3.1: Summary of Next Generation Sequencing data**

| NGS sequencing data | | coverage per individual alleles | | | |
|---|---|---|---|---|---|
| locus | # of alleles | mean | min | max | indels |
| vwf | 56 | 107 | 10 | 781 | 4 |
| eno | 49 | 144 | 11 | 8678 | 5 |
| nramp | 26 | 355 | 22 | 973 | 1 |

*Phylogenetic analyses*

The Bayesian tree of the complete cytb is shown in Fig. 3.5. The monophyly of the BLC is strongly supported (Bayesian PP=1.0). There was strong support for the monophyly of *E. coronatus, E. mongoz, E. macaco, E. flavifrons* and *E. rubriventer.* The relationships among clades were only poorly supported. Within the *BLC*, we found *E. rufus, E. rufifrons* and *E. collaris* to be monophyletic. *Eulemur cinereiceps, E. fulvus, E. sanfordi* and *E. albifrons* were polyphyletic. However, the individuals of *E. cinereiceps* from Andringitra are known to be hybrids (Delmore et al. 2011) of *E. rufifrons* and *E. cinereiceps*.

The phylogenetic tree including museum samples revealed the same pattern as the Bayesian phylogenetic tree without museum samples. Most individuals were found in the expected clade based on their museum labels. Museum samples of *E. albifrons,*

*E. sanfordi* and *E. fulvus* confirmed the polyphyletic pattern described above (Appendix Fig. 5).

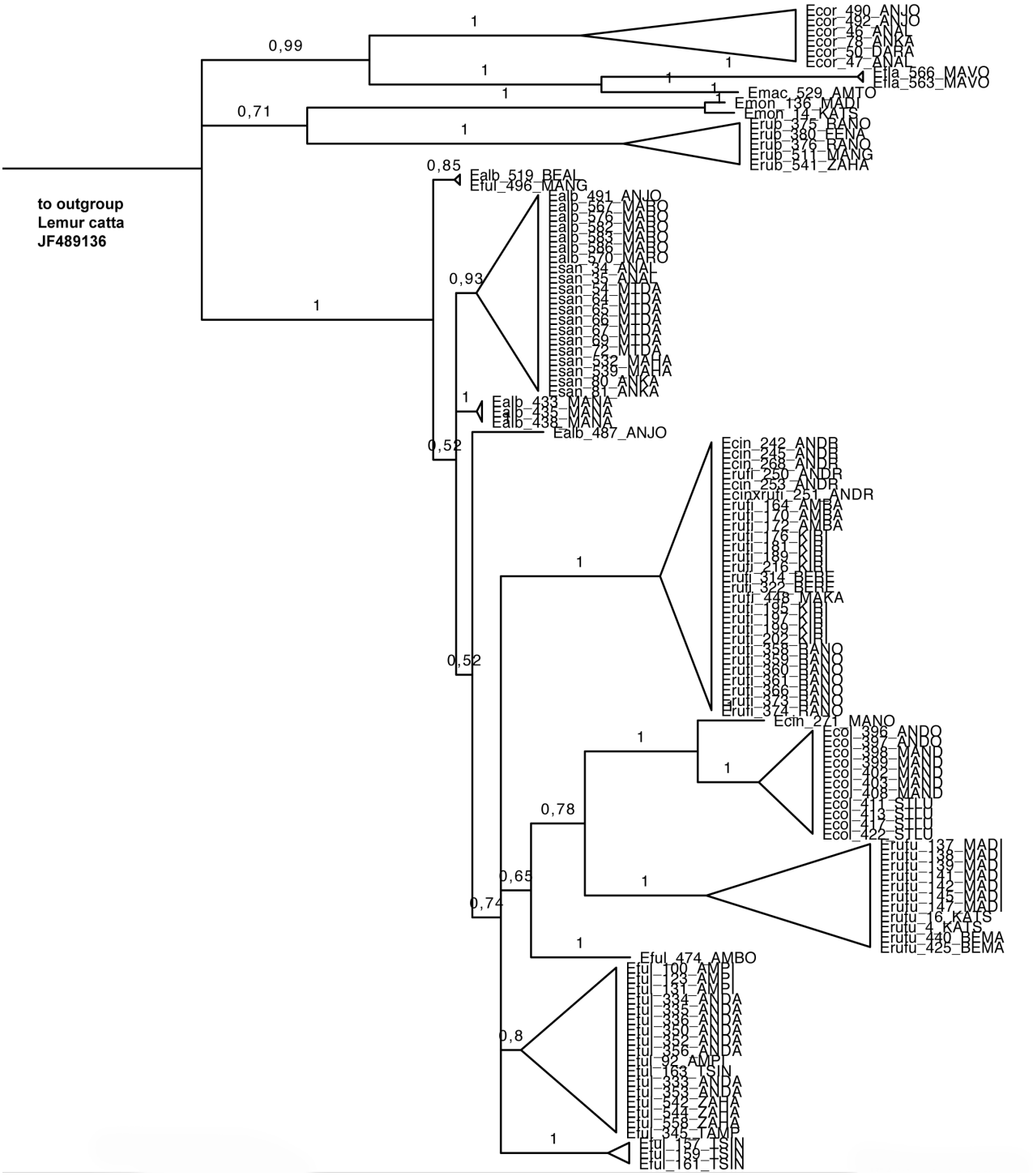



**Fig. 3.5: Simplified bayesian tree of the complete cytb gene of field samples.** Labels include the designated phenotype followed by an individual identifier and an abbreviation of the sampling locality. Bayesian posterior probabilities are give along branches of corresponding nodes.

Bayesian gene trees for the three nuclear loci (Appendix Fig. 6a-c) showed no congruence with phylogenetic relationships revealed by the cytb locus. Although *E. coronatus, E. mongoz, E. macaco*, and *E. rubriventer* clustered together for most of the nuclear loci, phylogenetic relationships among themselves and in relation to the BLC remained unresolved. This pattern was confirmed by the statistical parsimony haplotype networks depicted in Fig. 3.6 a-c for the three nuclear loci. *Eulemur coronatus, E. mongoz, E. rubriventer* and *E. macaco* showed more species-specific distinct haplotypes and did not cluster together in the network. One individual of *E. mongoz* (27) shared haplotypes with members of the BLC. This individual was sampled in Katsepy and is a hybrid *E. mongoz* x *E. rufus.* (see Pastorini et al. 2009). Some individuals labeled as *E. flavifrons* clustered within the BLC. However, we have no phenotypic information on these individuals form Manongarivo; thus they could also represent *E. fulvus.* Among the members of the BLC, we did not find any pattern corresponding to the relationships revealed by the mtDNA analyses. Several haplotypes are shared by members of different species, indicating incomplete lineage sorting for all three nuclear loci.

The genealogical sorting index showed considerable variation across loci and hypothesized lineages. Nonetheless, measures of exclusive ancestry over all loci (gsiT) were significant for all lineages except *E. cinereiceps* (Tab. 3.2.) and support lineage divergence. A gsi of 1 (= monophyly) was only estimated for several taxa for the cytb locus and for *E. mongoz* for the eno and vwf loci and for *E. rubriventer* and *E. coronatus* for the vwf locus, indicating substantial incomplete lineage sorting for our genetic loci.

**Tab. 3.2: Genealogical sorting index (gsi) and p- values based on 10.000 permutations for the Bayesian consensus trees of all 4 loci and the combined statistic gsiT over all loci.** x= no estimate.

| Species | gsi-cytb | p | gsi- eno | p | gsi-nramp | p | gsi- vwf | p | gsiT | pT |
|---|---|---|---|---|---|---|---|---|---|---|
| coronatus | 1,00 | < 0,001 | 0,04 | 0,09 | 0,79 | < 0,001 | 1,00 | < 0,001 | 0,71 | < 0,001 |
| flavifrons | 1,00 | 0,03 | 0,00 | 0,86 | 0,07 | 0,22 | 0,23 | < 0,01 | 0,33 | < 0,001 |
| mongoz | 1,00 | < 0,01 | 1,00 | < 0,001 | 0,50 | 0,01 | 1,00 | < 0,001 | 0,62 | < 0,001 |
| macaco | x | x | 0,01 | 0,69 | 0,24 | < 0,001 | 0,04 | 0,65 | 0,32 | < 0,001 |
| rubriventer | 1,00 | < 0,001 | 0,69 | < 0,001 | 0,74 | < 0,001 | 1,00 | < 0,001 | 0,86 | < 0,001 |
| albifrons | 0,70 | < 0,001 | 0,03 | 0,63 | 0,04 | 0,65 | 0,09 | 0,14 | 0,21 | < 0,001 |
| fulvus | 0,73 | < 0,001 | 0,12 | < 0,001 | 0,18 | < 0,001 | 0,18 | < 0,001 | 0,30 | < 0,001 |
| sanfordi | 0,91 | < 0,001 | 0,51 | < 0,001 | 0,06 | 0,10 | 0,20 | < 0,001 | 0,42 | < 0,001 |
| cinereiceps | 0,17 | 0,04 | 0,02 | 0,44 | 0,03 | 0,38 | 0,01 | 0,94 | 0,06 | 0,25 |
| rufifrons | 0,85 | < 0,001 | 0,38 | < 0,001 | 0,20 | < 0,001 | 0,28 | < 0,001 | 0,43 | < 0,001 |
| collaris | 1,00 | < 0,001 | 0,25 | < 0,001 | 0,29 | < 0,001 | 0,14 | < 0,01 | 0,42 | < 0,001 |
| rufus | 1,00 | < 0,001 | 0,33 | < 0,001 | 0,19 | < 0,001 | 0,23 | < 0,001 | 0,44 | < 0,001 |

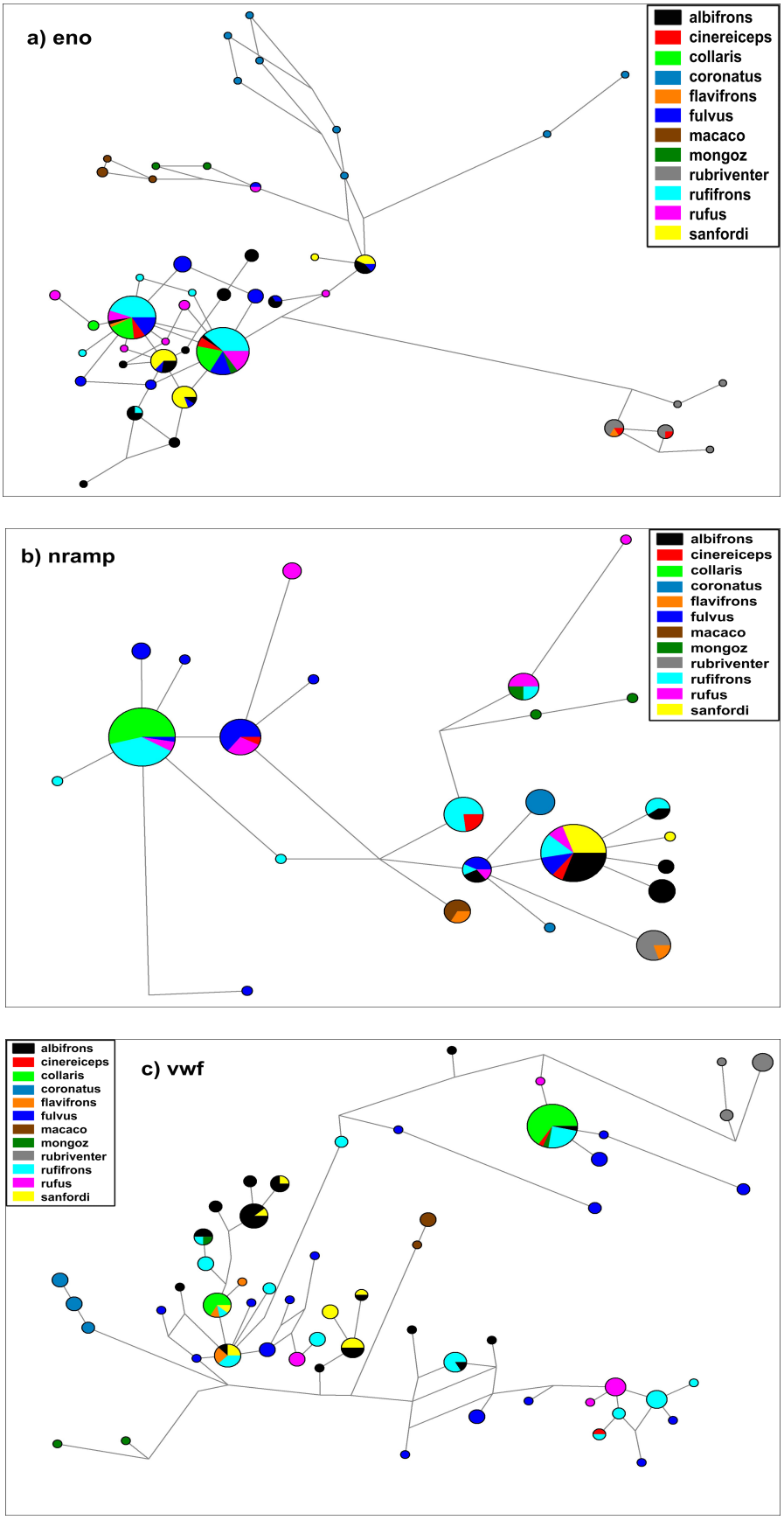



**Fig. 3.6a- c: Statistical parsimony haplotype networks.** Each circle represents a different haplotype. Colors indicate the species determined after phenotype or locality. Haplotype frequency corresponds to the size of the circles and length of the branches roughly correspond to the evolutionary distance between haplotypes.

*Population structure*

Bayesian population structure analysis for the members of the BLC favored a K=2 for the number of populations after the method of Evanno et al. (2005) and a K=3 after the estimated ln probability of the data. Assignment plots for both K are shown in Fig. 3.7. For K=2, with exception of individuals 271 and 322, all individuals of *E. albifrons, E. fulvus, E. rufus* and *E. sanfordi* were assigned to one cluster, and individuals of *E. cinereceps, E. collaris* and *E. rufifrons* formed a second cluster. For K=3, individuals of *E. albifrons* and *E. sanfordi* clustered together, and individuals of *E. cinereiceps, E.collaris* and *E. rufifrons* as well as individuals of *E. fulvus* and *E. rufus* showed east-west connections (see Fig. 3.1).

Results of the Discriminant Analysis of Principal Components (DAPC) on the haplotype matrix for the BLC are shown in Fig. 3.8. The optimal alpha score suggested retention of six principal components and five discriminate functions. Most individuals could be assigned with high probability to their respective taxon. However, there was also clear evidence for a mixed nuclear genetic composition of *E. albifrons*, *E. fulvus* and *E. sanfordi*, and *E. cinereiceps, E. fulvus, E. rufus* and *E. rufifrons. Eulemur collaris* were best discriminated; *E. cinereiceps* worst. However, three out of the four *E. cinereiceps* samples were from the hybrid zone of Andringitra.

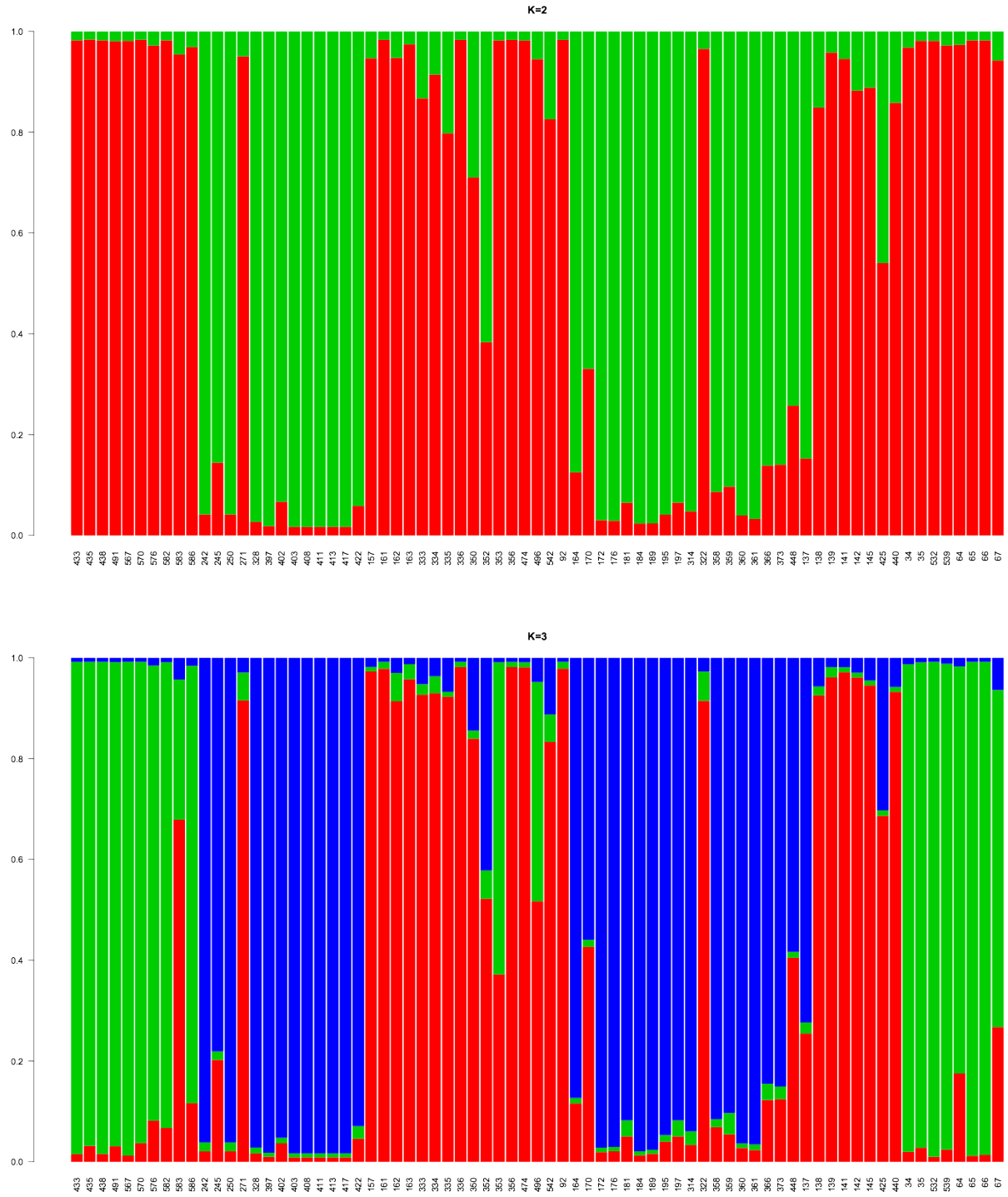
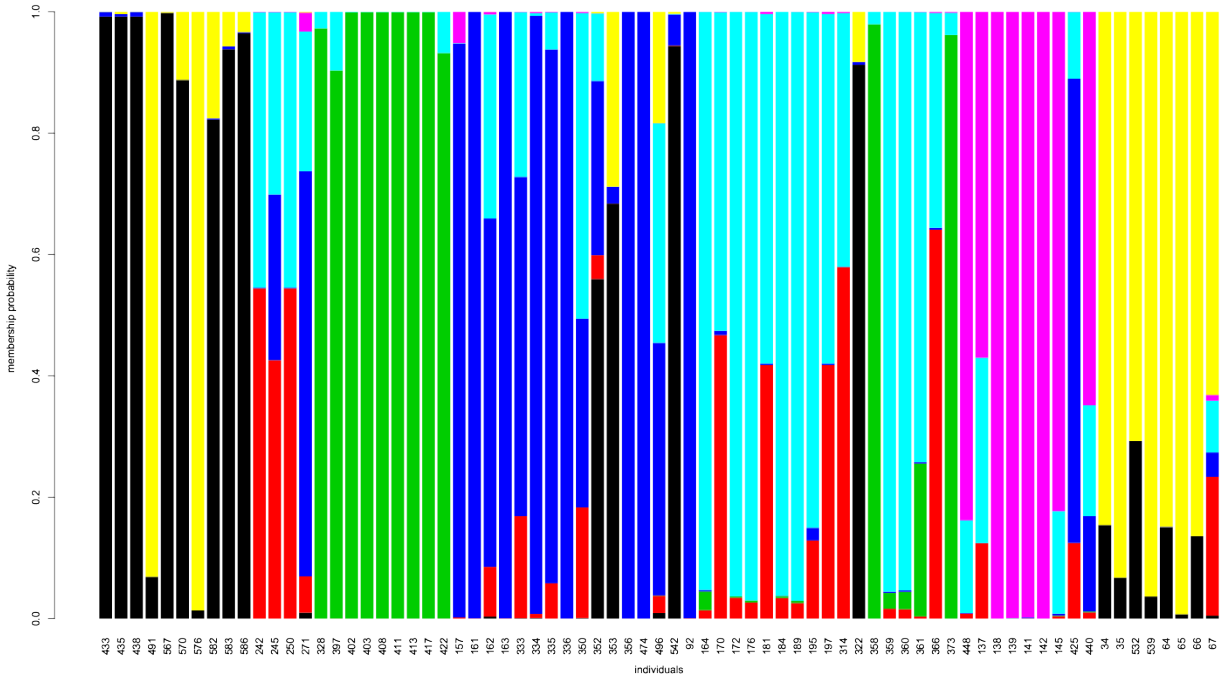



**Fig. 3.7:** Assignment probabilities of individual memberships to each cluster for K=2 and K=3. The y-axes depict the assignment probabilities of each individual to one of the clusters. The x-axes show individuals in alphabetical order from left to right. *E. albifrons* =433- 586, *E. cinereiceps*= 242-271, *E. collaris*= 328-422, *E. fulvus*= 157-92, *E. rufifrons*= 164- 448, *E. rufus*= 137-440 and *E. sanfordi*= 34-67**.**



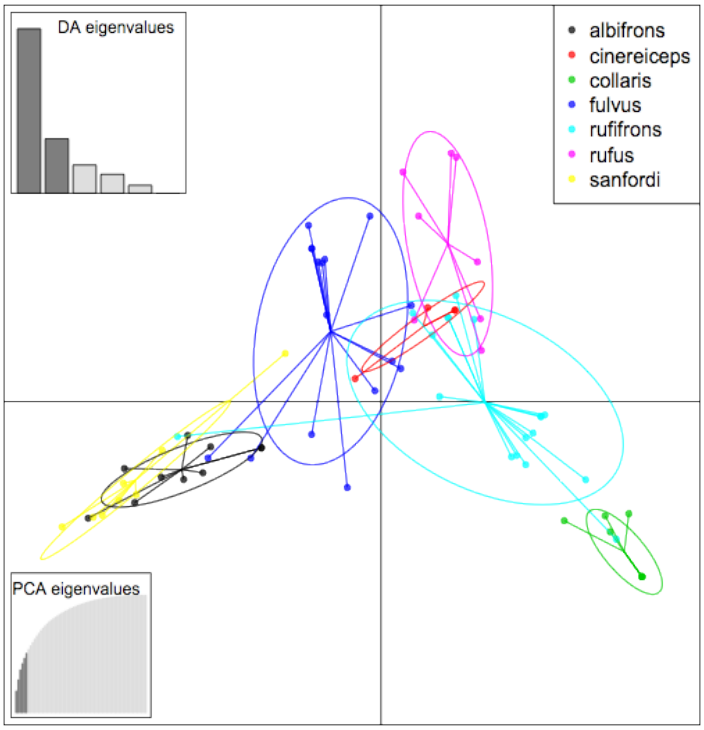




**Fig. 3.8: Discriminant analysis of principal components.**
Top: Assignment probabilities of individuals to their taxon based on 3 nuclear loci of the DAPC. The y-axis depicts the assignment probabilities of each individual. The x-axis shows individuals of taxa in alphabetical order from left to right. *E. albifrons* =433- 586, *E. cinereiceps*= 242-271, *E. collaris*= 328-422, *E. fulvus*= 157-92, *E. rufifrons*= 164- 448, *E. rufus*= 137-440 and *E. sanfordi*= 34-67.
Left: Scatterplot of DAPC with 95% confidence ellipses and number of retained principal components and discriminant functions. A color legend for both graphs is also depicted.

### Integration of all analyses

Table 3.3 summarizes the results of four different datasets and shows significant results of pairwise comparisons for morphological data, pelage coloration and acoustic parameters as well as the gsi statistic. Overall, our analyses revealed significant divergence between lineages of the BLC in all four datasets. However, the different datasets showed also considerable variation in their ability to discriminate between our predefined groups, especially in subsequent pairwise comparisons of taxa. BgPCAs of morphological shape and acoustic parameters showed that most variation in the data is explained by intraspecific variation.

**Tab. 3.3: Summary of the results of pairwise comparisons of four independent datasets.** Orange= Genetic (gsi), blue= morphology, green= pelage coloration, yellow= loud calls. Please note that we did not performed pairwise comparisons using genetic data. Therefore, we indicate significance of exclusive ancestry assessed by the gsi statistic. *E. cinereiceps* is indicated with a question mark as the gsi statistic was not significant, but this taxon was only poorly represented in our sampling and most samples were collected from the hybrid population at Andringitra.

| Species | *cinereiceps* | | *collaris* | | *fulvus* | | *rufifrons* | | *rufus* | | *sanfordi* | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *albifrons* | ? | | | | | | | | | | | |
| | | | | | | | | | | | | |
| *cinereiceps* | | | ? | | ? | | ? | | ? | | ? | |
| | | | | | | | | | | | | |
| *collaris* | | | | | | | | | | | | |
| | | | | | | | | | | | | |
| *fulvus* | | | | | | | | | | | | |
| | | | | | | | | | | | | |
| *rufifrons* | | | | | | | | | | | | |
| | | | | | | | | | | | | |
| *rufus* | | | | | | | | | | | | |
| | | | | | | | | | | | | |

For this reason, morphological shape and acoustic parameter analyses found also the smallest number of significant differences among species in pairwise comparisons. In contrast, variation in pelage coloration, especially in males, could be explained to a high degree by between-group variation, and consequently revealed significant differences between almost all species pairs. All species, except *E. cinereiceps* showed significant exclusive ancestry for the cytb locus, but also after inclusion of the three nuclear genetic loci. Monophyly of the species of the BLC for the cytb locus, however, is only evident for *E. collaris*, *E. rufus* and *E. rufifrons* (excluding the hybrids from Andringitra). Overall, results of the genetic analyses indicate a substantial amount of incomplete lineage sorting within the BLC, especially for the nuclear loci. This is shown independently by discordance among the Bayesian clustering results of STRUCTURE and the DAPC as well as in the nuclear gene trees and networks. Morphological (see Appendix. Fig. 4) and genetic divergence of *E. coronatus, E. mongoz, E. rubriventer, E. macaco and E. flavifrons* is much more pronounced than among the members of the BLC.

As geographic and phylogenetic relationships between taxa of the BLC are crucial for a taxonomic decision, we briefly summarize results for geographically adjacent populations.

*E. albifrons and E. fulvus have adjacent geographical populations* at the high plateau of Tsaratanana in central northern Madagascar and along the east coast between the National Parks Mananara Nord and Zahamena (see also Fig. 3.1). *Eulemur sanfordi* is supposed to be separated by the Maevarano du Nord river from western *E. fulvus* populations and by the Bemarivo river from southern populations of *E. albifrons.* All three can potentially meet at the headwaters of the Tasaratanana massif and/or crossing rivers. Individuals seen at Tsaratanana resemble phenotypically *E. fulvus,* but had a mixed genetic composition (ID 496). All three are significantly different in male and female coloration. Additionally, *E. albifrons* differs significantly from *E. fulvus* in shape. *Eulemur fulvus* and *E. sanfordi,* and *E. fulvus* and *E. albifrons* seem to differ also in shape, although not significantly so (p=0.05 and p=0.068). Additionally, *E. sanfordi* had high gsi values for the cytb and eno loci, suggesting independent evolution for this lineage.

*Eulemur rufifrons* is geographically adjacent to *E. rufus* in western Madagascar and to *E. fulvus* and *E. cinereiceps* in eastern Madagascar. Furthermore, *E. collaris* and *E. rufifrons* are supposed to hybridize at Berenty. Excluding hybrids from Andringitra,

*E. cinereiceps* is different in mtDNA from *E. collaris* and *E. rufifrons,* and differs from both in acoustic loud calls (chucks). Differences in female and male pelage coloration of *E. cinereiceps* and *E. rufifrons were also significant. Eulemur collaris* and *E. rufifrons* showed significant differences in all 4 datasets and *E. rufifrons and E. rufus* differed significantly in pelage coloration genetics and acoustic parameters. Finally, *E. rufus* and *E. fulvus* differed significantly in mtDNA, female and male coloration and loud calls (chucks).

## 3.4 Discussion

In this study, we investigated the ability of an integrative approach for the delimitation of species of a recently evolved radiation in order to falsify hypothesized lineages, in this case of the *Eulemur fulvus* (Groves 2001a, 2006). Results clearly indicate the difficulties and discordances that can arise among and within different criteria that are frequently used to delineate taxa. Moreover, our results highlight the necessity for a detailed and geographically broad sampling in order to effectively compare intra- and inter-specific variation of hypothesized lineages. In the following, we discuss our results in relation to the taxonomy of the BLC, as well as the significance of the discordances among data sets and their consequences for species delineation in this and other taxonomic groups.

### How many species of true lemurs are there?

Lineage divergence occurs when populations accumulate contingent properties, such as reciprocal monophyly for different genes, distinctive ecological or morphological characters, reproductive isolation or adaptive behavioral traits (de Queiroz 1998). As speciation is a temporal process, these different contingent properties may not begin to accumulate at the same during the lineage separation process. In fact, different contingent properties often yield conflicting results, especially in recent or adaptive radiations (Leaché et al. 2009; Wake 2006). Using different contingent properties to delimit species, however, can lead to more robust evidence of lineage separation when they are concordant (Dayrat 2005; de Queiroz 2007). In this study we combined multiple lines of evidence for the delimitation of seven allopatric populations of the BLC across the island of Madagascar. This evidence comprised data from mitochondrial and nuclear DNA as well as comparisons of phenotypes in skull shape, pelage coloration and call structure.

Under the general lineage concept of species, we found evidence for the lineage divergence of all seven taxa formerly considered as subspecies of *Eulemur fulvus.* These lineages seem to have diverged very recently in allopatry, probably triggered by climatic shifts during the late Pleistocene (Markolf et al., in prep). As eulemurs are ecologically highly flexible and occupy most biogeographic regions of Madagascar (Johnson 2006), it can be assumed that genetic drift is the main mechanism generating the observed divergence of those lineages and that ecological selective processes presumably played a less important role (Ossi & Kamilar 2006). Therefore, we cannot assume that lineages that are separated by hundreds of kilometers, such as *E. collaris* and *E. albifrons*, but occupy similar ecological niches necessarily accumulate strong differences in skull morphology or call structure. Hence, it seems reasonable to make taxonomic decisions based on lineage divergence of geographically adjacent and phylogenetically closer related lineages (see also Markolf et al., in prep). Following this approach, with the exception of *E. cinereiceps, E. albifrons* and *E. sanfordi,* we found evidence from three independent types of data supporting the delimitation of the taxa of the BLC as separate species.

However, *E. albifrons and E. sanfordi* were not only significantly different in male pelage coloration, but also in female coloration, a pattern not expected considering the fact that females of these two species can be hardly distinguished externally. Both species had significant gsi test statistics, indicating lineages divergence. Moreover, *E. sanfordi* had very high gsi values for the cytb and the eno loci, and DAPC could assign most *E. sanfordi* individuals with high probability to the respective cluster, suggesting exclusive ancestry for this taxon. A very recent split between these two taxa along with several past migration events (Markolf et al., in prep.) seem to be responsible for a high degree of incomplete lineage sorting and less divergence in other traits analyzed here. Individual 491, treated as *E. albifrons* in our analyses, was assigned with high probability to *E. sanfordi*. In fact, we lack phenotypic information for this sample, and it may well represent *E. sanfordi* as it was sampled north of the Bemarivo. Unfortunately, security issues did not allow us to sample the area north of the Bemarivo more extensively. Thus, it remains unresolved whether *E. sanfordi* is distributed south up to the Bemarivo river, but species status is warranted. At least the museum sample from Vohemar clusters with *E. sanfordi,* indicating that this taxon had a much larger distribution than assumed today.

A clear taxonomic decision based on our data for *E. cinereiceps* is difficult. The sample from Manombo (271) clustered as a sister group to *E. collaris* in the mtDNA gene

tree. The rest of our samples were collected from the hybrid population of Andringitra (Delmore et al. 2011) and had mitochondrial haplotypes introgressed from *E. rufifrons.* Thus, genetically we have only one sample of "pure" *E. cinereiceps* from one locality and demarcation of this taxon based on genetics is difficult. Additionally, sample size was also very small for the museum samples and could be one explanation why *E. cinereiceps* was not found to be significantly different from any of the other members of the BLC in skull shape. However, *E. cinereiceps* differed in the acoustic structure of their chucks from adjacent *E. rufifrons* and *E. collaris,* and from *E. rufifrons* additionally in pelage coloration. Furthermore, *E. cinereiceps* and *E. collaris* have different chromosome numbers. They can therefore not produce fertile offspring (Dutrillaux & Rumpler 1977) and would consequently qualify as species under the BSC. Further genetic investigations of the hybrid zone at Andringitra, which might shed additional light on the pattern of lineage divergence of *E. cinereiceps* in relation to *E. rufifrons* are under way (Johnson, pers. comm.).

*Discordance among data sets*

We found considerable differences in the ability of different datasets to delimit among members of the BLC. None of the four data sets alone could provide enough evidence for lineage separation of all species. Genetic analyses and pelage coloration could discriminate between most members of the BLC, followed by morphological shape analysis and acoustics.

The weak discriminatory ability and low interspecific variation of the acoustic data set might be due to the structure of the calls. Most studies that used acoustic signals for species discrimination in primates analyzed calls with several syllables or even songs (Nietsch & Kopp 1998; Meyer et al. 2012; Thinh et al. 2011). Those signals show necessarily more variation due to the inherent structure of the call. Furthermore, as allopatric populations normally never meet, selective pressure on calls, even those used during intergroup encounters, is probably very low. In fact, acoustic group distances and genetic group distances estimated for the cytb (data not shown) were positively correlated, indicating that genetic drift might be mostly responsible for the small divergence in acoustic parameters.

The same can be assumed for the divergence of morphological shape, as allopatric populations occupy similar ecological niches. The large overlap of the members of the BLC in the bgPCA including the three smaller eulemurs (Appendix Fig 4.) confirms the extensive homoplasy found in previous studies (Tattersall & Schwartz 1991, Viguier 2002).

It can be argued that variation in pelage coloration might be influenced by environmental factors (Santana et al. 2012) and storing or preparation conditions of skins sampled in different museums. The same might be the case for acoustic variables that can be highly influenced by the environment and the distance to the animal during recordings (Maciej et al. 2011). To control for these potential errors, we used only mean values and those acoustic parameters that should be less influenced by the distance to the animal during recording (Maciej et al. 2011). And, prior to bgPCA, we run general linear models for both data types and included habitat (western dry forest and eastern humid forest) as well as museum for the color analysis as factor in the model. None of them had significant effects on the variables (data not shown). In general, data acquisition and analyses were conservative, and we aimed to cover as much intraspecific variation as possible. Therefore, we included only 17 landmarks for the analysis of shape that could be easily reproduced and placed on all available specimens. Because facial and ventral areas of museum skins were often in bad shape, areas for color measurements were chosen only on the dorsal view of the skins in order to avoid non-homologous placement of the measurement area and to cover variation of as many specimen as possible. Hence, color differences of males are definitely underestimated. As such, however, the method can be easily reproduced by other researchers even for different species.

One obvious drawback of our approach is that all four kinds of data could not be collected for the same individuals. Therefore, direct comparison or even combined analysis of morphological and genetic data such as offered in the software Geneland (Guillot et al. 2012) could not be conducted. On the other hand we showed that species delimitation using several kinds of data is possible even with a complete non- invasive sampling. Especially the amount of samples for genetic analyses could not have been collected with an invasive approach.

To the best of our knowledge, this is the first study that uses Next Generation Sequencing Technology to sequence multiple independent genetic loci from feces to infer

species boundaries in endangered or critically endangered primates. Following the conservative approach above, we intentionally used a high threshold to sort out potential genotyping errors. Under the assumption that sequence variants with errors occur less frequently in the dataset than sequence variants without errors, and that false alleles occur less frequently in individuals than true alleles (Galan et al. 2010), our filtering approach and a mean coverage per allele per individual ranging from 107- 355 among the three loci is unlikely to have produced false genotypes. In fact, after discarding sequence reads without both MIDs and unmatched target primers, most sequences were already filtered out. Among the remaining sequences most sequence errors turned out to be chimeras of the two most abundant sequences for an individual. Finally, that the nuclear dataset is unlikely to be influenced by genotyping errors is simply evident because of biological reasons. Although members of the BLC show a substantial mixed nuclear composition, the remaining *Eulemur* taxa have distinct haplotypes. This pattern was not necessarily expected, but confirms phylogenetic results of previous studies (Pastorini et al. 2003; Yoder & Yang 2004) and underlines the validity of our genotyping results.

Although we had known hybrids in the data and these species can hybridize in the wild and in captivity, the mixed nuclear composition of members of the BLC is more likely be a consequence of incomplete lineage sorting. With the exception of the individuals from the Andringitra hybrid zone there is no indication of any geographic locality with more admixed individuals as would be expected, if hybridization was the primary cause for admixed ancestry (Hewitt 2001). Nevertheless, the structure results of K=3 revealed mixed ancestry for *E. albifrons-E. sanfordi, E. collaris-E. cinereiceps-E. rufifrons* and *E. fulvus-E. rufus.* However, whether this pattern is due to incomplete lineage sorting among phylogentically closer related species or ongoing gene flow is beyond the scope of this article (but see, Markolf et al., in prep).

**Delimiting species with multiple data sources**

Using multiple lines of evidence, we showed that delimitation of members of recent radiations can be particularly challenging. Because different datasets can come to different conclusions about the status of species, the use of several independent data is highly recommended in order to avoid false positives. Because taxonomic classification can be

treated as a hypothesis that can be modified as new evidence accumulates (Groves 2000), several independent data sets allow much stronger tests of a given hypothesis.

Species delimitation in lemurs, however, has been recently criticized for relying too strongly on evidence from mtDNA alone or for using different secondary species concepts (*sensu* de Queiroz 1998; Markolf et al. 2011; Tattersall 2007). It is obvious that species delimitation based on pelage coloration or morphology alone will not be very promising in cryptic species. Nevertheless, there are other methods one could think of to falsify taxonomic hypothesis in cryptic species. Although not intended to clarify species boundaries, delBarco-Trillo et al. (2012) recently showed that chemical composition in scent marks between some eulemurs are significantly different from each other. Integrating this approach into the methods for species delimitation in lemurs would be particularly useful for many of the cryptic species, as scent marks may play a role in species recognition (Smadja & Butlin 2009). The same applies to visual and acoustic signals, whose meaning and function to the animals in this context can be tested experimentally (e.g. Kappeler 2012).

Lemurs are not the only group of mammals that has been subjected to a substantial increase in species numbers. The number of primates in general more than tripled during the last two decades (Tattersall 2007). In fact, the order primates has been completely revisited following the PSC (Groves 2001a), resulting of the elevation of many taxa from subspecies to species level without new data. A similar trend can be observed in many other mammalian orders (Agapow et al. 2004; Zachos et al. 2013), where similar biases have been introduced by the use off the PSC, as e.g. in ungulates (Groves & Grubb 2011). Although a discussion of species concepts is way beyond the scope of this article, the PSC, which was also used to give species status to the members of the BLC, has several shortcomings that make its application inappropriate for theoretical and practical reasons. Although there are many versions of the PSC, they all emphasize a common descent, mostly referred to as monophyly, in conjunction with diagnosability, such as "A species is the smallest diagnosable cluster of individual organisms within which there is a parental pattern of ancestry and descent" (Cracraft 1983). Diagnosability, however, can be achieved even for the smallest possible units that might well represent demes, populations or even family groups due to limited dispersal and reproduction among geographically close individuals of the same species (Avise 2000). Therefore, the PSC is very prone to overestimating species diversity based on local genetic structure, as has recently been

demonstrated with genetic data from wild mouse lemurs (Markolf et al. 2011). Cracraft (1998), for example, applied the PSC and proposed species status for the Sumatran tiger based on three diagnostic characters of the cytochrome b unique to tigers from Sumatra and different from all tigers from the mainland. Our three samples of *E. rufifrons* from Ambadira have three sites diagnosably distinct from sequences of the cytochrome b of *E. rufifrons* ~20 km to the south along continuous forest. Do they qualify as distinct species? They could under the PSC, but they definitively do not, if we consider that haplotypes of the cytb are shared among individuals from Kirindy and Ranomafana, which is more than 200 km apart and separated by Madagascar's deforested central highlands.

As evolution below and at the species level is shaped by population-level processes, taxonomic decisions require sample sizes that cover the whole intraspecific variation (Zachos et al. 2013). Furthermore, it has been shown repeatedly that gene trees (although this does also apply for trees build form other kinds of data) can substantially differ from the species tree (Camargo & Sites 2013; Knowles & Kubatko 2011; Nichols 2001). Considering this and the fact that evolution at the species level is often reticulate, monophyly, especially of single genes, is in general not a good criterion for species delimitation. Using multiple genes to estimate phylogenies and delimit species is becoming popular due to advances in sequencing technology, and several new coalescent-based methods for species delimitation have recently been developed (Ence & Carstens 2011; Fujita et al. 2012; Rannala & Yang 2013). These methods seem very promising for reliably identifying recently diverged lineages. However, any deviation from the standard coalescent model (e.g. panmixia, no gene flow) is likely to overestimate species diversity, and these methods should therefore also be complemented with standard methods from morphology, ecology or behavior (Camargo & Sites 2013).

As conservation organizations and national governments are relying strongly on the decisions of taxonomists to assess the value of protected areas or the allocation of resources for conservation, describing and raising species based on insufficient data can also be a waste of resources and additionally lead to false decisions concerning captive or natural breeding for conservation (Zachos 2013).

## Conclusions

We conclude that members of the brown lemur complex (formerly *Eulemur fulvus* ssp.) are best classified as species according to the general lineage concept of species. As different contingent properties can arise at different times during the lineage separation process and potentially lead to ambiguous conclusions, we suggest, independent of the species concept, the utility of several independent lines of evidence, coupled with field sampling that covers intraspecific variation of the taxa under study for the delimitation of species.

# 4 Phylogeographic analysis of the true lemurs (genus *Eulemur*) underlines the role of river catchments for the evolution of micro-endemism in Madagascar

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**Abstract**

Due to its remarkable species diversity and micro-endemsim, Madagascar has recently been suggested to serve as a biogeographic model region. However, hypothesis-based tests of various diversification mechanisms that have been proposed for the evolution of the island's micro-endemic lineages are still limited. Here, we test the fit of several diversification hypotheses with new data on the broadly distributed genus *Eulemur* using coalescent-based phylogeographic analyses. Time-calibrated species tree analyses and population genetic clustering resolved the previously polytomic species relationships among eulemurs. The most recent common ancestor of eulemurs was estimated to have lived about 4.45 million years ago (mya). Divergence date estimates furthermore suggested a very recent diversification among the members of the "brown lemur complex", i.e. former subspecies of *E. fulvus*, during the Pleistocene (0.33-1.43 mya). Phylogeographic model comparisons of past migration rates showed significant levels of gene flow between lineages of neighboring river catchments as well as between eastern and western populations of the redfronted lemur (*E. rufifrons*). Together, our results are concordant with the centers of endemism hypothesis (Wilmé et al. 2006, Science 312:1063-1065), highlight the importance of river catchments for the evolution of Madagascar's micro-endemic biota, and they underline the usefulness of testing diversification mechanisms using coalescent-based phylogeographic methods.

## 4.1 Introduction

Although biodiversity is higher in the tropics, most of our knowledge of species dynamics in space and time come from the northern hemisphere (Hewitt 2001, Posada et al. 2013). Climatic changes during the ice ages, however, also had profound effects on the history and formation of tropical species because cooler and drier periods during the Quartenary caused reduction of tropical forests and expansion of savannahs (Burney et al. 2004; Hamilton & Taylor 1991; Primack & Corlett 2005). As tropical regions are the placeholders and producers of great parts of biodiversity, there is an urgent need to study those regions (Hewitt 2001), and hypothesis-based statistical phylogeographic methods are particularly appropriate methods for this purpose (Chan et al. 2011, Hickerson et al. 2010; Knowles & Carstens 2007).

The fourth-largest island of the world, Madagascar, is renowned for its exceptional biodiversity and levels of endemism (Mittermeier et al. 1998; Myers et al. 2000). New species are still being regularly discovered, including plants, reptiles, fishes and mammals

(Thompson 2011). One hundred percent of amphibians, 90% of plants, 92% of reptiles and a the primate suborder *Lemuriformes* are endemic to the island (Goodman & Benstead 2003), highlighting Madagascar's importance for biodiversity studies and conservation efforts (Ganzhorn 2001; Myers et al. 2000). In addition, a large proportion of Madagascar's extant fauna is micro-endemic to small ranges within the landmass of the island (Goodman & Benstead 2003; Wilmé 2006; Vences et al. 2009).

The current understanding of the origin of Madagascar's exceptional faunal biodiversity and endemism is that most of the endemic lineages at higher taxonomic levels (families and genera) resulted from oversea dispersal from the African or Indian mainland starting about 65 mya (Yoder & Nowak 2006), whereas other faunal elements are remnants of the Gondwanian fragmentation during the Cretaceous when India-Madagascar broke off from Africa around 158-160 mya, from Antarctica around 130 mya and the separation of Madagascar from India around 84-96 mya (Briggs 2003; Samonds et al. 2013, Vences et al. 2009). Whereas the origin of these endemic genera and families in Madagascar is well explained by irregular colonization events from the African and Indian mainlands, the origin of Madagascar's micro-endemic biota is still in debate (Wilmé 2006; Pearson & Raxworthy 2009). Several mechanisms have been proposed to explain the diversification of Madagascar's extant fauna, recently reviewed by Vences et al. (2009).

An early model to explain species distributions in Madagascar was based on phytogeography, bioclimatic zonation of the island and the distribution of lemur species communities (Martin 1972; Ganzhorn et al. 2006) (Fig. 4.1 c- d). Following this model, the island was separated into eight zoogeographic regions and specifically highlighted the importance of the western dry and eastern humid habitats, as well as major rivers, to further divide similar climatic regions (Pastorini et al. 2003). Additional new evidence and changing phylogenies for several taxonomic groups over the last two decades, however, revealed considerable discordance between these zoogeographic regions and the biogeographic separation of Madagascar into an eastern and western domain (Pastorini et al. 2003; Ganzhorn et al. 2006; Yoder et al. 2000), leading to the formulation of new hypotheses.

Wilmé et al. (2006) proposed one hypothesis to explain the evolutionary history and regional speciation of Madagascar's forest biota based on the extant distribution of 35.400 vertebrate taxa and the watersheds associated with the island's rivers. After this so-called centers of endemism hypothesis, quarternary paleoclimatic variation played an important

role for the distribution and speciation of the extant Malagasy fauna. During periods of glaciation, cooler and dryer climates resulted in more arid conditions, forcing animals to retreat to refugia along river catchments. For habitats with rivers at low altitudes this would have lead to extensive isolation of coastal areas, creating centers of endemism, which allowed for allopatric speciation and the evolution of micro-endemic taxa (Fig. 4.1f). In contrast, watersheds of rivers with sources at high elevation, defined as retreat-dispersal watersheds, would have allowed dispersal along the river catchments to neighboring retreat-dispersal watersheds. As Madagascar has three major mountains along the eastern highlands above 2000 m (Fig. 4.1e), and the largest rivers of the west (Betsiboka, Tsirihbihina and Mangoky) as well as of the east (e.g. Manangoro) have their headwaters at the summits of those mountains, gene flow from the west to the east and *vice versa* would have been possible.

Pearson and Raxworthy (2009) proposed a climatic gradient model to explain local speciation patterns based on current distributions of lemurs, geckos and chameleons, and compared it to the centers of endemism hypothesis and a biogeographical null model. They found concordant distributions with either the centers of endemism or their current climate hypothesis, and suggested that multiple sources have played a role in the diversification of Madagascar's micro-endemic fauna.

In 2009, Vences et al. reviewed all currently proposed diversification hypotheses for Madagascar and formulated specific predictions to investigate the role of each model for the evolution of Madagascar's micro-endemic biota. They included five different speciation mechanisms that are also relevant in other parts of the world, which are shortly explained in the following (see Vences et al. 2009 for details). The 'ecogeographic constraint' model is identical to the one formulated by Martin (1972, see above) and assumes that an ecologically tolerant species occurs in different eco-geographic regions, whereas younger sister lineages to the former are more specialized and restricted to one of the eco-geographic regions (Fig. 4.1c-d). Lineages should correspond to eco-geographic regions and a east-west pattern should be evident. A variant of the eco-geographic constraint model, the 'western rainforest refugia' model, assumes that eastern species spread into western Madagascar during more humid times and become subsequently isolated in rainforest relict areas, which allowed for vicariant speciation. No gene flow from west to east can be predicted for this mode of speciation. The 'riverine barrier' model assumes rivers to act as barriers and allows for allopatric speciation. No gene flow between populations or species on both sides of a river can be expected from this model, but

species on one side of the river should be sister species to the ones on the other side of the river. The 'montane refugia' hypothesis is based on the assumption that isolated populations of a widely distributed species on high mountains during dry periods later diversified due to vicariant divergence. Sister species in a phylogeny would be distributed on neighboring massifs according to this scenario. Finally, the 'river catchments' hypothesis corresponds the centers of endemism hypothesis as proposed by Wilmé et al. (2006). For species distributed in retreat dispersal watersheds we can expect that gene flow occurred several times during pleistocene climatic variations and that speciation therefore should have occurred within the last ~5 million years (Vences et al. 2009, Wilmé et al. 2006). As for the 'riverine barrier hypothesis' species distributed in neighboring retreat dispersal watersheds should be sister species in a phylogeny.

Given the various diversification mechanisms, explicit hypothesis testing using either the whole Malagasy system (Vences et al. 2009) and/or specific radiations within the extant fauna, is now possible (but see Chan et al. 2011; Chan et al. 2012; Craul et al. 2007; Pearson & Raxworthy 2009; Rakotoarisoa et al. 2013, Wollenberg et al. 2008).

The genus of true lemurs (*Eulemur,* Simons & Rumpler 1988) has already been subject to various phylogenetic and biogeographic analyses (Goodman & Ganzhorn 2004; Ganzhorn et al. 2006; Thalmann 2007; Pastorini et al. 2003; Yoder & Yang 2004). The genus contains 12 species that are distributed over the remaining forest fragments of almost the entire island of Madagascar (Fig. 4.1a-b) (Johnson 2006; Mittermeier et al. 2010). Seven species, namely *E. albifrons, E. cinereiceps, E. collaris, E. fulvus, E. rufifrons, E. rufus and E. sanfordi,* long had unresolved phylogenetic relationships among each other and were traditionally classified as subspecies of the common brown lemur (*E. fulvus*) and collectively referred to as the 'brown lemur complex' (Wyner et al. 1999). Using multiple lines of evidence, Markolf et al. (in prep.) could recently show that all members of the 'brown lemur complex' qualify as true species under the general lineage concept of species (de Queiroz 1998), supporting an earlier suggestion by Groves (2001a).

The species of the 'brown lemur complex' are distributed in allopatric populations in a circle-like pattern along the remaining forest fragments of the island (Fig. 4.1a). The only biogeographic zones not inhabited by members of the 'brown lemur complex' are the central highlands and the south-western spiny forests (Johnson 2006). *Eulemur rufifrons* and *E. fulvus* have disjunct populations in eastern as well as western parts of the island. The remaining members of the genus (Fig. 4.1b), *E. coronatus, E. mongoz, E. rubriventer, E. macaco and E. flavifrons* occur in sympatry with one of the members of the 'brown

lemur complex' and exhibit much greater genetic divergence among each other and to the members of the brown lemur complex (Markolf et al., in prep.).

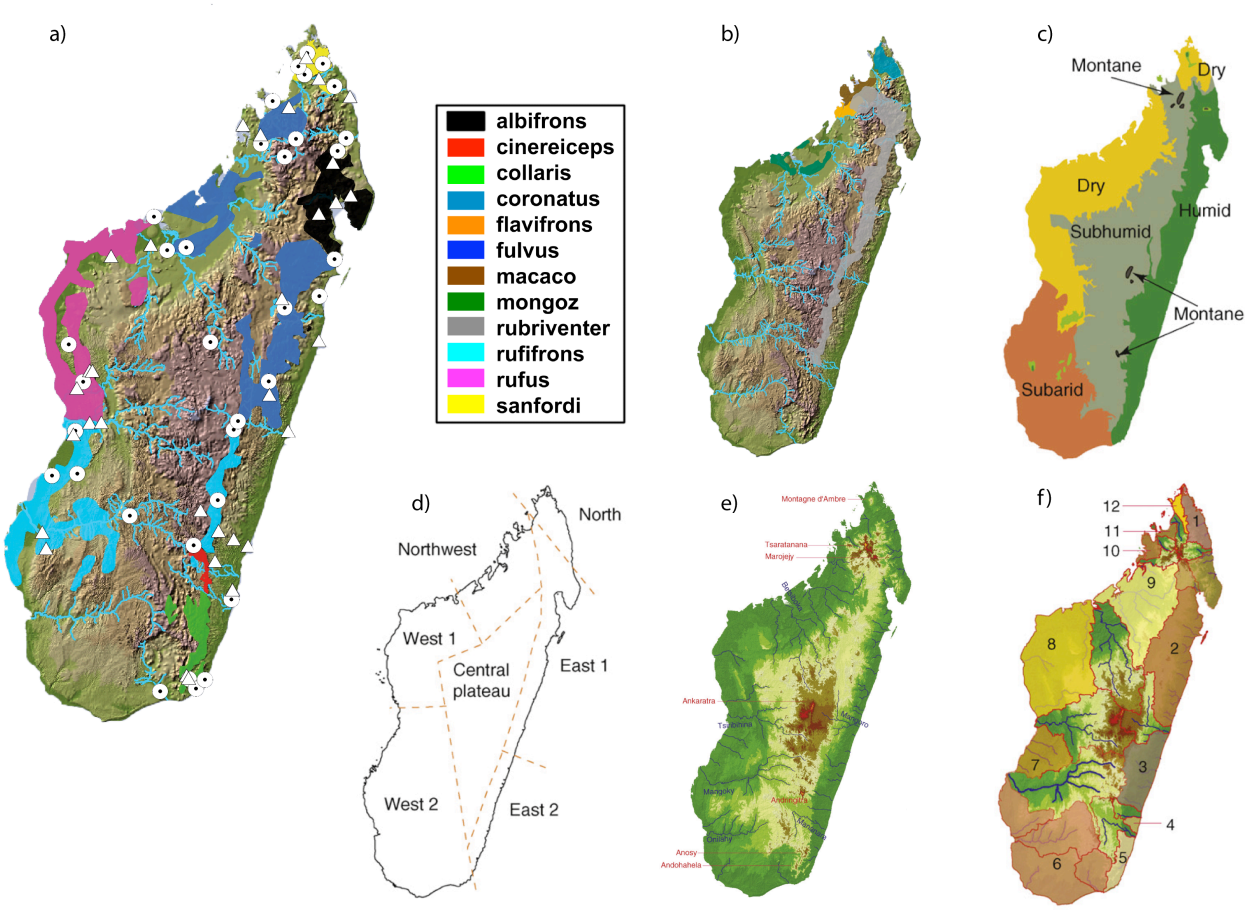


**FIg. 4.1.: Maps of Madagascar showing the distribution of *Eulemur* species, sampling sites and relevant information for different diversification hypotheses.** a) Distribution of species of the brown lemur complex, formerly considered subspecies of *E. fulvus* and sampling sites. Circles= sampling sites for individuals used in this study, Triangles= Sampling sites of museum specimens. b) Distribution of *E. flavifrons*, *E. macaco*, *E. rubriventer* and *E. mongoz.* c) Major climatic zones of Madagascar. d) Major eco-geographic regions based on climatic zones. e) Illustration of the three highest mountains of Madagascar and associated rivers that are at the base of the centers of endemism (river catchments) hypothesis. f) Map showing the centers of endemism (numbered from 1-12) and the retreat dispersal watersheds in between. c), d), e) and f) adapted after Vences et al. (2009).

Given the broad geographic distribution of eulemurs, it is not surprising that the genus *Eulemur* had an influence on the development of several of the above-mentioned hypotheses, including the role of rivers (Goodman & Ganzhorn 2004; Pastorini et al. 2003), the zonation into zoogeographic regions (Martin 1972) or the centers of endemism (Wilmé et al. 2006).. As the distributions of some species, e.g. *E. coronatus*, *E. fulvus*,

*E. sanfordi* and *E. albifrons* (Goodman & Ganzhorn 2004), are still poorly defined, and contemporary distributions do not necessarily correspond to distributions during times of speciation, incorporation of phylogeographic approaches such as gene flow models and divergence estimates of species will help to illuminate diversification mechanisms. Thus, the aims of this study were two-fold. First, we aimed to resolve the phylogeny of the genus *Eulemur* using multi-locus coalescent-based species tree analyses. Second, we wanted to infer the predominant speciation mechanisms that shaped the evolutionary history of this genus in space and time, using coalescent-based phylogeographic methods.

To this end, we tested the following predictions (see also Table 4.1). For the eco-geographic constraint hypothesis, we predicted that distribution of lineages should coincide with major Malagasy eco-geographic zones. Furthermore, the youngest sister lineage of a group or species should be a generalist and occur in different eco-geographic zones, whereas older sister lineages should be more specialized and show restricted distributions. We also predicted an east-west phylo-geographic pattern corresponding to the humid eastern rain forest and the western dry forests.

According to the western refugia hypothesis, we predicted no gene flow from west to east. However, this model is only relevant for *E. fulvus* and *E. rufifrons*, which both have populations in the east and the west, as well as for *E. rufus*, which might be a relict population of *E. rufifrons* expanding from the east to the west.

The riverine barrier hypothesis predicted that sister lineages are neighbors and separated by a major river. Gene flow between sister lineages should be small or absent, if rivers are the primary cause of geographic separation and divergence. The riverine barrier hypothesis allowed specific predictions for all species except *E. rubrivente*r.

Finally, the river catchment hypothesis predicted that lineages occurring in retreat dispersal watersheds are sister lineages to lineages in neighboring retreat-dispersal watersheds. If Pleistocene glacial maxima and minima have been the driving factor for the retreat of populations along watersheds, lineages of the brown lemur complex must have diverged very recently ( < 5 mya; Vences et al. 2009) and watersheds would have allowed for gene flow among sister lineages or populations of species that occur in eastern as well as in western parts of Madagascar, such as *E. fulvus* and *E. rufifrons* (Wilmé et al. 2006).

**Tab. 4.1.** Species-specific predictions (left) and support (right) for different diversification hypotheses modified after Vences et al. (2009) for the genus *Eulemur.* The western refugia hypothesis allowed only to formulate predictions for some of the species. RDW: Retreat dispersal watershed (after Wilmé et al. 2006)

| **Species** | **Eco-geographic constraints** | Support | **Western refugia** | Support | **Riverine barrier** | Support | **Centers of endemism (river catchments)** | Support |
|---|---|---|---|---|---|---|---|---|
| **General** | Youngest sister lineage is generalists and occurs in different eco-geographic zones; Older sister linages are more specialized; East (humid)-west (dry) phylo-geographic pattern; lineages correspond to eco-geographic regions | Partly | No gene flow from west to east | No | Sister lineages occur on either side of a river, low gene flow between sister lineages | Partly | Lineages occurring in retreat dispersal watersheds (RDW) are sister lineages to lineages occurring in neighboring RDW; glacial cycles during the Pleistocene (< 2.8 mya) allowed gene flow among RDW; sister lineages occur in neighboring river catchments when their headwaters are at low elevations | Yes |
| ***Eulemur albifrons*** | Lineage distribution corresponds to eco-geographic regions; east-west division | No | - | - | Sister lineage to *E. sanfordi* (Bemarivo), but low gene flow | Partly | Sister lineage to *sanfordi* (*fulvus*) + recent divergence + gene flow to *albifrons* and *fulvus* via retreat dispersal watersheds | Yes |
| ***Eulemur cinereiceps*** | Lineage distribution corresponds to eco-geographic regions; east-west division | No | - | - | Sister lineage to *E. collaris* (Mananara) | Yes | Sister lineage to *collaris* + recent divergence | Yes |
| ***Eulemur collaris*** | Lineage distribution corresponds to eco-geographic regions; east-west division | No | - | - | Sister lineage to *E. cinereiceps* (Mananara) | Yes | Sister lineage to *cinereiceps* + recent divergence | Yes |
| ***Eulemur fulvus*** | Lineage distribution corresponds to eco-geographic regions; east-west division | No | No gene flow from west to east | Probably no (few data) | Sister lineage to *E. rufifrons* (Manangoro), *E. rufus* (Betsiboka), *E. albifrons* (Mananara), but low gene flow | Partly | Gene flow between east and west | Probably (not tested) |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| ***Eulemur rufifrons*** | Lineage distribution corresponds to eco-geographic regions; east-west division | No | No gene flow from west to east | No | Sister lineage to *E. rufus* (Tsirihbihina), but low gene flow | Partly | Gene flow between east and west | Yes |
| ***Eulemur rufus*** | Lineage distribution corresponds to eco-geographic regions; east-west division | Yes | No gene flow from *E. rufus* to *E. rufifrons* | - | Sister lineage to *E. rufifrons* (Tsiribihina) or *fulvus* (Betsiboka), but low gene flow | Partly | Sister linage to *rufifrons* or *fulvus* + recent divergence + gene flow to *fulvus* and/or *rufus* | Yes |
| ***Eulemur sanfordi*** | Lineage distribution corresponds to eco-geographic regions; east-west division | Yes | - | - | Sister lineage to *E. albifrons* (Bemarivo) or fulvus (Mahavavy du Nord), but low gene flow | Partly | Sister lineage to *albifrons (fulvus)* + recent divergence + gene flow to *albifrons* via retreat dispersal watersheds | Yes |
| ***Eulemur rubriventer*** | Sister lineages to *fulvus* group | Yes | - | - | - | - | - | - |
| ***Eulemur macaco*** | Lineage distribution corresponds to eco-geographic regions; east-west division | No | - | - | Sister lineage to *E. flavifrons* (Maeverano) | Yes | Sister lineage to *E. flavifrons* + recent divergence | Yes |
| ***Eulemur flavifrons*** | Lineage distribution corresponds to eco-geographic regions; east-west division | No | - | - | Sister lineage to *E. macaco* (Maeverano) | Yes | Sister lineage to *E. macaco* + recent divergence | Yes |
| ***Eulemur coronatus*** | Lineage distribution corresponds to eco-geographic regions; east-west division | Yes | - | - | Sister species to *E. macaco + E. flavifrons* (Mahavavy du nord) | yes | Sister lineage to *macaco/flavifrons* + recent divergence | Yes |

## 4.2 Methods

Genetic data of wild populations of eulemurs collected by Markolf et al. (in prep.) and Pastorini et al. (2003) were used to estimate divergence times and phylogenetic relationships for single gene trees as well as for a multi-locus species tree. Details of DNA extraction and sequencing have been described in detail elsewhere Markolf et al. (in prep.). Nuclear population structure of the brown lemur complex as estimated in Markolf et al. (in prep.) was plotted on a map of Madagascar and gene flow models were compared using a Bayesian approach as implemented in migrate-n (Beerli 2006).

### Divergence date estimation and mtDNA phylogeny

Sequence data of the complete cytochrome b (1140bp) of 121 *Eulemur* individuals were used to simultaneously estimate phylogeny and divergence times in a Bayesian MCMC approach using a relaxed molecular clock as implemented in Beast version 1.7.5 (Drummond & Rambaut 2007). Seven additional outgroup taxa were included in the analysis. As there are no fossil calibration points available for lemurs (Horvath et al. 2008; Yoder & Yang 2004), calibrations were based on molecular evidence from a phylogeny of complete mitochondrial genomes of primates (Finstermeyer et al., in press) as depicted in Tab. 4.2. A HKY+I+G substitution model was chosen as suggested by Akaike's Information Criterion of JModeltest v2 (Darriba et al. 2012). A birth-death process and an uncorrelated log-normal relaxed clock with a broad normal prior distribution for the mean of the branch rates (ulcd.mean = 0 - ∞) was assumed. Fifty million generations were run with parameter sampling at every 5.000 generation resulting in 10.001 trees.

The adequacy of the burn-in was assessed by visual inspection of the trace of the parameters using Tracer v.1.5 (Rambaut & Drummond 2007). Tree Annotator v1.7.5 was used to discard 2.500 trees as burn-in and to calculate a maximum clade credibility tree of the remaining 7.501 trees.

**Tab 4.2. Calibrated nodes, means, standard deviation (sd) and 95% confidence intervals in million of years used for divergence date estimates of the cytochrome b tree.**

| Calibration node | Mean +/- sd | 95 % range |
|---|---|---|
| *Chiromyiformes + Lemuriformes- Lorisiformes* | 57.09 +/- 4.2 | 50.18- 64 |
| *Chiromyiformes* - Lemuriformes | 47.38 +/- 3.99 | 40.82- 53.94 |
| *Propithecus- Lemuridae* | 27.76 +/- 3.1 | 22.66- 32.86 |

**Time calibrated multi-locus species tree**

The multi-species coalescent approach implemented in *BEAST v1.7.5 was used to infer a species tree for the genus *Eulemur* based on one mitochondrial, three nuclear loci published by Markolf et al. (in prep.) and one mitochondrial locus published by Pastorini et al. (2003). The numbers of sequences included were 109 for the cytb locus, 147 for the eno locus, 125 for the vwf locus, 120 for the nramp locus and 53 for the past fragment, resulting in a total number of 554 sequences. Both alleles were used for all nuclear loci. *BEAST simultaneously estimates gene trees and species trees under the multi species coalescent (Heled & Drummond 2010). As the model assumes that discordance of gene trees is based solely on incomplete lineage sorting, we had to exclude potential and known hybrids prior to analysis (see appendix Tab. 1). Potential hybrids were determined via discriminant analysis of principal components (DAPC), as described in Markolf et al. (in prep.). Exclusion of individuals resulted in incomplete taxon sampling for some of the loci for *E. cinereiceps* and *E. flavifrons.* As *BEAST requires at least one sequences per species per locus, we included the 2.400 bp (PAST) fragment of mtDNA published by Pastorini et al. (2001, 2003) to have sufficient genetic information for *E. cinereiceps* and *E. flavifrons*. Dummy sequences ( ? = unknown state) were coded for the nramp and vwf loci for *E. cinereiceps* and for all three nuclear loci for *E. flavifrons*. Tree, substitution and clock models were unlinked for all partitions. As tree partitions of two mitochondrial genes should be linked in *BEAST analyses, because mtNDA lacks recombination among genes, we calculated two separate species trees, once with and once without the PAST fragment. Linking tree partitions for the two mtDNA genes was not possible, because sample sizes of the cytochrome B of Markolf et al. (in prep.) and Pastorini et al. (2003) were too different.

To calibrate the species tree in units of million of years, we set the clock rate of the cytb locus to the estimated substitution rate (0.0138) as revealed by the previous divergence time analysis of the cytb locus. The analyses were run with a Birth-Death prior and substitution models as indicated by jModeltest v2 (cytb=HKY+I+G, eno + vwf= GTR +I , nramp=HKY+G, PAST=GTR+G). For both analyses, we ran four separate runs of 30 million generations each and a sampling of parameters every 1.000 generation, resulting in 30.001 trees for each run. Convergence of the MCMC runs, adequacy of the burn-in and effective sample size (ESS >200) were assessed using the combined log.files in Tracer v. 1.5. Trees of separate runs were combined using LogCombiner v.1.7.5 discarding one third (10.000) of the trees as burn-in for each run. Trees of the four separate runs were combined using LogCombiner, and TreeAnnotator was used to calculate the final species tree from 80.004 trees. DensiTree (Bouckaert 2010) was additionally used to visualize gene tree species tree discordance using 10.000 trees from the posterior distribution.

**Geographical visualization of nuclear population structure**

Nuclear genetic population structure of the members of the brown lemur complex estimated in Markolf et al. (in prep.) based on a genotype matrix of three nuclear genetic loci was plotted on a map of Madagascar, using the online platform PhyloGeoViz (www.phylogeoviz.org)(Tsai 2011). PhyloGeoViz was originally designed to plot haplotype or allele frequencies as proportions of pies on a map. However, geo-referenced pie charts can also be constructed using assignment probabilities of individuals to populations inferred from genetic clustering methods such as STRUCTURE (Pritchard et al. 2000) or Discriminant Analysis on Principal Components (DAPC) (Jombart et al. 2010). Individual assignment probabilities of STRUCTURE for K=3 and DAPC (see Markolf et al., in prep.) were plotted separately on a map of Madagascar to geographically visualize nuclear genetic population structure. Due to the difficulties of visualizing multiple individuals from the same location, the geographic positions of pie charts correspond only roughly with the sampling site.

**Model-based phylogeography**

Log marginal likelihood comparisons (Bayes factors) of coalescent simulations were used to assess the fit of the data to different phylogeographic models following the approach of Beerli & Palczewski (2010) implemented in the software MIGRATE-n v3.5.1 (Beerli 2006). Three different model comparisons were conducted following the species

tree relationships among eulemurs. Model comparisons were conducted between western and eastern populations of *E. rufifrons*, between *E. fulvus, E. rufifrons and E. rufus* and finally between the three northern species of *E. fulvus, E. albifrons* and *E. sanfordi.* The three nuclear genetic loci and the complete cytb locus of Markolf et al. (in prep.) were used for the analyses. The mutation rate for the three nuclear loci were scaled to 0.25, comparable to mtDNA, using the inheritance scalar in MIGRATE-n to allow for easy interpretation of multi-locus parameters. Markers were run with a F84 substitution model and transition/transversion ratios of 13.1 (cytB), 2.3 (eno), 2.3 (nramp) and 3.1(vwf) as indicated by jModeltest v2. Mutation rate was set to constant, as suggested for most analyses by the user manual of migrate-n (Beerli 2006). Bayesian analysis consisted of one long chain with 10.000 recorded parameter steps, a sampling interval of 100 and a burn-in of 250.000 (25%). We used Metropolis Hastings sampling and eight statically heated chains at their default temperatures simultaneously in each run to effectively explore the parameter space. Uniform prior distributions for Θ and M were assumed.

To compare models, scaled log Bayes factors were calculated by subtracting the highest value of the log marginal likelihoods (lmL) (Bezier curve approximation) from lmL values of each model. The probability of the model in relation to all other models tested was then calculated by dividing the Bayes factor by the sum of all Bayes factor scores from all models following Kass & Raftery (1995). For all three model combinations, we tested all possible combinations, however report and describe only those that are biological meaningful in terms of the species distribution and the island geography. Those were a full migration matrix model (gene flow in all directions among all populations), a panmixia model, where populations are treated as one panmictic population, and a no gene flow model by setting M to a constant value of 0.1 migrant per generation (as suggested by the author of the program, P. Beerli personal comm.). For eastern and western populations of *E. rufifrons*, we additionally included a model with asymmetrical gene flow between east and west. For the three species comparison of *E. rufifrons, E. fulvus* and *E. rufifrons*, we additionally included models that predict only gene flow between two of these populations, which could be equally likely to a full migration matrix model based on the distribution of the three species. For the three northern species of *E. fulvus, E. albifrons* and *E. sanford*i we included an additional model of only panmixia of

*E. albifrons* and *E. sanfordi* and only gene flow among the latter two species, as suggested by the results of the species tree (Fig. 4.3) and the nuclear genetic structure (Fig. 4.4)

## 4.3 Results

Detailed description of the genetic loci used in this study are given in Markolf et al. (in prep.).

### Divergence dates estimation and phylogeny of mtDNA

Phylogenetic relationships and divergence dates as estimated from the Bayesian MCMC approach for the complete cytb locus are shown in Fig. 4.2. Details about divergence dates and node support are summarized in Tab. 4.3. Phylogenetic relationships among higher clades are well supported and in agreement with recently published phylogenetic relationships among major lineages of the *Lemuriformes* based on multiple genetic loci (Perelman et al. 2011). Our divergence dates, however, are considerably younger for deeper nodes than estimated by Perelman et al. (2011), but correspond to the estimates based on whole mtDNA genomes of Finstermeyer et al. (submitted) that were also used to calibrate three of deeper nodes in the present analysis. The most recent common ancestor (MRCA) of all eulemurs is estimated to have lived at about 6.15 mya. Monophyly is highly supported for the genus *Eulemur* as well as for brown lemur complex (posterior probability (pp)= 1) and sister species relationships of *E. macaco-E. flavifrons* (pp= 1) and *E. cinereiceps-E. collaris* (pp= 1). *Eulemur rubriventer* is the sister lineage to the brown lemur complex, However, this node is only poorly supported (pp= 0.22). The brown lemur complex began to diversify at the Pliocene-Pleistocene boundary around 1.22- 3.26 (mean= 2.18) mya. Whereas *E. albifrons*, *E. fulvus* and *E. sanfordi* are polyphyletic, the remaining lineages of the brown lemur complex, i.e. *E. cinereiceps, E. collaris, E. rufifrons* and *E. rufus,* are monophyletic for the cytb locus (see also Markolf et al., in prep.)

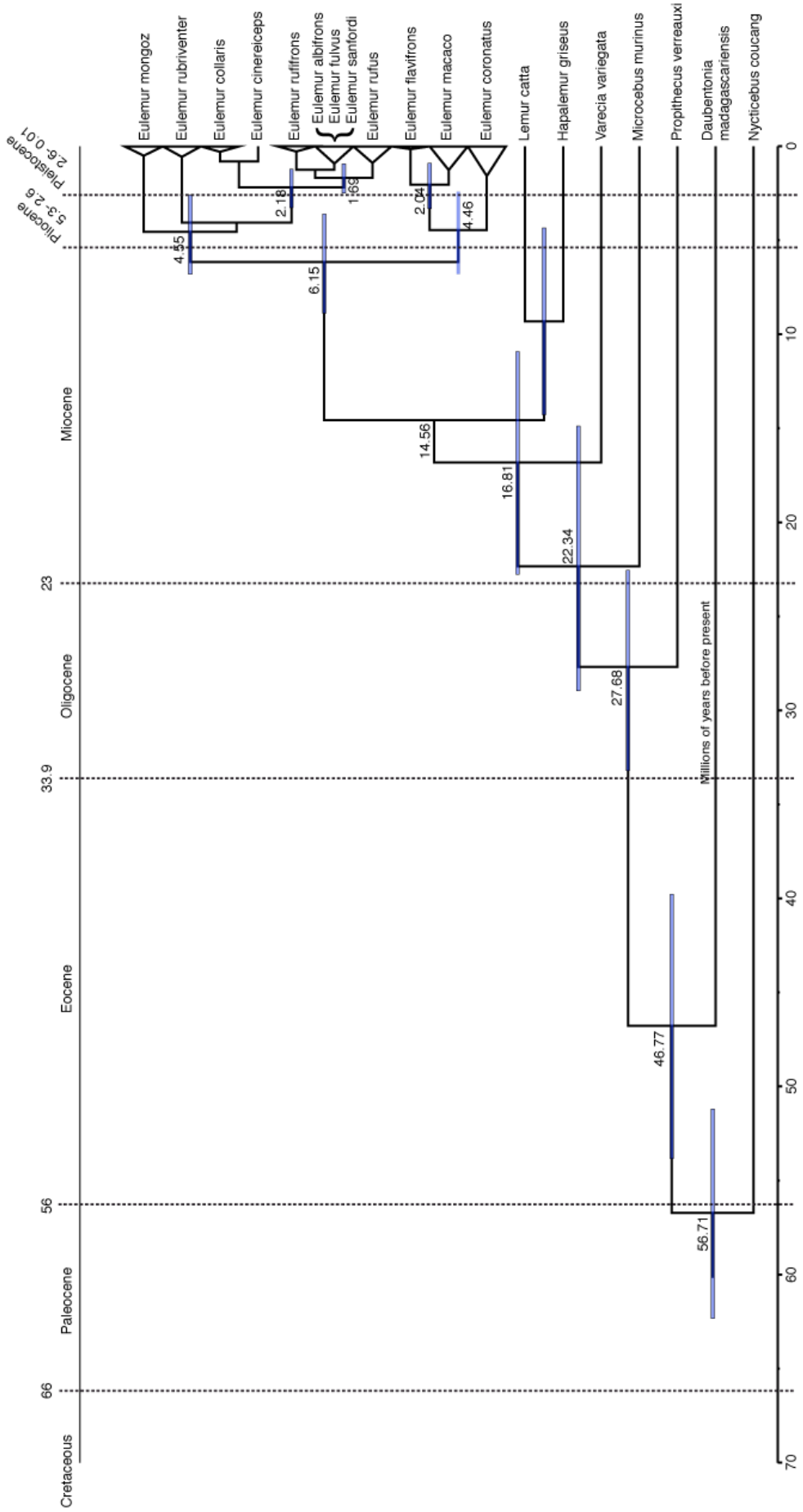


**Fig. 4.2: Simplified Bayesian tree of 121 Eulemur individuals and seven outgroups of the cytochrome B with divergence date estimates for well supported nodes.** The mean age is given in millions of years and 95 % credibility intervals are indicated by blue bars. A geological time scale is shown at the top. Details of divergence date estimates are given in Table 4.3.

**Tab. 4.3. Bayesian divergence date estimates in million of years.** The mean, 95% credibility intervals (95% HDP) and node supports (Prob) are given for the analyses of the cytochrome B and the species tree estimation from multiple loci. Missing values (-) are due to taxa that were not included in the species tree estimation, low support or discordance among the gene tree of the cytochrome B and nodes estimated from the combined analysis of multiple loci. MRCA= Most Recent Common Ancestor, *= time calibrated nodes from Tab. 4.1

| Node | Cytochrome B | | | Species tree | | |
|---|---|---|---|---|---|---|
| | Mean | 95% HPD | Prob | Mean | 95% HPD | Prob |
| *Chiromyifromes + Lemuriformes-Lorisiformes* * | 56,71 | 51,2- 62,34 | 1 | - | - | - |
| *Chiromyifromes - Lemuriformes* * | 46,77 | 39,77- 53,84 | 1 | - | - | - |
| *Propithecus - Lemuridae + Cheirogaleidae* * | 27,68 | 22,54- 33,21 | 1 | - | - | - |
| *Lemuridae - Cheirogaleidae* | 22,34 | 14,88- 28,95 | 0,85 | - | - | - |
| *Lemuridae* | 14,56 | 10,92- 22,76 | 0,84 | - | - | - |
| *Lemur catta- Hapalemur griseus* | 9,31 | 14,35- 14,27 | 0,89 | - | - | - |
| MRCA *Eulemur* | 6,15 | 3,6- 8,89 | 1 | 4,45 | 3,26- 5,68 | 1 |
| MRCA *E. coronatus + E. macaco + E. flavifrons* | 4,46 | 2,42- 6,8 | 0,87 | 3,84 | 2,65- 5,05 | 0,58 |
| MRCA *E. macaco + E. flavifrons* | 2,04 | 0,91- 3,31 | 1 | 1,15 | 0,6- 1,71 | 1 |
| MRCA *fulvus* group + *E. rubriventer + E. mongoz* | 4,55 | 2,61- 6,81 | 0,96 | 2,86 | 1,83- 3,91 | 1 |
| MRCA *fulvus* group + *E. rubriventer* | 4,06 | - | 0,22 | 2,24 | 1,16- 3,32 | 0,6 |
| MRCA fulvus group | 2,18 | 1,22- 3,26 | 1 | 0,93 | 0,33- 1,43 | 0,98 |
| MRCA *E. albifrons, E. fulvus, E. sanfordi, E. rufifrons, E. rufus* | - | - | - | 0,35 | 0,22- 0,51 | 0,9 |
| MRCA *E. cinereiceps + E. collaris* | 0,8 | 0,3-1,38 | 1 | 0,51 | 0,22- 0,79 | 0,91 |
| MRCA *E. rufifrons + E. rufus* | - | - | - | 0,17 | 0,08- 0,28 | 0,98 |
| MRCA *E. fulvus + E. albifrons + E. sanfordi* | - | - | - | 0,27 | 0,19- 0,36 | 0,86 |
| MRCA *E. albifrons + E. sanfordi* | - | - | - | 0,09 | 0,04- 0,14 | 1 |

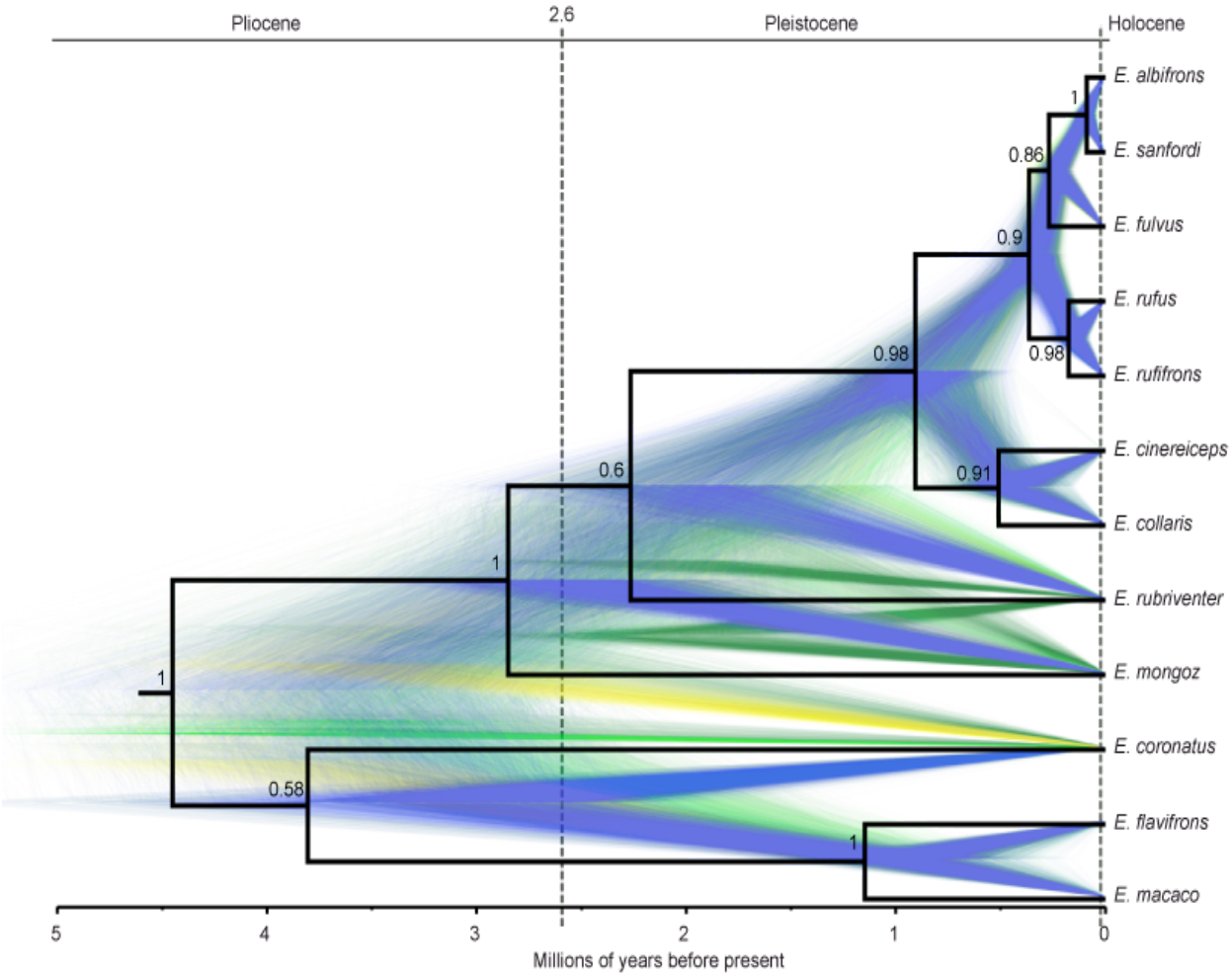


**Fig. 4.3: Time-calibrated species tree of the genus *Eulemur* based on two mitochondrial and three nuclear loci.** Black solid lines show a single combined tree estimated from 80.004 species trees. Numbers depict posterior probabilities of each node. Gene tree species tree discordance is illustrated by 10.000 colored trees of the posterior distribution in the background. Blue: Most popular topologies, Yellow: 2nd most popular topologies, Green: 3rd most popular topologies. A geological time scale is given at the top. Details of species divergence dates are given in Table 4.3.

### Time-calibrated multi locus species tree

The time-calibrated species tree for the genus *Eulemur* is depicted in Fig. 4.3. Detailed divergence dates and posterior probabilities for all clades are given in Tab. 4.3. Relationships among deeper nodes of the species tree correspond to the phylogenetic relationships estimated for the cytb locus. *Eulemur coronatus, E. macaco* and *E. flavifrons* form a sister clade to the remaining eulemurs. *Eulemur rubriventer* is the sister lineage to the species of the brown lemur complex. However, this node is also not well supported. The monophyly of the brown lemur complex is well supported (pp= 1) as are the sister group relationships of *E. collaris* and *E. cinereiceps* (pp= 0.91), *E. rufus* and *E. rufifrons*

(pp= 0.96), and *E. albifrons* and *E. sanfordi* (pp= 1). The sister group relationship of *E. fulvus to E. albifrons* and *E. sanfordi* is supported by a posterior probability of pp= 0.86. Species divergence dates are similar but slightly younger compared to the cytb locus and 95% credibility intervals are smaller for the multi locus analysis. The most recent common ancestor of the genus *Eulemur* was estimated to have lived at about 4.45 (3.26-5.68) mya. *Eulemur macaco* and *E. flavifrons* diverged from *E. coronatus* about 3.84 (2.65-5.05) mya. *Eulemur macaco* and *E. flavifrons* diverged about 1.15 (0.6-1.71) mya. *Eulemur mongoz* diverged from *E. rubriventer* and the members of the brown lemur complex about 2.86 (1.83-3.91) mya. The split between *E. rubriventer* and the members of the brown lemur complex was dated at 2.24 (1.16- 3.32) mya. The MRCA of the brown lemur complex was estimated at 0.93 (0.33-1.43) mya. The clade was then split into the two most southern species, *E. cinereiceps* and *E. collaris* that diverged 0.51 (0.22-0.79) mya, and the remaining species of the brown lemur complex that diverged 0.35 (0.22 0.51) mya into two groups, one containing *E. rufus* and *E. rufifrons* and one containing *E. albifrons, E. fulvus* and *E. sanfordi.* Splits of *E. rufus-E. rufifrons* and *E. albifrons-E. sanfordi* were estimated at only 0.17 (0.08-0.28) mya and 0.09 (0.04 0.14) mya, respectively. Diversification of the brown lemur complex occurred during the last ~1.5 million years of the late Pleistocene. The species tree estimated without the PAST fragment resulted in similar divergence date estimates and similar phylogenetic relationships among most of the clades (see appendix Fig. 7). However, the positions of *E. cinereiceps, E. collaris* and *E. fulvus* were different, and posterior probabilities for all clades are considerably lower.

**Nuclear genetic population structure**

Genetic population structure of three nuclear loci of the members of the brown lemur complex as estimated with STRUCTURE and DAPC in Markolf et al. (in prep.) plotted on a map of Madagascar is depicted in Fig. 4.4. For the STRUCTURE results of K= 3 populations, individuals from the east cluster with individuals from the west, and a clear south to north structure is evident. Assignment probabilities of the DAPC supports the sister group relationship of *E. sanfordi* and *E. albifrons* as estimated in our species tree in northern Madagascar as well as significant differentiation of nuclear genes of *E. fulvus* and *E. rufus.* Western and eastern populations of *E. rufifrons* show mixed nuclear genetic composition. *Eulemur collaris* individuals in the southeast are best separated from the others based on nuclear genetic data although some admixture exists with eastern *E. rufifrons.*

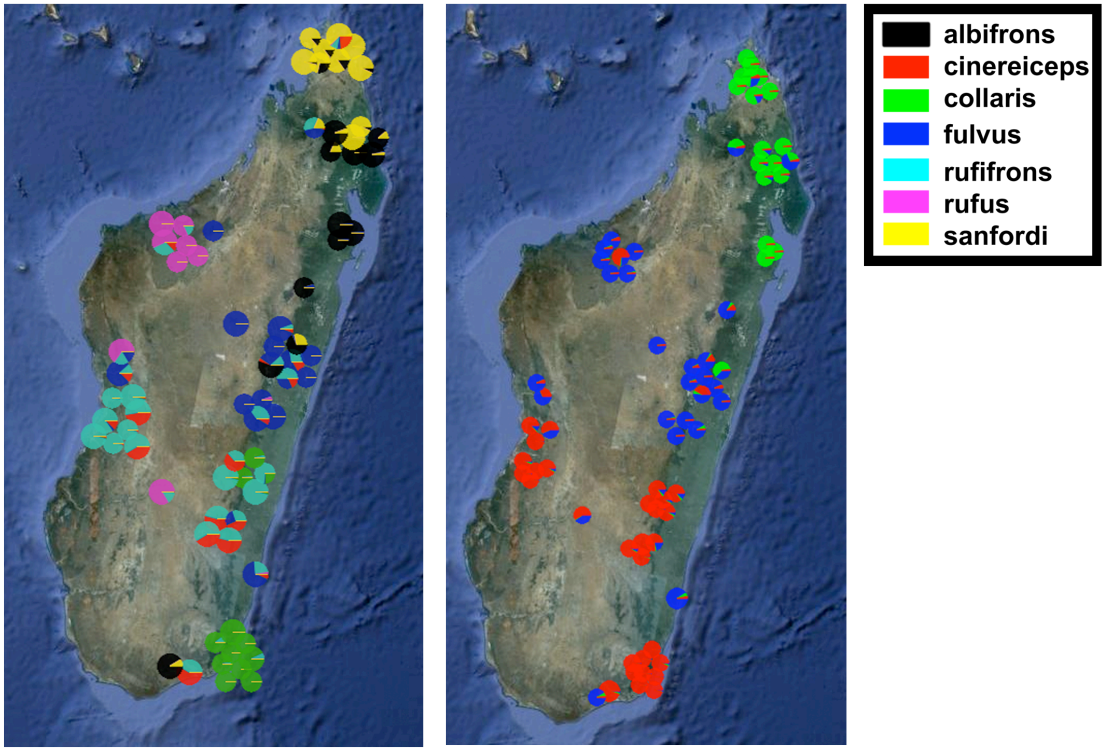


**Fig. 4.4: Geographic plot of nuclear genetic population structure of species of the brown lemur complex as inferred by Markolf et al. (in prep.) using STRUCTURE (K=3) and Discriminant Analysis of Principal Components (DAPC).** Pies represent individuals. Colors represent assignment probabilities of individuals to populations (STRUCTURE, left) or species (DAPC, right). Species colors for the DAPC analysis are given in the color legend. Please note that the color legend is only relevant for the map on the right. Pies correspond only roughly to the sampling locality.

### Model-based phylgeography

Marginal likelihoods corresponding Bayes factors and relative model probabilities of the different migration models for three population/species combinations are reported in Tab. 4.4 a-c. In all cases coalescent simulations favored the more complex model of a full a migration matrix between populations/species over more simpler models of panmixia, uni-directional or no gene flow models. Although we tested all possible combinations for the dyads or triads, we only report the models that had biological relevance in terms of the potential speciation mechanisms mentioned above. Past immigration rates were high, especially for the migration model of eastern and western populations of *E. rufifrons.* However, as we did not aim to interpret and assess the exact number of migrants or the effective population sizes, demographic parameters of Θ and M over all loci for the best models are reported in appendix Tab. 9. Here, our aim was to test the prediction of past gene flow between sister lineages of the species tree or species that occur in disjunct

populations on both sides of the island. All three models clearly rejected panmixia or the no gene flow models ($p < 0.001$) and favored a full migration matrix model with a relative probability to all other models of 1.

**Tab 4.4 a-c)** Log marginal likelihoods (lmL) and log Bayes factor (LBF) comparisons for different migration models for a) western and eastern populations of *E. rufifrons*, b) *E. fulvus, E. rufifrons* and *E. fulvus* and c) *E. albifrons, E. fulvus* and *E. sanfordi*. The log marginal likelihood is given as Bezier approximation score (BA lmL). LBF shows differences between the best and all other models. The model probability (Model prob) shows the probability of each model being the correct model relative to the others.

a)

| Model | BA lmL | LBF | Model prob | Model rank |
|---|---|---|---|---|
| **full migration matrix** | -3056,85 | 0 | 1 | 1 |
| **panmixia** | -3129,01 | -72,16 | <0,001 | 4 |
| **no gene flow** | -3193,61 | -136,76 | <0,001 | 5 |
| **west to east** | -3085,35 | -28,5 | <0,001 | 3 |
| **east to west** | -3084,74 | -27,89 | <0,001 | 2 |

b)

| Model | BA lmL | LBF | Model prob | Model rank |
|---|---|---|---|---|
| **full migration matrix** | -4786,19 | 0 | 1 | 1 |
| **panmixia** | -5032,93 | -246,74 | <0,001 | 2 |
| **no gene flow** | -5137,37 | -351,18 | <0,001 | 4 |
| **rufifrons<>fulvus** | -5190,23 | -404,04 | <0,001 | 5 |
| **fulvus<>rufus** | -5084,62 | -298,43 | <0,001 | 3 |
| **rufifrons<>rufus** | -5227,18 | -440,99 | <0.001 | 6 |

c)

| Model | BA lmL | LBF | Model prob | Model rank |
|---|---|---|---|---|
| **full migration matrix** | -4278,23 | 0 | 1 | 1 |
| **panmixia** | -4498,92 | -220,69 | <0,001 | 3 |
| **panmixia albifrons/ sanfordi** | -4403,69 | -125,46 | <0,001 | 2 |
| **no gene flow** | -4887,55 | -609,32 | <0,001 | 5 |
| **E. albifrons <> E. sanfordi** | -4518,64 | -240,41 | <0,001 | 4 |

Results for the specific predictions for different diversification hypotheses are summarized in Tab. 4.2. The combination of species divergence dates, which correspond well to the climatic variations during glacial cycles in the late Pleistocene, sister group relationships as estimated from the species tree, and Bayes Factor comparisons of gene flow models are highly concordant with the center of endemism hypothesis. In contrast, we found no or only limited support for any of the other hypotheses.

## 4.4 Discussion

In this study we explored the evolutionary history of the genus *Eulemur* in space and time and could resolve the previously polytomic phylogenetic relationships among members of the group. Divergence date estimates indicate that the MRCA of the genus *Eulemur* is estimated to have lived ~4.45 mya and that diversification among the members of the *fulvus* group happened during the Pleistocene. Additional comparisons of gene flow models among sister lineages favored full migration models over panmixia, uni-directional or no gene flow models. After discussing the validity of our phylo-geographic analyses we will discuss the fit of our data to the different diversification hypotheses proposed for the evolution of microendemsim in Madagascar as well as the suitability of our approach to other radiations endemic to the island.

**Phylogeography of eulemurs**

The present analyses clearly suggest a Pleistocene origin for members of the brown lemur complex as well as for *E. macaco* and *E. flavifrons.* Divergence dates estimated for the cytb locus were slightly older than divergence dates for the species tree analysis. This can be explained by the smaller effective population size of mtDNA compared to nuclear DNA (Smith & Klicka 2013) and the fact that gene divergence will occur prior to species divergence, and divergence dates estimated from single gene trees will necessarily overestimate divergence times (Edwards & Beerli 2000; Carstens & Knowles 2007). As time-calibrated species trees provide more realistic estimates of species divergence (McCormack et al. 2010) our divergence date estimates provide a more realistic picture than previous analyses based on single genes or concatenated genes.

As there are no fossil calibrations points available for lemurs (Horvath et al. 2008; Yoder & Yang 2004), we used calibrations points from a recent study based on complete mitochondrial genomes (Finstermeier et al, in press) to calibrate our tree for the cytb locus and used the estimated clock rate from this analysis for the calibration of the species tree. As calibration points in Finstermeier et al. (in press) were based on several dated primate fossils, the clock rate was allowed to vary among the remaining loci and the applied substitution rate of 0.0138 substitutions/per site/per million years is close to the 2% evolutionary rate for vertebrate mtDNA (Brown et al. 1979). The present divergence date estimates should therefore not be dramatically over- or underestimated. Although accuracy of molecular divergence dates should not be taken as obsolete, because divergence date estimations are particularly difficult for lemurs due to branch rate variation and the lack of lemur fossils (Yoder 2013), a very recent divergence of the brown lemur complex in the last four million years is in agreement with other recently published studies. (e.g. 3.34 mya (2.54- 4.38) in Finstermeier et al. (in press); 3.1 mya (2.77- 4.04) in Horvath et al. 2008; 2.91 mya (1.57- 4.27) in Perelman et al. 2011).

Simulation studies revealed that three loci combined with multiple gene copies per lineage are sufficient to resolve a species tree with high accuracy even of recently diverged radiations (Heled & Drummond 2010; Knowles & Kubatko 2011; Maddison & Knowles 2006; McCormack et al. 2010). Furthermore, it has been shown that concatenation of different genes can lead to substantial errors in phylogeny estimation (Weisrock et al. 2012). Although the number of gene copies per lineage varied considerably between lineages (see appendix Tab.1) because our sampling was focused

on the members of the brown lemur complex, the present species tree analysis using five loci represents the most complete phylogeny for the genus *Eulemur* so far. Posterior probability values ranged from 0.86-1.00 for the phylogenetic relationships among the species of the brown lemur complex, which could not be resolved in previous studies based on single gene trees or concatenated genes (Horvath et al. 2008; Delpero et al. 2006; Pastorini et al. 2003; Yoder & Yang 2004). Inclusion of the PAST fragment without linking the tree partitions, as suggested for mitochondrial DNA in species tree analyses, did not introduce any bias to the present phylogeny. As depicted in Appendix Fig. 1, the phylogenetic relationships of the PAST fragment are completely concordant with the phylogenetic relationships estimated for the cytb locus. *Eulemur albifrons, E. fulvus* and *E. sanfordi* are polyphyletic and *E. rufus* is a sister group to a clade consisting of *E. rufifrons, E. albifrons, E. fulvus* and *E. sanfordi.* Exclusion of the PAST fragment in species tree analysis, however, resulted in a different topology, but consistent pattern for deeper nodes. Although both mitochondrial genes did neither find a sister group relationship between *E. albifrons* and *E. sanfordi* nor between *E. rufifrons* and *E. rufus*, the inclusion of three nuclear loci seems to support the close relationships among these taxa. A sister group relationship of *E. albifrons* and *E. sanfordi* is also supported by Bayesian nuclear structure analysis for K=3 as shown in Fig. 4.4.

Bayes factor comparisons of coalescent simulations for different phylogeographic models among sister groups left little room for misinterpretations of the prevailing migration pattern. All three model comparisons consistently rejected panmixia and the no gene flow model in favor of a full migration model among lineages. This is highly consistent with several events of gene flow between members of adjacent retreat dispersal watersheds and the centers of endemism hypothesis (Wilmé et al. 2006). Rejection of panmixia furthermore supports the delimitation of the members of the brown lemur complex as distinct species, as suggested recently by Markolf et al. (in prep.), despite a high degree of incomplete lineage sorting due to past migration events among lineages during the Pleistocene.

**Eco-geographic factors**

The eco-geographic constraints hypothesis can be rejected as a general model for the diversification of the genus *Eulemur.* Only three species, *E. coronatus, E. rufus* and *E. sanfordi* are exclusively distributed in one of the eco-geographic zones (Fig. 4.1). However, the position of *E. rubriventer* as the sister lineage to all species of the brown

lemur complex, and the fact that *E. rubriventer* is distributed along the entire east coast, suggest the possibility that ecological factors also played a role during the initial diversification of the brown lemur complex. If the phylogenetic position of *E. rubriventer* is true, one could hypothesize that populations of the much more broadly distributed *E. rubriventer* had to retreat to isolated mountain refugia during cooler and drier periods. Individuals adapted to more arid conditions, however, could have descended from mountain refugia to lower elevations, forming the MRCA of the members of brown lemur complex. This is highly speculative, but is supported by the fact that *E. rubriventer* is normally found at higher elevations than sympatric species of the brown lemur complex (Markolf, pers. observation). However, with the current data at hand, this is impossible to test and the position of *E. rubriventer* was also one of the least supported in the present phylogeny. Although we did not include any ecological variables in the present analysis, the adaption of *E. fulvus* and *E. rufifrons* to eastern and western regions with very different climatic conditions does not support the model of ecogeographic constraints as a general model for *Eulemur* diversification.

**Western refugia**

The western refugia hypothesis predicted no gene flow from western to eastern populations. In the present dataset, this hypothesis was only biologically relevant for western and eastern populations of *E. rufifrons*, *E. fulvus* and *E. rufus*, which could potentially be a western relict population of eastern *E. rufifrons.* However, the gene flow models clearly reject the predictions of no gene flow from west to east for *E. rufifrons* and *E. rufus.* Unfortunately, we could not test gene flow between eastern and western populations of *E. fulvus,* because we had only two geographically disjunct individuals from the west. However the nuclear genetic structure results and the phylogeny of the cytb locus (see also Markolf et al., in prep.) suggested gene flow between east and west also for *E. fulvus*.

**Riverine barriers**

The riverine barrier hypothesis predicted sister lineages on either side of a river. This pattern is true for all Eulemurs based on our genetic sampling and the species tree except for *E. rubriventer*. However, the amount of gene flow between sister species that occur on both sides of the river is not concordant with a hypothesis that predicts rivers as the primary force for the physical separation of species. Furthermore, there is evidence

that large rivers do not form a barrier for several species. *Eulemur mongoz,* for example, is distributed on both sides of the Betsiboka, the largest river of Madagascar. Goodman and Ganzhorn (2004) evaluated the role of rivers and the distribution of eulemurs in the eastern rainforest and also found no support for the riverine barrier hypothesis based on eulemur distributions for most taxa. *Eulemur albifrons* and *E. fulvus,* for example, do not have a riverine barrier and might occur in parapatric or sympatric populations (Goodman and Ganzhorn 2004, Mittermeier et al. 2010), and *E. fulvus* occurs south of its supposed riverine barrier, the Manangoro (Lehmann & Wright 2000). Therefore, it is highly unlikely that the riverine barrier hypothesis can explain the diversification and present distribution of the genus *Eulemur* alone.

**Centers of endemism**

Our data broadly support the centers of endemism hypothesis as the main force in driving *Eulemur* diversity. The prediction of sister species relationships among neighboring retreat-dispersal watersheds could be confirmed with high support for all higher nodes in the *Eulemur* phylogeny. Furthermore, the timing of speciation is concordant with the time of climatic variations during glacial cycles of the Pleistocene. As retreat and dispersal to refugia at higher elevations would have happened several times during the Pleistocene (Wilmé et al. 2006), high levels of gene flow among sister species occurring in neighboring retreat-dispersal watersheds can be expected and were confirmed by our phylogeographic models. *Eulemur rubriventer* is again the only taxon that shows no concordance whatsoever with river catchment hypothesis. Fine scale genetic sampling of *E. rubriventer* along its distribution would be necessary to test whether mountain refugia shaped the demographic history of this species.

The lack of concordance of *E. rubriventer* with the center of endemism hypothesis also highlights an unrealistic assumption that one speciation mechanism or diversification hypothesis can and must explain the diversification pattern of an entire genus or all radiations endemic to Madagascar. Although it might be less important for the diversification of the genus *Eulemur,* the montane refugia hypothesis, for example, could be shown to explain patterns of species richness and endemism in Malagasy cophyline frogs (Wollenberg et al. 2008). Furthermore, climatic gradients had probably important influences on the diversification of several chameleons, geckos and also lemurs (Pearson & Raxworthy 2009).

*Testing diversification mechanisms with unknown ancestral distributions*

It has been shown repeatedly in all major primate radiations that climatic fluctuations during the Quarternary had a fundamental influence on the diversification of several primate genera (Haus et al. 2013; Liedigk et al. 2012; Matauschek et al. 2011; Meyer et al. 2011; Roos et al. 2008; Zinner et al. 2012). This study, however, represents the first example of explicit hypothesis-based testing of the diversification mechanism of a primate radiation endemic to the island of Madagascar. Our approach using coalescent simulations was particularly useful because exact distributions of *Eulemur* species are still poorly defined and today's distribution must not necessarily correspond to the distribution of lineages during speciation events. Our geographically broad-scale genetic sampling, however, should compensate for uncertainty of ancestral lineage distributions. *Eulemur sanfordi's* distribution, for example, is supposed to be restricted to the centers of endemism 1 and 12 of Wilmé et al. (2006) (Ankarana and Vohimarina after (Wilmé et al. 2012)) with the Manambato river as its southern limit (Mittermeier et al. 2010). However, it can be assumed that *E. sanfordi* had a much bigger distribution in the past. Evidence comes from a museum sample collected south of the Manambato close to Vohemar that corresponds phenotypically to *E. sanfordi* and clusters with *E. sanfordi/E. albifrons* based on mitochondrial DNA (Markolf et al., in prep.) as well as a sample (ID= 491, Appendix Tab. 1) north of the Bemarivo, which is more likely to be *E. sanfordi* based on nuclear genetic assignment probability (Markolf et al., chapter 3). Unfortunately, we do not have phenotypic information of this individual. Additionally, *E. coronatus,* which occurs in sympatry with *E. sanfordi,* and is also supposed to have its southern distributional limit at the Manambato river (Mittermeier et al. 2010), was found at the same locality (Anjombalava, samples 490 and 494, Appendix Tab. 1) north of the Bemarivo. We can therefore assume that the distribution of *E. sanfordi* was extended to adjacent RDWs Mahavavy and Bemarivo (Wilmé et al. 2012), which allowed gene flow to neighboring RDWs during the Pleistocene. Our data clearly favored a gene flow model over a model of panmixia of *E. albifrons and E. sanfordi* or a model of complete isolation of the latter two, illustrating the power of molecular coalescent-based approaches despite unknown ancestral distribution to test phylogeographic hypotheses.

Methods to test phylogeographic hypotheses a diversifying rapidly (Hickerson et al. 2010; Chan et al. 2011), and we are aware of the fact that there are several methods, e.g. ecological niche modeling approaches (Carstens & Richards. 2007; Lozier & Mills 2009),

approximate Bayesian computations (ABC) (Cornuet et al. 2008; Csillery et al. 2010) or isolation with migration models (IMa) (Hey 2010), that could be additionally applied to the present data set to further explore the evolutionary history of this group. However, time-calibrated species tree analyses and Bayes factor comparisons of gene flow models as applied here, using several different model comparisons, could clearly answer our questions concerning diversification of the genus *Eulemur* in space and time and had the advantage over other methods in reducing the amount of demographic parameters that have to be estimated in parallel from the data, especially when the number of species is high and computational effort would be immense (Beerli & Palczewski 2010).

*Madagascar as biogeographic model region*

As previously suggested (Vences et al. 2009), the time is overdue to use Madagascar as a biogeographic model region, and to conduct hypotheses-based testing of phylogeographic pattern among the many endemic lineages to infer speciation mechanisms that shaped this island's stunning biodiversity. Madagascar is in particular suitable as a model region of species diversification. Although this has not any biological relevance, data collection to test phylogeographic hypotheses can be done within the borders of one country, which has definitely practical advantages for researches concerning the administrative procedures necessary to sample biological material of CITES listed taxa (Vences et al. 2009). Furthermore, its high species richness and endemism, together with a relatively simple geographic structure of the island, but pronounced climatic variations from east to west, together with pronounced regional ecotones allows to test recurring patterns in several different animal and plant radiations in a relatively small geographical area isolated from other continental landmasses for a long time. As different taxa diversify at different times, several diversification mechanisms may have influenced even single radiations as was also evident from our analysis.

Our approach, however, could be easily adapted to other endemic radiations of the island that have been less involved in the initial formulation of different biogeographic models for Madagascar. It would be particularly interesting for species that have more restricted distributions than the *Eulemur* species. Genetic data already exists for various lineages and genomic resources for non-model organisms are increasing rapidly (Perry et al. 2012; Yoder 2013). Sister lineages of mouse lemurs for example showed considerable correspondence with the center of endemism (Weisrock et al 2010), however we don't

know yet the time of species divergences and, if they correspond to major climatic events during the Pleistocene. Although the accuracy of species trees, for example, depend on a optimal range of the number of loci, individuals and sequence length (Camargo et al. 2012), phylogeographic studies can also test diversification hypotheses on a smaller geographical scale, as recently shown for northern populations of *Daubentonia madagascariensis* (Perry et al. 2013) or frogs of the genus *Mantella* (Crottini et al. 2012).

The application of hypothesis based tests on speciation mechanisms to more single Malagasy radiations in the future will allow to infer the "global" patterns of diversification of Madagascar's biodiversity by integrating multi-locus phylogenies, ecological niche modeling and GIS approaches in a comparative framework (Chan et al. 2011). This in turn could help to understand the many ways that shaped biological diversity in other regions of the planet. The future of phylogeography seems promising due to the advances in sequencing technology and statistical modeling techniques (Hickerson et al. 2010). However, investigating mechanisms of species diversification needs case-specific formulations of predictions, which can then be tested with coalescent-based phylogeographic techniques (Knowles & Maddison 2002; Knowles & Carstens 2007) and/ or GIS modeling techniques (Carstens & Richards 2007; Chan et al. 2011).

**Conclusions**

We conclude that the diversification of the genus *Eulemur* was shaped by climatic variation during the Pleistocene, as suggested by the centers of endemism hypothesis (Wilmé et al. 2006). This result highlights the importance of river catchments for the evolution of Madagascar's large number of microendemic lineages. Nevertheless, other diversification mechanisms, such as the role of montane refugia, local or regional climatic variations or a combination of several different forces should not be neglected and could well have played a role in the diversification of other radiations on the island. However, testing these models with genetic data requires *a priori* formulated predictions as well as a dense sampling design for the lineages under investigation.

# 5 General discussion

## 5.1 Summary of results

This dissertation addressed general aspects of species delimitation of the endemic Malagasy order *Lemuriformes* as well as a more detailed investigation of the taxonomy and phylogeography of the genus *Eulemur.* Data from GenBank, genetic and acoustic data collected from various field sites in Madagascar as well as morphometric, molecular and pelage coloration data from various museums were used to answer the following questions.

**Chapter 2:**

*Are approaches based on genetic distance or diagnosability of mitochondrial DNA a valid method for species delimitation in lemurs and how does sampling influence these approaches?*

Based on comparisons of intra- and interspecific genetic distances in various lemur taxa and a simulation of a fictive taxonomic study, we showed that genetic distance estimates as well as Population Aggregation Analysis (PAA) are inappropriate for species delimitation in lemurs. Intra- and interspecific distances overlapped in 14 of 17 cases independent of the mitochondrial marker used. The simulation of a fictive taxonomic study indicated that for the mitochondrial d-loop the minimum required number of individuals sampled per locations is 10 in order to avoid false positives via PAA.

**Chapter 3:**

*How many* Eulemur *species can be delineated and are members of the brown lemur complex valid species or subspecies?*

Using several independent lines of evidence, our results confirmed the species status of the members of the "brown lemur complex" under the general linage concept of species. With the exception of *E. cinereiceps* we found evidence from at least two independent types of data supporting the delimitation of the taxa of the brown lemur

complex as separate species. *Post hoc* statistical tests between pairs of populations, however, showed considerable discordance among different data sets for different pairs of populations and nuclear genetic loci revealed a high degree of incomplete lineage sorting. Our results highlight the importance of integrating multiple types of evidence in making taxonomic decisions.

**Chapter 4:**

*Phylogeography of the genus Eulemur?*

Our phylo-geographic analyses revealed that *E. coronatus* and its sister group containing *E. macaco* and *E. flavirons* are the most basal clades in the *Eulemur* phylogeny. *Eulemur mongoz* diverged next and *E. rubriventer* is the sister taxon to the brown lemur complex. Within the brown lemur complex, *E. collaris* and *E. cinereiceps* are sister lineages to the rest. *E. rufifrons* and *E. rufus* are sister linages to a clade containing *E. fulvus* as sister lineages to *E. albifrons* and *E. sanfordi*. The most recent common ancestor of eulemurs was estimated to have lived about 4.45 mya. Divergence date estimates furthermore suggested a very recent diversification among the members of the brown lemur complex during the Pleistocene (0.33-1.43 mya).

Is *Eulemur* phylogeography concordant with major biogeographic hypotheses proposed to explain the present distribution of taxa across Madagascar?

Phylogeographic model comparisons of past migration rates showed significant levels of gene flow between lineages of neighboring river catchments as well as between eastern and western populations of the redfronted lemur (*E. rufifrons*). Together with species divergence times that coincide with climatic variations during the Pleistocene, our results confirmed the role of river catchments for the evolution of Madagascar's microendemic biota. The diversification of the brown lemur complex is highly concordant with the centers of endemism hypothesis proposed by Wilmé et al (2006).

## 5.2 A personal view on species concepts and their influence on science and conservation

Throughout this dissertation, it has been stated repeatedly that species are the fundamental units of evolutionary biology. During a recent discussion about the socioecological model at a small workshop on primate behavior, I counted the mentioning of the word 'species'. After 30 minutes, the word 'species' had already been mentioned more than 50 times. This observation highlights the importance of species for evolutionary biologists who want to draw conclusions about the evolution of social behavior, ecological adaptions, cognitive capabilities, diseases and all other sorts of other biological phenomena. Does this mean that we need a universal definition of the term species, because otherwise biologists will talk about biological phenomena at different levels of organization? Yes, at least to a certain degree. However, the problem is certainly more important for specific disciplines, such as comparative and conservation biology, that highly depend on species lists to draw the correct conclusions from their data (Isaac & Purvis 2004).

As it is hypothesized that sociality played a major role in the evolution of primate facial variation (Santana et al. 2012), let us assume that we want to analyze the evolution of facial color patterns and diversity with respect to sociality in the lemurs of Madagascar. The genus *Eulemur* shows one of the most pronounced examples of primate facial variation and is without any doubt the most diverse group of Malagasy primates in terms of facial variation. Thus, excluding seven eulemur taxa by treating them as subspecies could severely underestimate the influence of sociality on the evolution of facial color variation in lemurs. A potential solution for this and for other comparative studies would be not to use species as the units of comparisons, but phylogenetically well supported lineages, whether they are species, subspecies or any other kind of level of biological organization. However, for most lineages within the *Lemuriformes,* whether they can be treated as species or not, we lack information on their biology, including variation in sociality or facial color variation. This is particularly true for many nocturnal species of the genera *Microcebus* and *Lepilemur,* but will also apply to many other species outside of Madagascar. Thus, without some detailed knowledge about the biology of lineages, species status remains questionable and the influence of various classifications on comparative phylogenetic analysis can be profound.

Taxonomic inflation through the application of different species concepts can have also profound effects on global and regional conservation efforts. The assessment of local

and global areas of conservation priority is highly dependent on the classification of organisms through taxonomists and the estimation of the number of existing species (Agapow et al. 2004, Isaac & Purvis 2004, Zachos et al. 2013). In my opinion, this problem can be reduced to one simple question. In light of the general aim to protect biodiversity of the planet, which I assume is a desirable concern for most biologists, this question is, whether we are better off elevating all possibly unique groups of organisms to species level and potentially 'downgrade' them after we have collected more detailed information about their biology, or whether a slower, but more detailed analysis of potential species followed by a taxonomic decision is a more efficient way to protect global biodiversity.

There are arguments for both approaches. Increasing the number of species even without detailed knowledge about their biology can have benefits for a species in terms of conservation because it will be easier to obtain funding for its conservation. A general increase in the numbers of species in a certain geographical area will also have important consequences for the allocation of global resources for conservation. During the last decades, intensive research on various taxonomic groups has uncovered Madagascar's exceptional biodiversity and endemism, and consequently qualified the island as one of the global biodiversity hotspots (Myers 2000). As a consequence, Madagascar has come into focus of conservation efforts and funding (Bode et al. 2008), which is certainly a good thing. Finally, as long as species limits are treated as a hypothesis formulated within the context of available evidence, the existence of species whose status is not entirely clear should not be particularly disturbing (Cracraft 1992, Hazevoet 1996)

However, taxonomic inflation can also have some negative effects on conservation. Acceptance of invalid species may hinder conservation and management plans and can lead to inappropriate translocation or captive breeding decisions (Zachos et al. 2013). Moreover, the number of species is often used to define the conservation value of protected areas and to allocate resources for local conservation projects. As resources for conservation are limited this can result in a waste of limited resources. The term 'species' is also the basis for all political decisions concerning conservation, animal trade, landscape protection, etc.. Considering the extinction of many species and growing threats to biodiversity, it is necessary to find a practical solution to the species problem to provide decision makers with a sound basis of organismic classification, while keeping in mind that taxonomy should be independent of political decisions.

Taxonomists may never agree about what a species is and what is not, but this is a simple consequence of the continuous process of evolution (Zachos et al. 2013). Hence,

species might also not be the best way to evaluate conservation priorities. Although I must admit that I do not have the 'ultimate' measurement for the assessment of conservation priority, there are at least several approaches that are more objective than species lists. These priority indices are based on a combination of phylogenetic distinctiveness or taxonomic uniqueness and are combined with protection or IUCN Red List status (see Lehman 2006, Isaac et al. 2007). As genetic data are accumulating rapidly, these measures might be a better way to allocate conservation efforts and resources and would even allow including subspecies into the estimation.

In Chapters 2 and 3, we expressed strong criticism about the use of the phylogenetic species concept (PSC) for species delimitation. I want to stress that this criticism is neither directed at the concept of a phylogenetic species itself, nor to the applicability of diagnosability and common ancestry for species delimitation. The PSC is indeed very useful as a secondary species concept (*sensu* de Quieroz 1998) as most lineages share a common descent or are even already reciprocally monophyletic for some genes and/or have diagnosable distinct phenotypes. The criticism mainly relates to the misuse of the PSC by several authors, in particular in the lemurs of Madagascar. Two major advocates of the PSC wrote: *"Phylogenetic species are basal, diagnosably distinct taxa; that is, they are comprised of one or more populations that share a combination of characters that distinguish them from other such units" (*Cracraft et al. 1998, p. 148) and *"The other major reason why mtDNA should not be used by itself as a taxonomic criterion is that it tells us only about matrilines, not about the population as whole." (*Groves 2001b, p. 197). Both authors stress the population or even more populations as the unit of phylogenetic species and also a combination of characters that distinguish phylogenetic species. This notion, however, as outlined in Chapter 2 was ignored by numerous authors who described several species solely based on mitochondrial DNA of a few samples sometimes from a single locality. I can not stress more that this does not represent an appropriate sample size to diagnose phylogenetic species, as we completely lack information on intraspecific variation of the mitochondrial DNA in those cases.

Moreover, I argue that as long as we sample enough individuals at an appropriate geographical scale in order to have a good idea about the intraspecific variation for the taxa in question, different species concepts will come to similar conclusions. Although phenotypic character states overlapped considerably among the members of the brown lemur complex, our insights about intraspecific variation allowed us to statistically test the

independent evolution of those linages under GLC. However, the same conclusions could be derived under the PSC with our dataset.

So why use the GLC? Most criticism of the GLC applies to the vague definition of "separate or independent evolving metapopulation linages" (Wilkins 2009). De Quieroz (1998, 2005, 2007) did not specifically define what is a (meta)population and what is "separate" or "independent". To me that actually appears to be one of the benefits of the GLC. This more or less vague definition of the term species highlights the continuous process of evolution that acts on populations and might result in the divergence of populations as well as the possibility of two or more populations to admix in secondary contact and eventually become one. Whether biologists see species as 'real' objects of biological organization or just as an arbitrary human categorization, it is hard to find any argument against the possibility to adopt this general definition of species as segments of separately evolving metapopulation lineages. Moreover, the GLC clearly emphasizes the separation of the theoretical concept of species from operational criteria that are used to empirically delimit them. There are no necessary properties such as reproductive isolation or monophyly to be an independent evolving lineage, but empirical analyses of many different properties will serve as stronger evidence for lineage separation (Dayrat 2005; De Quieroz 2007, Leaché et al. 2009). The sticking point here is that we should not concentrate the debate on what a species is in nature, but rather how we use a word, choose our favorite definition and continue with science (Brookfield 2002).

## 5.3 Uncovering the origin of Madagascar's species richness and endemism

While the origin of most endemic Malagasy lineages at higher taxonomic levels is well supported to be the result of oversea dispersal throughout the Cenozoic from African and Indian landmasses (Vences et al. 2009), explaining the subsequent diversification of those lineages in many micro-endemic taxa is still in its infancy. Molecular methods are increasingly used to elucidate biogeographic events in space and time. As a consequence of advances in sequencing technology the amount of molecular data even for non-model organisms is increasing rapidly. At the time of writing, whole genomes of the Aye- aye (*Daubentonia madagascariensis*), and the mouse lemur, (genus *Microcebus*) have been sequenced (Perry et al. 2012, Yoder 2013) and will accelerate the development of new genetic markers to infer demographic parameters of populations over time and species divergence times for many endemic Malagasy lineages. In Chapter 3, I demonstrated one possible approach to explore the diversification pattern of a single radiation endemic to

Madagascar using a combination of molecular genetic and distributional data in order to test lineage-specific predictions derived from different diversification hypotheses. In order to achieve a more detailed understanding of the origin of Madagascar's species richness and local endemism, we need more phylogeographic studies at the intra- and interspecific level on different Malagasy animals and plants. In the future this will allow to us to obtain a better picture of the general patterns that shaped the diversification of Madagascar's fauna and flora. Considering Madagascar's exceptional species richness and recent advances in phylogeographic methods, which allow the integration of multi-locus phylogenies, ecological niche modeling and GIS approaches in a comparative framework (Chan et al. 2011), phylogeographers working in Madagascar are facing exiting times.

## 5.4 Methodology

In this dissertation several kinds of data were used to infer the taxonomic status of members of the brown lemur complex. Morphological, genetic and data on pelage coloration were collected from various museum specimens. As museum specimens can be labeled incorrectly, there is always the possibility to introduce errors in the analysis. However, we used only those specimen that could be clearly assigned to one of the seven populations based on phenotype, sampling locality or subsequent genetic analysis and can therefore exclude significant bias in the dataset because of mislabeled museum specimen.

As our morphological data analysis was based on two dimensions only, we can expect that already available three dimensional geometric morphometric approaches (e.g. see Fleagle & Gilbert 2010) would uncover even more variation among closely related species. However, until now all morphological studies of the genus *Eulemur* revealed extensive homoplasy among the members of the brown lemur complex whether they were based on qualitative traits (Tattersall & Schwartz 1931; Groves & Eaglen 1988; Groves & Trueman 1995) or quantitative geometric morphometrics (Viguier 2002, this study), indicating that ecological adaptions played a small role in the diversification of the brown lemur complex.

Variation of pelage color is severely underestimated. This was mainly due to bad conditions of the facial area of museum skins and the fact that standardized pictures are simply impossible to get from mostly arboreal wild animals without trapping them. On the other hand, we introduced a method that quantifies phenotypic divergence of lineages

based on pelage coloration. This method could be applied to other free-ranging animals, where this is possible, or to museum specimens of other lineages under taxonomic debate.

Although we can quantitatively distinguish communication cues or signals such as color or vocalizations between species, we still know very little about the significance of these differences in the context of species recognition (Kappeler 2012).

Genetic data used in this study were collected using a non-invasive approach. The rapid degradation of DNA is a serious problem in this context. Due to highly degraded DNA samples we were only able to sequence short fragments, especially of the nuclear loci. This limitation could have influenced the phylogenetic resolution of the nuclear markers as the numbers of polymorphisms found in a single marker might just be too low. However, combining multiple short nuclear and mitochondrial loci and estimating gene trees within a species tree revealed well supported relationships among the most important nodes.

A major part of this study is based on a very comprehensive field sampling for the members of the brown lemur complex. Without this extensive field sampling we could not have uncovered the evolutionary history of the BLC. Detailed field surveys and samplings are not only important to delimit species or to resolve phylogenetic relationships among recently evolved radiations, they also enable us to determine exact distributions, population sizes and genetic diversity of species, which is very important for conservation-planning.

## 5.5 Outlook

a. Assessment of the role of species recognition and sociality for the evolution of communication signals such as facial color patterns and vocalizations in lemurs. Behavioral experiments with wild ranging eulemurs to infer whether eulemurs can distinguish con- and heterospecific visual and acoustic signals are already on the way along with a comparative study to determine the underlining mechanisms (e.g. genetic drift, social organization, ecological pressures, etc.) that gave rise to the diversification of facial variation in lemurs.

b. Molecular evolution of pelage coloration. Along with the previous study, it would be particularly interesting to analyze genes known to be involved in the production of pelage coloration, such as the melanocortin receptor (MC1R) gene or the agouti signaling protein (ASIP) using next-generation sequencing

to assess variation at the population level. Diversifying selection should act on these or other genes responsible for variation in pelage coloration in eulemurs.

c. Distinguishing between incomplete lineage sorting or ongoing hybridization is particularly difficult. Inclusion of fast evolving markers such as microsatellites might confirm our interpretation that nuclear admixed ancestry is due to incomplete lineage sorting and not due to ongoing hybridization. Y-chromosomal markers would also be interesting to study in the context of hybridization.

d. Determining the exact distribution of eulemurs. Two areas are particularly interesting and unexplored. One area is the region around the Ambatovaky Special Reserve, where we lack information on the geographical extent of the distribution of *E. albifrons* and *E. fulvus*. The other area is north of the Bemarivo river with extension to the west towards the Tsaratanana Massif.

e. The inclusion of more museum samples for genetic analysis would be very helpful to determine original distributions of taxa as the current distributions are too much influenced by degradation of suitable habitats. This could also clarify taxonomic status of species such as *E. cinereiceps* that almost disappeared in their natural environment. Unfortunately, two of the main specimen holders, the American National History Museum as well as the Musée Nationale Histoire de Paris did not allow us to take any samples for genetic analysis.

f. Phylogeography of *E. rubriventer.* It remains unresolved why *E. rubriventer* is distributed along the entire east coast. A detailed geographic sampling of populations of *E. rubriventer* along the east coast might shed some light on its role in the diversification of the brown lemur complex.

# 6 Summary

Species are the fundamental units of comparison in all subfields of biology. Moreover, species are the currency of biological classification and used to define areas of conservation priority. Hence, central questions of evolutionary biology are “what is a species?”, “how can we delimit species?”, “how many species exist?” and “how did species evolve in space and time?”. These questions are the subject of this dissertation.

The first part of this thesis questions the use of the phylogenetic species concept to delimit species via mtDNA-based methods, such as comparisons of intra- and interspecific distances or diagnostic characters, in the lemurs of Madagascar. The number of lemur species has almost tripled during the last two decades. Many of the newly described species were solely delimited on the basis of mitochondrial DNA under the Phylogenetic Species Concept (PSC) using the above-mentioned methods. We used published sequence data collected from GenBank to compare intra- and interspecific distances among lemur genera for different mtDNA loci. Fourteen out of 17 comparisons showed overlapping intra- and interspecific genetic distances independent of the loci used. A simulation of a fictive taxonomic study furthermore revealed that the minimum required number of samples for the mitochondrial D-loop is 10 per population in order to avoid false positives via Population Aggregation Analysis. The results indicate that both methods are inappropriate to delimit species. We therefore recommend the use of nuclear and mtDNA genetic loci as well as multiple independent datasets (e.g. morphological, acoustic, ecological, etc.) to delimit species.

The second part of this thesis revisits the taxonomy of the brown lemur complex endemic to Madagascar using several types of data. Seven species of the genus *Eulemur* formerly treated as subspecies of the common brown lemur (*Eulemur fulvus*) and grouped into the brown lemur complex (BLC) were previously elevated to species level without the collection of new data. Between-group analyses of principal components revealed significant heterogeneity in skull shape, pelage color variation and loud calls across all seven populations. Furthermore, *post hoc* statistical tests between pairs of populations revealed considerable discordance among different data sets for different dyads. Despite a high degree of incomplete lineage sorting among nuclear genetic loci, significant exclusive ancestry was found for all populations, except for *E. cinereiceps,* based on one mitochondrial and three nuclear genetic loci. Under the general linage concept of species, using several independent lines of evidence, our results confirmed the species status of

the members of the BLC. More generally, this analysis highlights the importance and value of integrating several types of evidence in delimiting recently evolved radiations.

In the third chapter we explored the evolution of the genus *Eulemur* in space and time and the concordance of *Eulemur* diversification with major biogeographic hypotheses proposed for Madagascar. Due to its remarkable species diversity and micro-endemsim, Madagascar has recently been proposed as a biogeographic model region. However, hypothesis-based tests of various diversification mechanisms that have been proposed for the evolution of the island's micro-endemic lineages are still limited. Here, we tested the concordance of several diversification hypotheses with new data on the broadly distributed genus *Eulemur* using coalescent-based phylogeographic analyses. Time-calibrated species tree analyses and population genetic clustering resolved the previously polytomic relationships among eulemurs. The most recent common ancestor of eulemurs was dated about 4.45 million years ago (mya). Estimates of divergence dates furthermore suggested a very recent diversification among the members of the "brown lemur complex", i.e. former subspecies of *E. fulvus*, during the Pleistocene (0.33-1.43 mya). Phylogeographic model comparisons of past migration rates showed significant levels of gene flow between lineages of neighboring river catchments as well as between eastern and western populations of the redfronted lemur (*E. rufifrons*). Together, our results are concordant with the centers of endemism hypothesis, underline the importance of watersheds for the evolution of Madagascar's micro-endemic biota, and they highlight the utility of testing diversification mechanisms using coalescent-based phylogeographic methods.

# 7 Zusammenfassung

Arten sind die fundamentalen Einheiten für vergleichende Fragestellungen in allen Bereichen der Biologie. Darüber hinaus dienen Arten als Maß für die taxonomische Klassifizierung und bestimmen den Stellenwert von Organismen und Regionen der Erde im Bereich des Naturschutzes. Zentrale Fragen der Evolutionsbiologie sind daher, „was ist eine Art?“, „mit welchen Methoden können wir Arten voneinander abgrenzen?“, „wie viele Arten existieren?“ und „wie sind Arten in Raum und Zeit evolviert?“. Mit diesen allgemeinen Fragen beschäftigt sich die vorliegende Arbeit.

Der erste Teil dieser Dissertation hinterfragt kritisch die Anwendung des phylogenetischen Artkonzeptes (PSC) im Zusammenhang mit auf mitochondrialer DNS basierten Methoden, wie beispielsweise die Heranziehung von Vergleichen von intra- und interspezifischen genetischen Distanzen oder von populationsspezifischen Polymorphismen (Populationsaggregationsanalyse) zur Abgrenzung von Arten innerhalb der Lemuren Madagaskars. Die Anzahl von Lemurenarten hat sich in den letzten zwei Jahrzehnten fast verdreifacht. Viele dieser neuen Arten wurden allein auf der Grundlage von genetischen Distanzen und/oder populationsspezifischen Polymorphismen innerhalb der mitochondrialen DNA unter dem PSC beschrieben. Zur Verfügung stehende Sequenzen aus der öffentlichen Datenbank des National Center for Biotechnology Information (NCBI) wurden gesammelt, und die intra- und interspezifischen genetischen Distanzen verschiedenster mitochondrialer Marker von Lemurengattungen gegeneinander aufgetragen. In 14 von 17 Fällen, unabhängig vom genetischen Marker, überlappten die intra- und interspezifischen genetischen Distanzen. Des weiteren zeigte die Simulation einer fiktiven taxonomischen Studie, dass für den mitochondrialen D-loop mindestens 10 Individuen pro Population untersucht werden müssen, um falsch-positive Schlussfolgerungen mit der Populationsaggregationsanalyse (PAA) zu vermeiden. Unsere Ergebnisse zeigen, dass beide Methoden nicht zur Abgrenzung von Arten geeignet sind. Wir empfehlen daher die Heranziehung von verschiedenen Merkmalen (genetische, morphologische, verhaltensbiologische, ökologische, etc.), sowie verschiedener genetischer Marker der nukleären und mitochondrialen DNS zur Abgrenzung von Arten.

Der zweite Teil dieser Dissertation untersucht und prüft die aktuelle Taxonomie der endemischen *fulvus*-Gruppe von Madagaskar mit Hilfe von mehreren unabhängigen Datensätzen. Sieben allopatrisch verbreitete Arten der Gattung *Eulemur*, welche zuvor als Unterarten des braunen Makis (*Eulemur fulvus*) angesehen wurden, wurden ohne

Hinzuziehung von neuen Daten und Erkenntnissen auf Grundlage des phylogenetischen Artkonzeptes zu Arten erhoben. Hauptkomponentenanalysen zwischen und innerhalb von Gruppen ergaben, dass Schädelform, Fellfarbe sowie Vokalisationen extrem heterogen zwischen allen sieben Gruppen sind. Darüber hinaus zeigten anschließende paarweise Vergleiche, dass die verschiedenen Datensätze zu unterschiedlichen Ergebnissen in verschiedenen Dyaden kommen. Basierend auf einem mitochondrialen und drei nukleären Markern fanden wir trotz hohem Maß an inkompletter Aufspaltung von Abstammungslinien (incomplete lineage sorting) zwischen den nukleären Loci, außer für *E. cinereiceps,* Hinweise auf signifikante exklusive Abstammung für alle Populationen. Auf Grundlage des "general lineage concept of species" und der Heranziehung von vier unabhängigen Datensätzen können wir daher den Artstatus aller Mitglieder der *fulvus*-Gruppe bestätigen. Im allgemeinen zeigen unsere Ergebnisse die Wichtigkeit taxonomische Entscheidungen auf der Basis von mehreren unabhängigen Datensätzen zu treffen, im Speziellen, wenn es sich um Arten handelt, die erst in jüngerer Zeit evolviert sind.

Der letzte Teil dieser Dissertation beschäftigt sich mit der Entstehungsgeschichte der Gattung *Eulemur* in Raum und Zeit sowie mit verschiedenen biogeographischen Hypothesen, welche zur Erklärung des überaus reichen lokalen Endemismus von Madagaskar's Flora und Fauna angeführt worden sind. Obwohl es zahlreiche Hypothesen gibt, die versuchen die mikro-endemische Vielfalt in Madagaskar zu erklären, wurden bisher wenig auf Hypothesen basierte Tests anhand wissenschaftlicher Daten durchgeführt. Mit Hilfe von Koaleszenz-basierten phylogeograpischen Methoden untersuchten wir die Übereinstimmung von mehreren Diversifikations-Hypothesen mit der Evolution der Gattung *Eulemur.* Eine zeit-kalibrierte Artphylogenie und populationsgenetische "cluster" Methoden konnten die bisher polytomen Verwandtschaftsbeziehungen klären. Der letzte gemeinsame Vorfahre der Gattung *Eulemur* wurde demnach auf 4.45 Millionen Jahre datiert. Darüber hinaus deuteten Aufspaltungszeiten auf eine sehr zeitnahe Diversifikation der Arten der *fulvus*-Gruppe (zuvor Unterarten von *E. fulvus*) innerhalb des Pleistozäns (0.33 bis 1.43 Millionen Jahre) hin. Phylogeographische Modelvergleiche von Migrationsraten zeigten signifikanten genetischen Austausch zwischen Abstammungslinien von benachbarten Flusseinzugsgebieten sowie zwischen östlichen und westlichen Populationen des Rotstirnmakis (*E. rufifrons)* in der Vergangenheit. Unsere Ergebnisse stimmen mit der "centers of endemism"-Hypothese überein und bestätigen die Nützlichkeit von koaleszenz-

basierten phylogeographischen Methoden zur Überprüfung von Diversifikationsmechanismen.

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## Appendix Figures

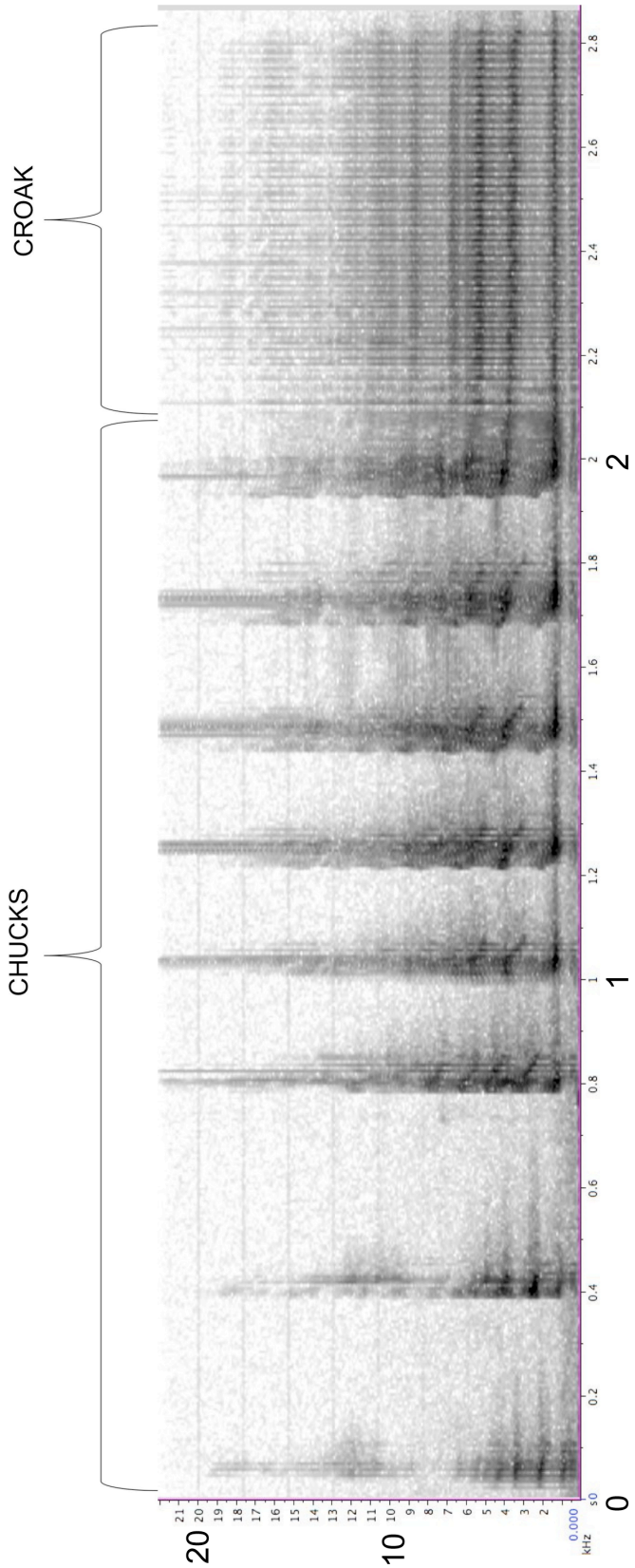



**Fig. 1: Spectrogram of typical disturbance and advertisement call of members of the fulvus group.** X-axes=Time in seconds, y-axes= Frequency in kHz.

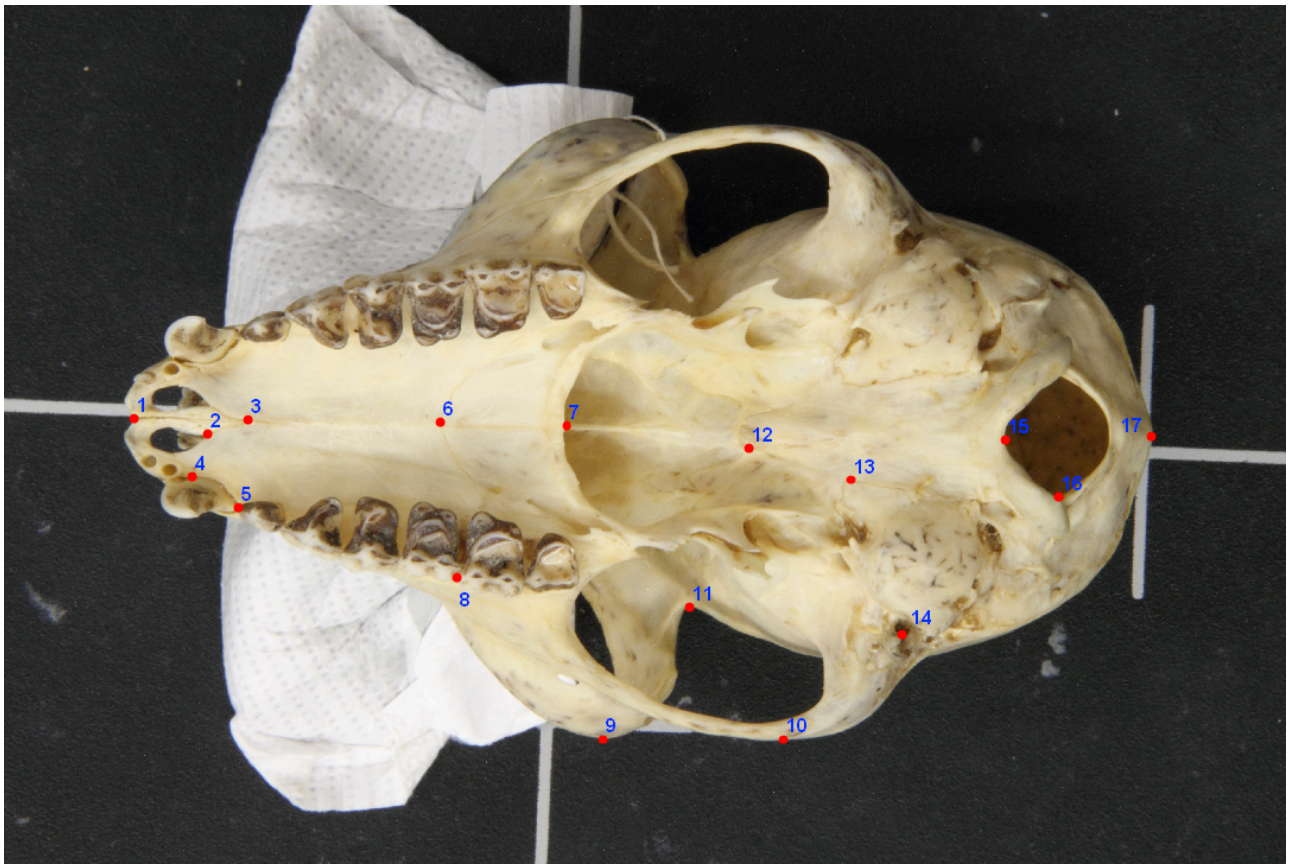



**Fig. 2: 17 homologous landmarks used for geometric morphometric analyses.** 1= Prosthion, 2= Posteriormost point of the left incisive foramen, 3= Posteriormost point of premaxilla- maxilla suture, 4= Meeting point of premaxilla- maxilla suture and canine alveolus, 5= Posteriormost point of canine alveolus, 6= Maxilla- palatine suture, 7=Staphilion, 8= Posterior-jugal contact of alveolar ridge and 1st molar, 9= Lateralmost point of orbitum, 10= Lateralmostpoint of jugale, 11= Medialmostpoint of the braincase, 12= Lateralmostpoint of basisphenoid- vomer suture, 13= Lateralmostpoint of basioccipitale- basisphenoid suture, 14= Lateralmostpoint of the meatus acousticus externus, 15= Basion, 16= Lateralmostpoint of foramen magnum, 17= Inion

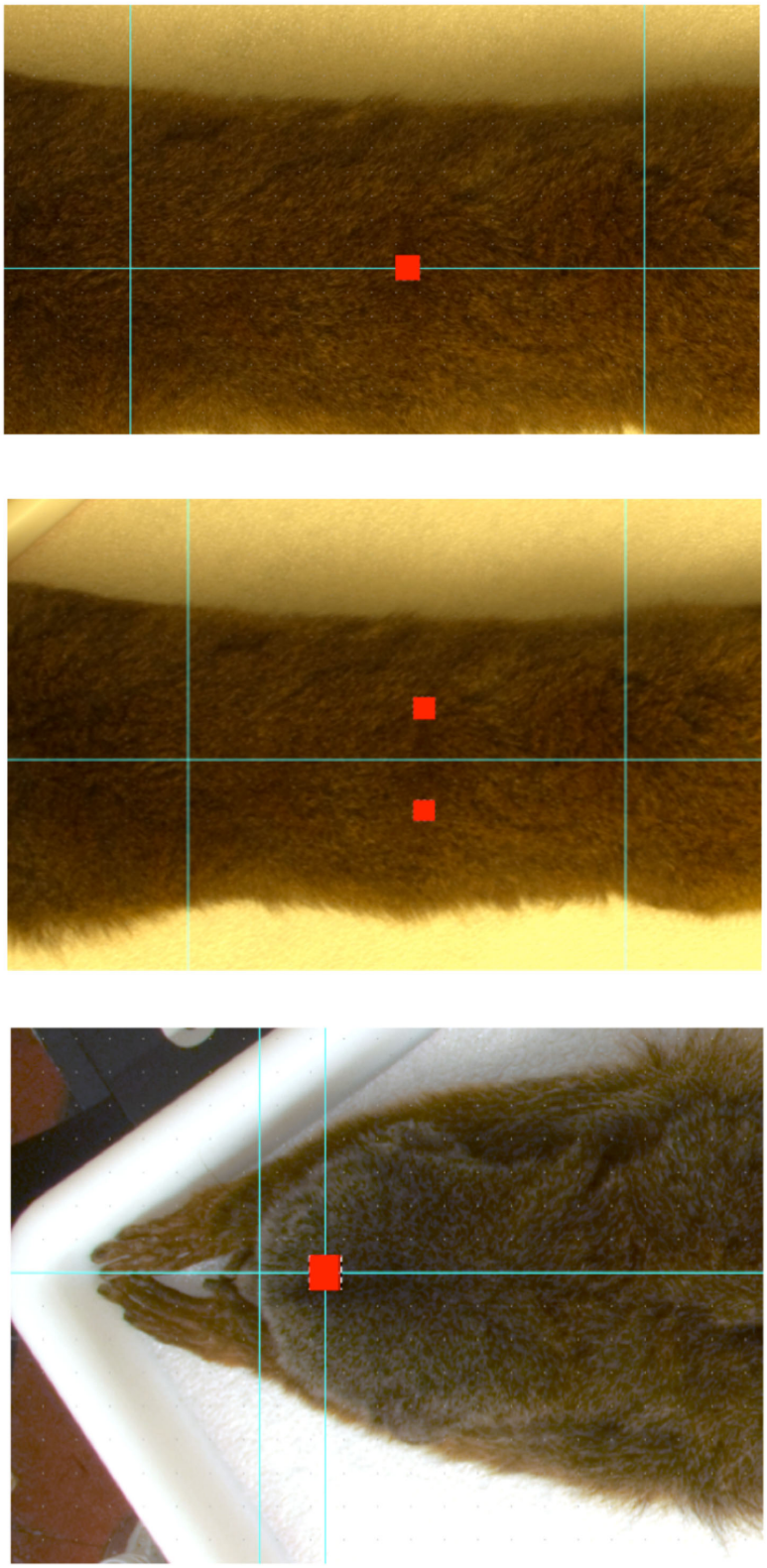


**Fig. 3: 50 x 50 pixel areas used for pelage color anaylsis.** Left= head, center= dorsolateral torso, right= meso-dorsal stripe.

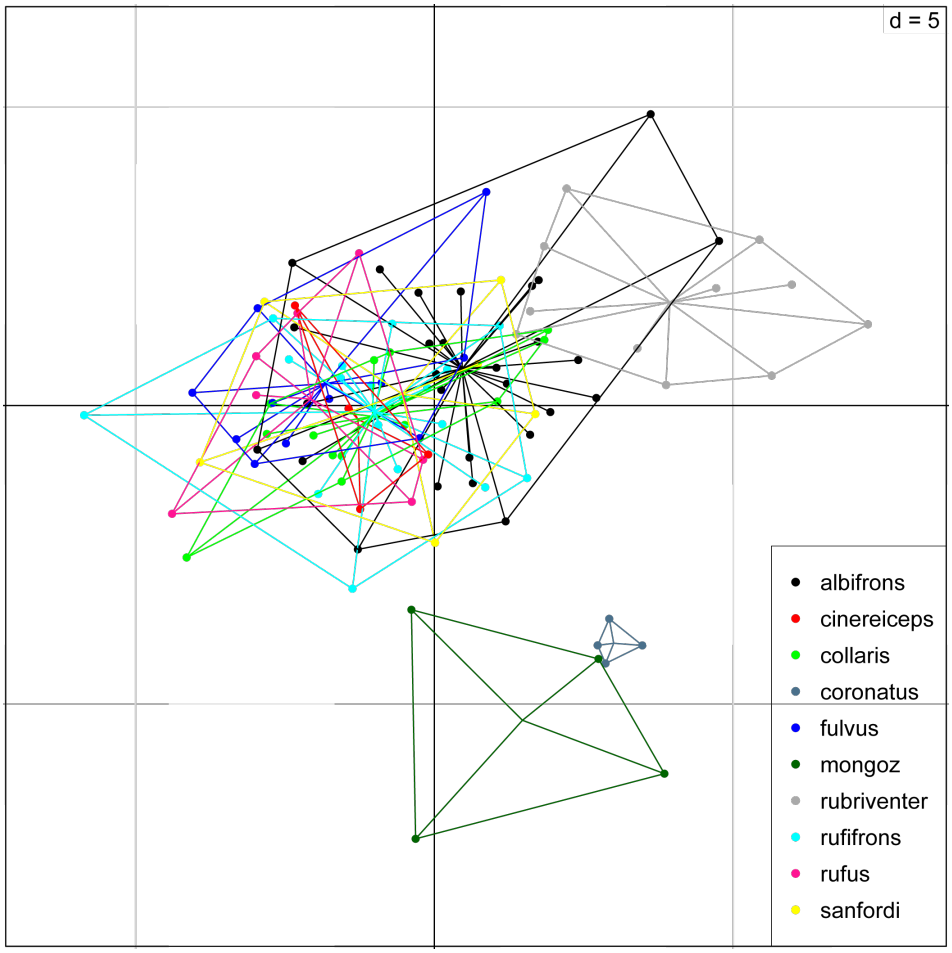



**Fig. 4: Scatterplot of bgPCA of morphological shape analysis including *E. coronatus, E. mongoz* and *E. rubriventer.***
Points represent individuals along the first and second principal component. A color legend for the different species is given inside the plot. p= < 0.001 (999 randomizations).

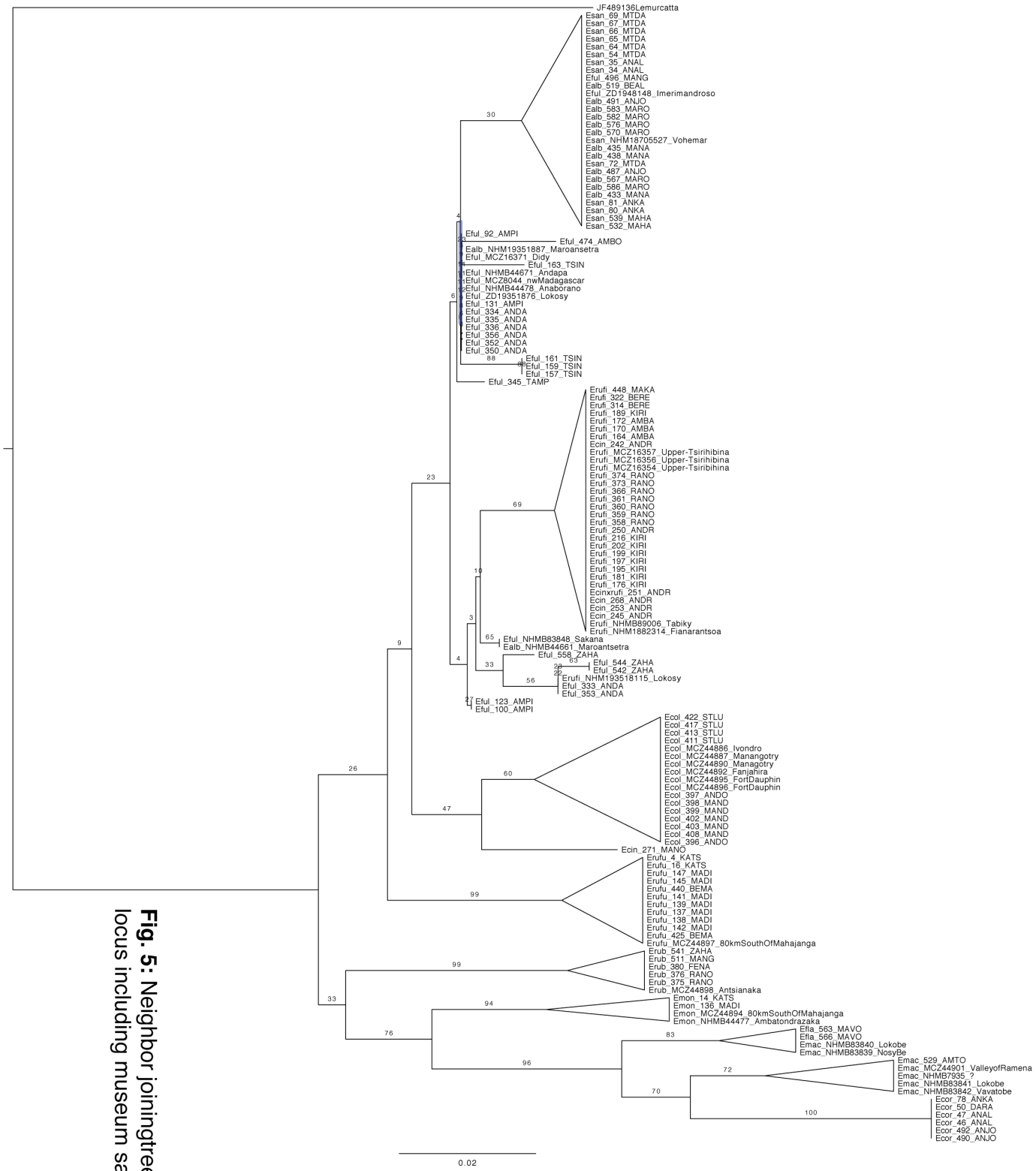
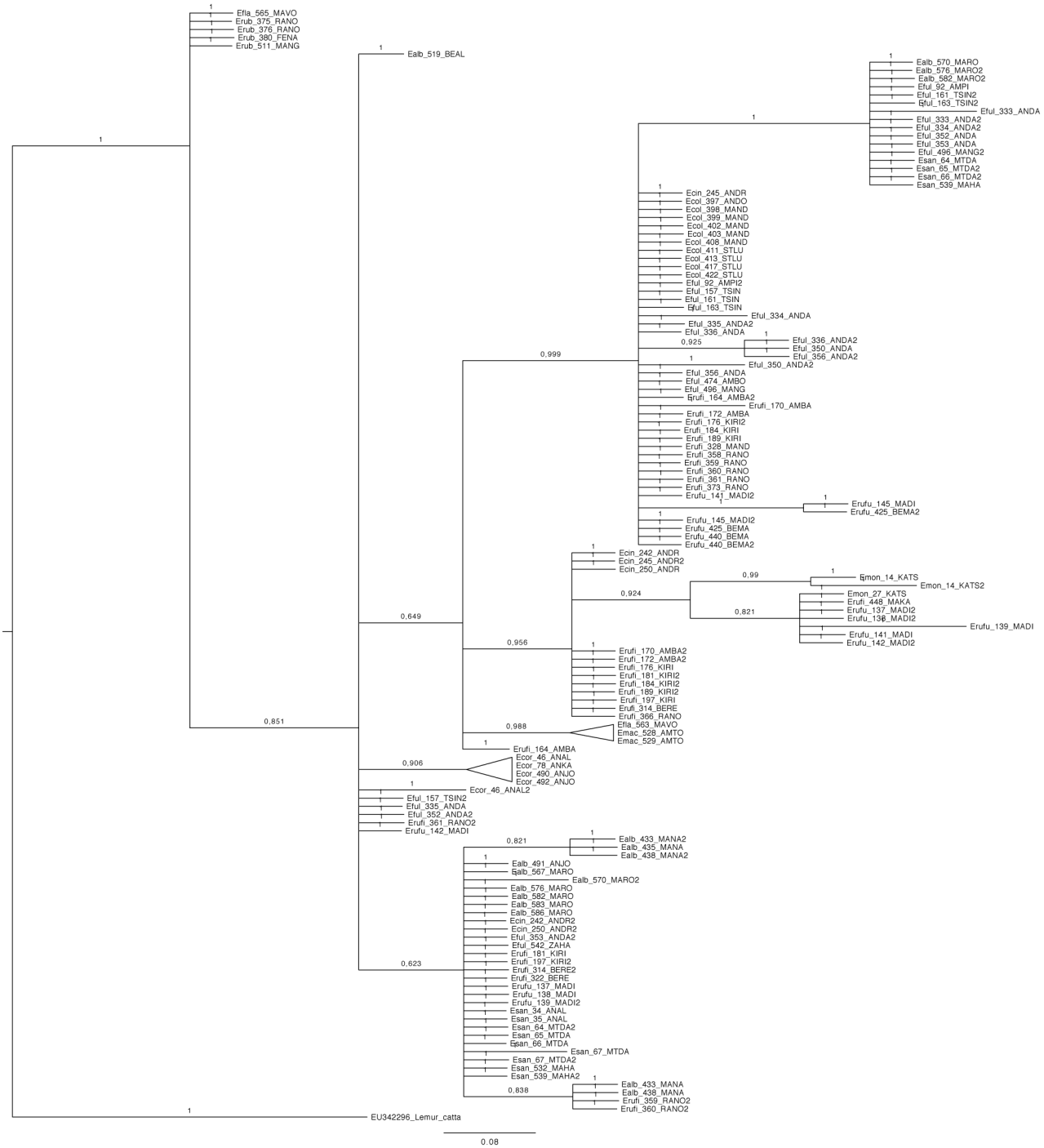
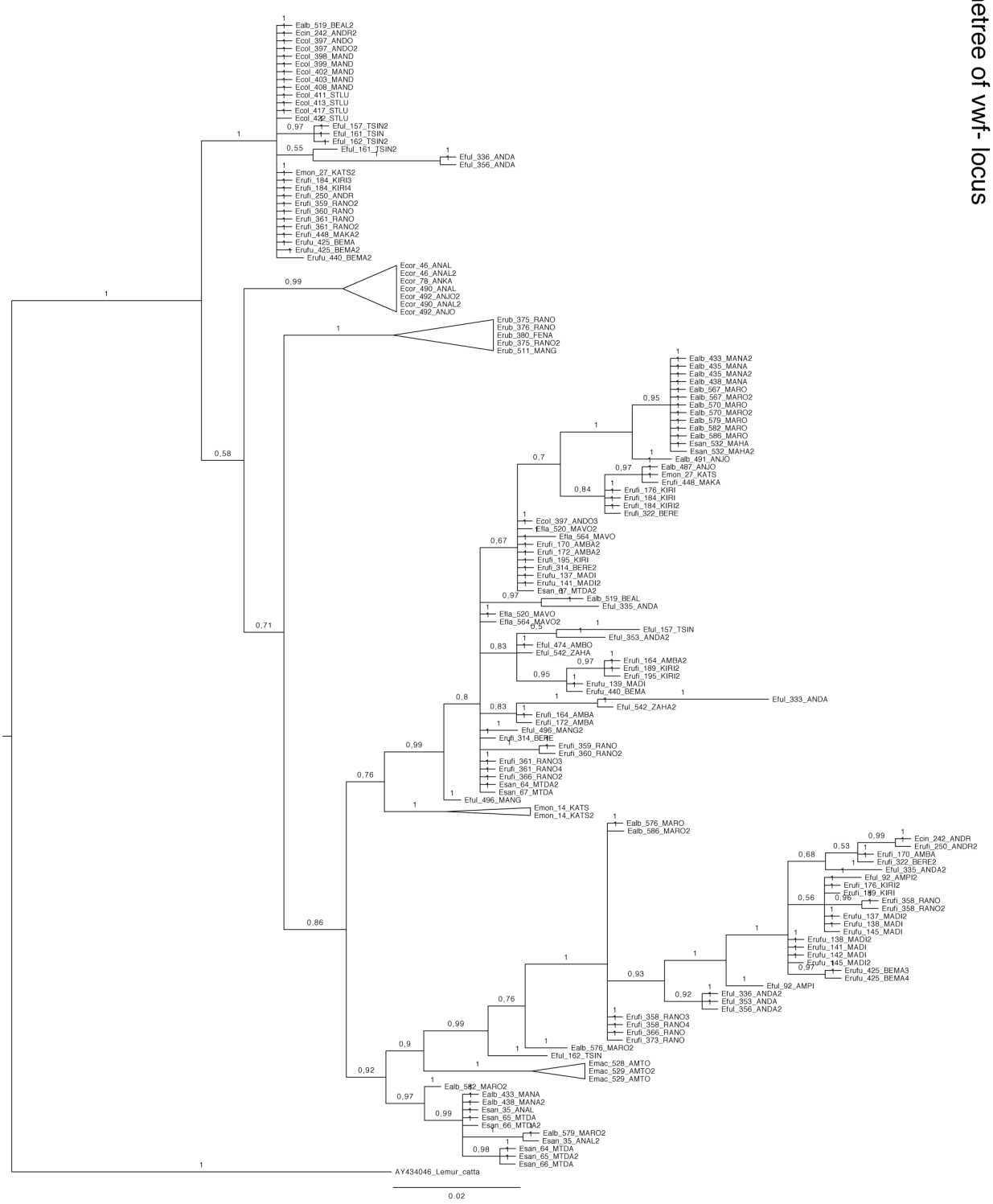



**Fig. 5:** Neighbor joiningtree of the cyt
locus including museum samples

6a) Genetree of nramp locus



6b) Genetree of vwf- locus



6c) Gene tree of eno locus

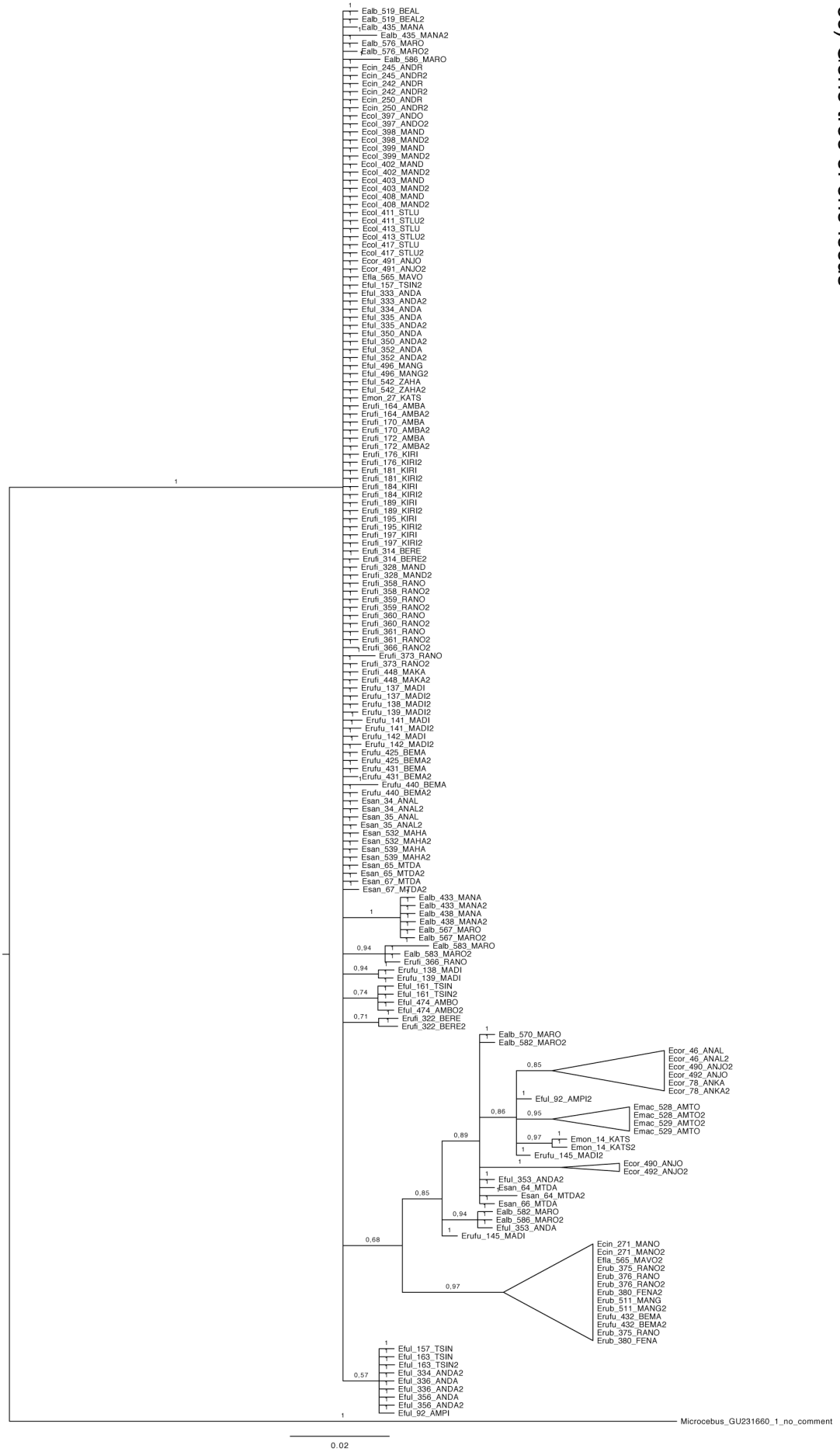



**Fig. 6a-c):** Bayesian gene trees of nuclear loci.

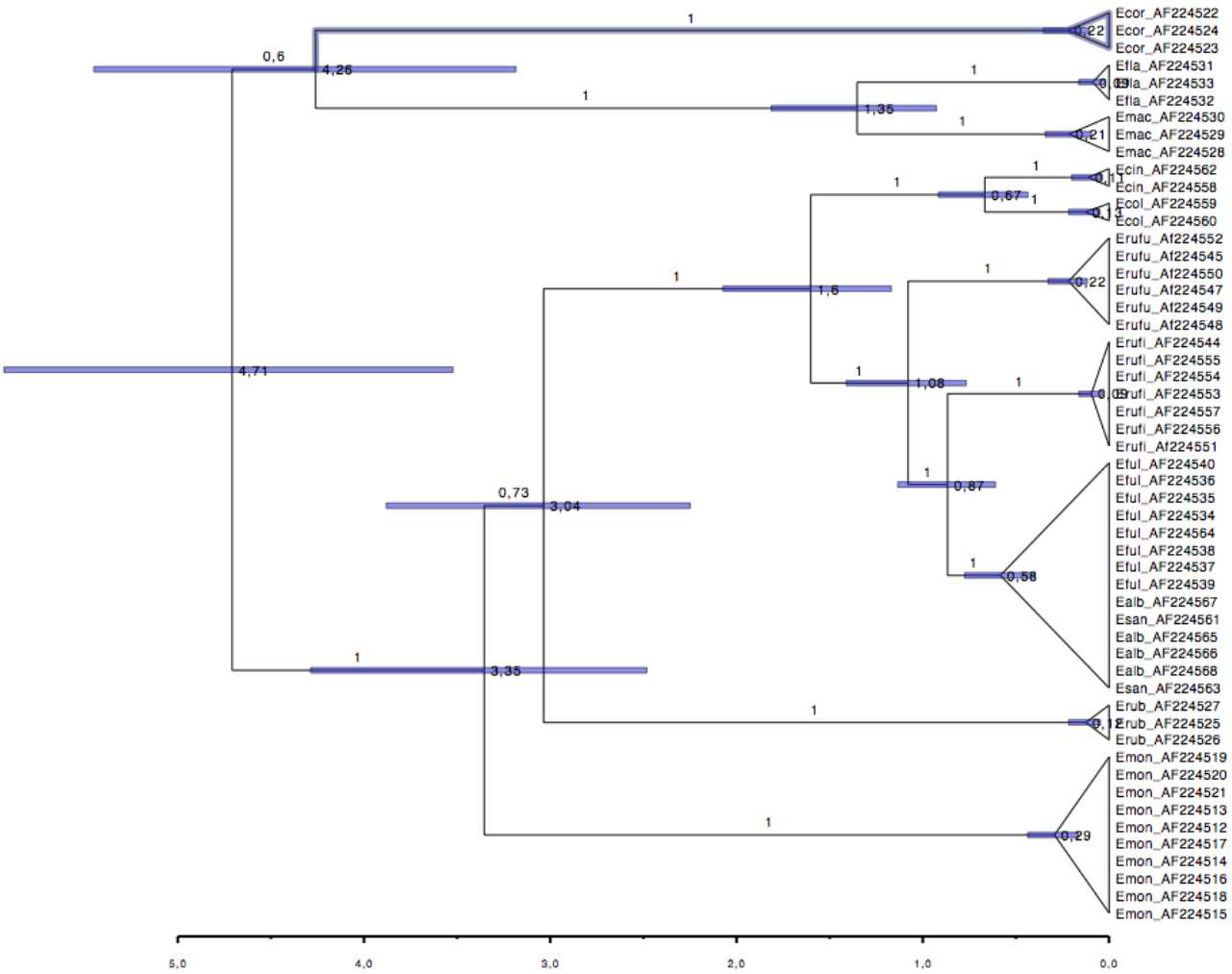



**Fig. 7: Simplified combined Bayesian tree of 53 *Eulemur* individuals of the PAST fragment (Pastorini et al. 2003) with divergence date estimates and node support as estimated from the *BEAST.** The mean age is given in million of years at the nodes and 95 % credibility intervals are indicated by the blue bars. Values along the branches show posterior probabilities. A time scale is shown at the bottom.

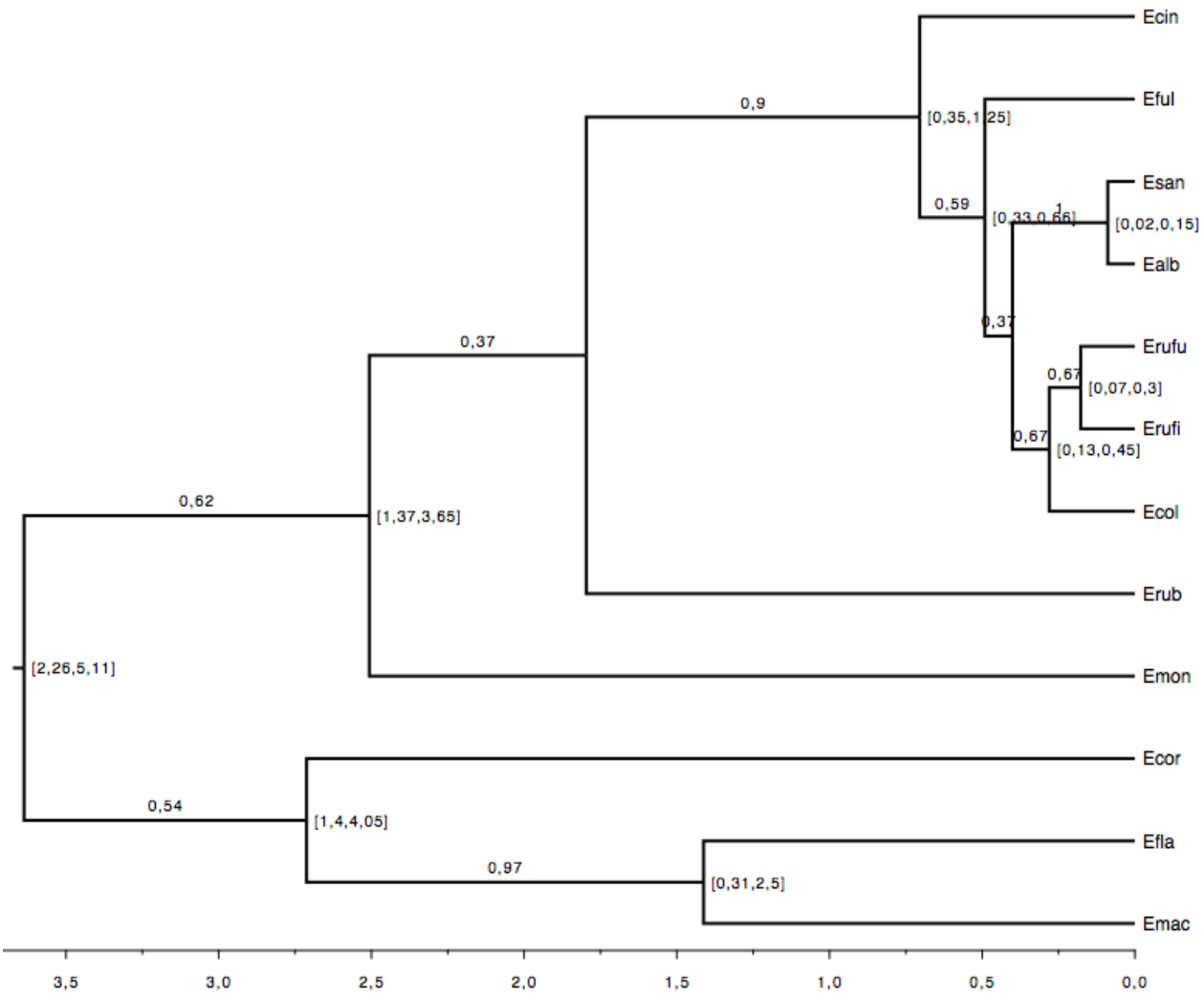



**Fig. 8: Time calibrated species tree of the genus *Eulemur* based on one mitochondrial (without PAST fragment) and three nuclear genetic loci.** Posterior probabilities are given at the branches. 95% credibility intervals for divergence date estimates are given at each node. A time scale in millions of years is given at the bottom.

## Appendix Tables

**Tab. 1: List of genetic samples used in this study.** # = Genbank accession number, x/y = GPS coordinates, ID= field or museum number (NHM= National History Museum, NHMB= Naturhistorisches Museum Berlin, MCZ= Museum of Comparative Zoology), POP= Population (IVOL=Parc Ivoloina, MANA=Mananara National Parc, ANJO= Anjombalava, BEAL= Bealanana, MARO=Marojejy, ANDR= Andringitra, MANO= Manombo Special Reserve, ANDO= Andohahela, MAND= Mandena, STLU= St.Luce, ANAL= Analamerana, DARA= Daraina, ANKA= Ankarana, MAVO= Manongarivo, AMPI= Ampijoroa, TSIN= Tsinjoarivo, ANDA= Andasibe, AMBO= Ambohitantely, MANG= Mangindrano, ZAHA= Zahamena, AMTO= Ambato, KATS= Katsepy, MADI= Madirovalo, RANO= Ranomafana, FENA= Fenarive Est, AMBA= Ambadira, KIRI= Kirindy, BERE= Berenty, MAKA= Massif du Makay, BEMA= Tsingy de Bemaraha, MTDA=Montagne D'Ambre, MAHA=Mahagaga), #= Genebank Accession Number

| ID | Pheno-type | Species | POP | eno | nramp | vwf | cytb | x | y | comment |
|---|---|---|---|---|---|---|---|---|---|---|
| 384 | Ealb | hybrid | IVOL | # | # | NA | NA | S18 03.405 | E49 21.599 | removed |
| 389 | Ealb | hybrid | IVOL | # | # | # | NA | S18 03.405 | E49 21.599 | removed |
| 390 | Ealb | hybrid | IVOL | # | # | # | NA | S18 03.405 | E49 21.599 | removed |
| 433 | Ealb | albifrons | MANA | # | # | # | # | S16 18.715 | E49 47.338 | |
| 435 | Ealb | albifrons | MANA | # | # | # | # | S16 18.715 | E49 47.338 | |
| 438 | Ealb | albifrons | MANA | # | # | # | # | S16 18.715 | E49 47.338 | |
| 487 | Ealb | albifrons | ANJO | NA | NA | # | # | S14 11.095 | E49 56.000 | removed |
| 491 | Ealb | albifrons | ANJO | # | # | # | # | S14 11.095 | E49 56.000 | Phenotype inferred from CytB and locality |
| 519 | Ealb | albifrons | BEAL | # | # | # | # | | | removed for *BEAST, pet |
| 567 | Ealb | albifrons | MARO | # | # | # | # | S14 11.095 | E49 56.000 | |
| 570 | Ealb | albifrons | MARO | # | # | # | # | S14 26.252 | E49 46.612 | |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| 576 | Ealb | albifrons | MARO | # | # | # | # | S14 26.071 | E49 45.641 | |
| 582 | Ealb | albifrons | MARO | # | # | # | # | S14 26.099 | E49 46.023 | |
| 583 | Ealb | albifrons | MARO | # | # | NA | # | S14 26.099 | E49 46.023 | |
| 586 | Ealb | albifrons | MARO | # | # | # | # | S14 26.252 | E49 46.612 | |
| 242 | Ecin | hybrid | ANDR | # | # | # | # | S22 08.714 | E46 56.926 | removed for *BEAST |
| 245 | Ecin | hybrid | ANDR | # | # | NA | # | S22 08.714 | E46 56.926 | removed for *BEAST |
| 250 | Ecin | hybrid | ANDR | # | # | # | # | S22 09.854 | E46 56.367 | removed for *BEAST |
| 251 | Ecinxrufi | hybrid | ANDR | NA | NA | NA | # | S22 09.854 | E46 56.367 | removed for *BEAST |
| 253 | Ecin | hybrid | ANDR | NA | NA | NA | # | S22 09.854 | E46 56.367 | removed for *BEAST |
| 271 | Ecin | cinereiceps | MANO | # | NA | NA | # | S23 01.200 | E47 41.569 | |
| 396 | Ecol | collaris | ANDO | NA | NA | NA | # | S24 45.330 | E46 51.418 | |
| 397 | Ecol | collaris | ANDO | # | # | # | # | S24 45.330 | E46 51.418 | |
| 398 | Ecol | collaris | MAND | # | # | # | # | S24 56.717 | E46 59.752 | |
| 399 | Ecol | collaris | MAND | # | # | # | # | S24 56.717 | E46 59.752 | |
| 402 | Ecol | collaris | MAND | # | # | # | # | S24 56.717 | E46 59.752 | |
| 403 | Ecol | collaris | MAND | # | # | # | # | S24 56.717 | E46 59.752 | |
| 408 | Ecol | collaris | MAND | # | # | # | # | S24 56.717 | E46 59.752 | |
| 411 | Ecol | collaris | STLU | # | # | # | # | S24 46.484 | E47 10.354 | |
| 413 | Ecol | collaris | STLU | # | # | # | # | S24 46.484 | E47 10.354 | |
| 417 | Ecol | collaris | STLU | # | # | # | # | S24 46.484 | E47 10.354 | |
| 422 | Ecol | collaris | STLU | NA | # | NA | # | S24 46.484 | E47 10.354 | |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| 46 | Ecor | coronatus | ANAL | # | # | # | # | S12 44.399 | E49 29.076 | |
| 47 | Ecor | coronatus | ANAL | NA | NA | NA | # | S12 44.399 | E49 29.076 | |
| 50 | Ecor | coronatus | DARA | NA | NA | NA | # | S13 10.026 | E49 42.408 | |
| 78 | Ecor | coronatus | ANKA | # | # | # | # | S12 56.389 | E49 07.242 | |
| 490 | Ecor | coronatus | ANJO | # | # | # | # | S14 11.095 | E49 56.000 | Phenotype inferred from CytB + vocalization |
| 492 | Ecor | coronatus | ANJO | # | # | # | # | S14 11.095 | E49 56.000 | Phenotype inferred from CytB + vocalization |
| 520 | Efla | flavifrons | MAVO | NA | NA | # | NA | S14 01.776 | E48 16.149 | removed |
| 563 | Efla | flavifrons | MAVO | NA | NA | NA | # | S14 01.661 | E48 16.603 | removed for *BEAST, Phenotype inferred from CytB |
| 565 | Efla | flavifrons | MAVO | # | # | NA | # | S14 01.661 | E48 16.603 | removed for *BEAST, unknown Phenotype |
| 566 | Efla | flavifrons | MAVO | NA | NA | NA | # | S14 01.661 | E48 16.603 | removed for *BEAST, Phenotype inferred from CytB |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| 92 | Eful | fulvus | AMPI | # | # | # | # | S16 19.163 | E46 48.374 | |
| 100 | Eful | fulvus | AMPI | NA | NA | NA | # | S16 19.163 | E46 48.374 | |
| 123 | Eful | fulvus | AMPI | NA | NA | NA | # | S16 19.163 | E46 48.374 | |
| 131 | Eful | fulvus | AMPI | NA | NA | NA | # | S16 19.163 | E46 48.374 | |
| 157 | Eful | fulvus | TSIN | # | # | # | # | S19 43.121 | E47 49.163 | |
| 159 | Eful | fulvus | TSIN | NA | NA | NA | # | S19 43.121 | E47 49.163 | |
| 161 | Eful | fulvus | TSIN | # | # | # | # | S19 43.121 | E47 49.163 | |
| 162 | Eful | fulvus | TSIN | NA | NA | # | NA | S19 43.203 | E47 49.078 | |
| 163 | Eful | fulvus | TSIN | # | # | NA | # | S19 43.203 | E47 49.078 | |
| 333 | Eful | fulvus | ANDA | # | # | # | # | S18 56.298 | E48 25.153 | |
| 334 | Eful | fulvus | ANDA | # | # | NA | # | S18 56.298 | E48 25.153 | |
| 335 | Eful | fulvus | ANDA | # | # | # | # | S18 56.298 | E48 25.153 | |
| 336 | Eful | fulvus | ANDA | # | # | # | # | S18 56.298 | E48 25.153 | |
| 345 | Eful | fulvus | TAMP | NA | NA | NA | # | S17 17.163 | E49 24.522 | |
| 350 | Eful | fulvus | ANDA | NA | NA | NA | # | S18 56.298 | E48 25.153 | |
| 352 | Eful | fulvus | ANDA | NA | NA | NA | # | S18 56.298 | E48 25.153 | |
| 353 | Eful | fulvus | ANDA | # | # | # | # | S18 56.298 | E48 25.153 | |
| 356 | Eful | fulvus | ANDA | # | # | # | # | S18 56.298 | E48 25.153 | |
| 474 | Eful | fulvus | AMBO | # | # | # | # | S18 11.906 | E47 16.929 | |
| 496 | Eful | fulvus | MANG | # | # | # | # | S14 11.955 | E48 57.093 | removed for *BEAST |
| 542 | Eful | fulvus | ZAHA | # | # | # | # | S17 29.167 | E48 44.007 | |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| 544 | Eful | fulvus | ZAHA | NA | NA | NA | # | S17 28.987 | E48 44.120 | |
| 558 | Eful | fulvus | ZAHA | NA | NA | NA | # | S17 29.167 | E48 44.007 | |
| 528 | Emac | macaco | AMTO | # | # | # | NA | 13 27.565 | 48 29.722 | |
| 529 | Emac | macaco | AMTO | # | # | # | # | 13 27.565 | 48 29.722 | |
| 14 | Emon | mongoz | KATS | # | # | # | # | S15 42.917 | E46 10.238 | |
| 27 | Emon | mongoz | KATS | # | # | # | NA | S15 42.917 | E46 10.238 | removed for *BEAST |
| 136 | Emon | mongoz | MADI | NA | NA | NA | # | S16 22.626 | E46 26.219 | |
| 375 | Erub | rubriventer | RANO | # | # | # | # | S21 15.838 | E47 25.329 | |
| 376 | Erub | rubriventer | RANO | # | # | # | # | S21 15.838 | E47 25.329 | |
| 380 | Erub | rubriventer | FENA | # | # | # | # | S17 22.558 | E49 24.319 | |
| 511 | Erub | rubriventer | MANG | # | # | # | # | S14 11.955 | E48 57.093 | |
| 164 | Erufi | rufifrons | AMBA | # | # | # | # | S19 47.569 | E44 39.331 | |
| 170 | Erufi | rufifrons | AMBA | # | # | # | # | S19 54.574 | E44 38.410 | |
| 172 | Erufi | rufifrons | AMBA | # | # | # | # | S19 54.574 | E44 38.410 | |
| 176 | Erufi | rufifrons | KIRI | # | # | # | # | S20 04.290 | E44 41.058 | |
| 181 | Erufi | rufifrons | KIRI | # | # | NA | # | S20 04.290 | E44 41.058 | |
| 184 | Erufi | rufifrons | KIRI | # | # | # | NA | S20 04.290 | E44 41.058 | |
| 189 | Erufi | rufifrons | KIRI | # | # | # | # | S20 04.290 | E44 41.058 | |
| 195 | Erufi | rufifrons | KIRI | # | NA | # | # | S20 04.290 | E44 41.058 | |
| 197 | Erufi | rufifrons | KIRI | # | # | NA | # | S20 04.290 | E44 41.058 | |
| 199 | Erufi | rufifrons | KIRI | NA | NA | NA | # | S20 04.290 | E44 41.058 | |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| 202 | Erufi | rufifrons | KIRI | NA | NA | NA | # | S20 04.290 | E44 41.058 | |
| 216 | Erufi | rufifrons | KIRI | NA | NA | NA | # | S20 04.290 | E44 41.058 | |
| 314 | Erufi | rufifrons | BERE | # | # | # | # | S24 59.932 | E46 17.874 | removed for migrate |
| 322 | Erufi | rufifrons | BERE | # | # | # | # | S24 59.932 | E46 17.874 | removed for *BEAST/migrate |
| 328 | Erufi | rufifrons | MAND | # | # | NA | NA | S24 56.717 | E46 59.752 | removed for *BEAST/migrate |
| 358 | Erufi | rufifrons | RANO | # | # | # | # | S21 15.762 | E47 25.364 | |
| 359 | Erufi | rufifrons | RANO | # | # | # | # | S21 15.781 | E47 25.429 | |
| 360 | Erufi | rufifrons | RANO | # | # | # | # | S21 15.781 | E47 25.429 | |
| 361 | Erufi | rufifrons | RANO | # | # | # | # | S21 15.762 | E47 25.364 | |
| 366 | Erufi | rufifrons | RANO | # | # | # | # | S21 15.838 | E47 25.329 | removed for *BEAST |
| 373 | Erufi | rufifrons | RANO | # | # | # | # | S21 15.838 | E47 25.329 | |
| 374 | Erufi | rufifrons | RANO | NA | NA | NA | # | S21 15.838 | E47 25.329 | |
| 448 | Erufi | rufifrons | MAKA | # | # | # | # | S21 34.036 | E45 04.228 | |
| 4 | Erufu | rufus | KATS | NA | NA | NA | # | S15 42.955 | E46 10.240 | |
| 16 | Erufu | rufus | KATS | NA | NA | NA | # | S15 42.955 | E46 10.240 | |
| 137 | Erufu | rufus | MADI | # | # | # | # | S16 22.409 | E46 26.118 | |
| 138 | Erufu | rufus | MADI | # | # | # | # | S16 22.409 | E46 26.118 | |
| 139 | Erufu | rufus | MADI | # | # | # | # | S16 22.409 | E46 26.118 | |
| 141 | Erufu | rufus | MADI | # | # | # | # | S16 22.519 | E46 26.285 | |
| 142 | Erufu | rufus | MADI | # | # | # | # | S16 22.519 | E46 26.285 | |

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|---|---|---|---|---|---|---|---|---|---|---|
| 145 | Erufu | rufus | MADI | # | # | # | # | S16 22.766 | E46 26.384 | |
| 147 | Erufu | rufus | MADI | NA | NA | NA | # | S16 22.935 | E46 25.799 | |
| 425 | Erufu | rufus | BEMA | NA | NA | NA | # | S19 01.134 | E44 46.444 | removed for *BEAST |
| 431 | Erufu | rufus | BEMA | # | NA | NA | NA | S19 01.134 | E44 46.444 | removed |
| 432 | Erufu | rufus | BEMA | # | NA | NA | NA | S19 01.134 | E44 46.444 | removed |
| 440 | Erufu | rufus | BEMA | # | # | # | # | S19 01.134 | E44 46.444 | |
| 34 | Esan | sanfordi | ANAL | # | # | NA | # | S12 44.241 | E49 28.859 | |
| 35 | Esan | sanfordi | ANAL | # | # | # | # | S12 44.241 | E49 28.859 | |
| 54 | Esan | sanfordi | MTDA | NA | NA | NA | # | S12 31.346 | E49 10.398 | |
| 64 | Esan | sanfordi | MTDA | # | # | # | # | S12 31.346 | E49 10.398 | |
| 65 | Esan | sanfordi | MTDA | # | # | # | # | S12 31.346 | E49 10.398 | |
| 66 | Esan | sanfordi | MTDA | # | # | # | # | S12 31.346 | E49 10.398 | |
| 67 | Esan | sanfordi | MTDA | # | # | # | # | S12 31.381 | E49 10.395 | |
| 69 | Esan | sanfordi | MTDA | NA | NA | NA | # | S12 31.346 | E49 10.398 | |
| 72 | Esan | sanfordi | MTDA | NA | NA | NA | # | S12 31.175 | E49 10.488 | |
| 80 | Esan | sanfordi | ANKA | NA | NA | NA | # | S12 56.486 | E49 07.268 | |
| 81 | Esan | sanfordi | ANKA | NA | NA | NA | # | S12 56.486 | E49 07.268 | |
| 532 | Esan | sanfordi | MAHA | # | # | # | # | S12 46.019 | E48 59.966 | |
| 539 | Esan | sanfordi | MAHA | # | # | NA | # | S12 46.019 | E48 59.966 | |
| NHM(ZD)1935 1887 | Ealb | albifrons | NA | NA | NA | NA | # | Maroantsetra | | |
| NHMB44661 | Ealb | albifrons | NA | NA | NA | NA | # | Maroantsetra | | |

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|---|---|---|---|---|---|---|---|---|---|
| MCZ44886 | Ecol | collaris | NA | NA | NA | NA | # | Ivondro | |
| MCZ44887 | Ecol | collaris | NA | NA | NA | NA | # | Manangotry | |
| MCZ44890 | Ecol | collaris | NA | NA | NA | NA | # | Manangotry | |
| MCZ44892 | Ecol | collaris | NA | NA | NA | NA | # | Fanjahira | |
| MCZ44895 | Ecol | collaris | NA | NA | NA | NA | # | Fort Dauphin | |
| MCZ44896 | Ecol | collaris | NA | NA | NA | NA | # | Fort Dauphin | |
| NHMB44671 | Eful | fulvus | NA | NA | NA | NA | # | Andapa | |
| MCZ16371 | Eful | fulvus | NA | NA | NA | NA | # | Didy | |
| MCZ8044 | Eful | fulvus | NA | NA | NA | NA | # | northwest Madagascar | |
| NHMB44478 | Eful | fulvus | NA | NA | NA | NA | # | Anaborano | |
| NHMB83848 | Eful | fulvus | NA | NA | NA | NA | # | Sakana | |
| NHM(ZD)1935 1876 | Eful | fulvus | NA | NA | NA | NA | # | Lokosy | |
| NHM(ZD)1948 148 | Eful | fulvus | NA | NA | NA | NA | # | Imerimandroso | |
| MCZ44901 | Emac | macaco | NA | NA | NA | NA | # | Valley of Ramena | |
| NHMB7935 | Emac | macaco | NA | NA | NA | NA | # | NA | |
| NHMB83839 | Emac | macaco | NA | NA | NA | NA | # | Nosy Be | |
| NHMB83840 | Emac | macaco | NA | NA | NA | NA | # | Nosy Be | |
| NHMB83841 | Emac | macaco | NA | NA | NA | NA | # | Lokobe | |
| NHMB83842 | Emac | macaco | NA | NA | NA | NA | # | Vavatobe | |
| MCZ44894 | Emon | mongoz | NA | NA | NA | NA | # | 80 km south of Mahajnaga | |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| NHMB44477 | Emon | mongoz | NA | NA | NA | NA | # | Lokobe | |
| MCZ44898 | Erub | rubriventer | NA | NA | NA | NA | # | Antsianaka | |
| NHM193518115 | Erufi | rufifrons | NA | NA | NA | NA | # | Lokosy | |
| MCZ16354 | Erufi | rufifrons | NA | NA | NA | NA | # | Upper Tsiribihina | |
| MCZ16356 | Erufi | rufifrons | NA | NA | NA | NA | # | Upper Tsiribihina | |
| MCZ16357 | Erufi | rufifrons | NA | NA | NA | NA | # | Upper Tsiribihina | |
| NHM(ZD)1882314 | Erufi | rufifrons | NA | NA | NA | NA | # | Fianarantsoa | |
| NHMB89006 | Erufi | rufifrons | NA | NA | NA | NA | # | Tabiky | |
| MCZ44897 | Erufu | rufus | NA | NA | NA | NA | # | 80 km south of Mahajanga | |
| NHM(ZD)18705527 | Esan | sanfordi | NA | NA | NA | NA | # | Vohemar | |
| | Lcat | Lemur catta | | | | | JF489136 | | |

**Tab. 2: Museum specimen used for morphometric analysis.** AMNH= American Museum of National History, New York; USNM= Smithsonian Institution Washington D.C.; NHM= National History Museum, London; MCZ= Museum of Comparative Zoology, Boston. m= male, f= female.

| ID | Species | Sex | Locality | Museum |
|---|---|---|---|---|
| AMNH100566 | albifrons | f | Maroantsetra | AMNH |
| AMNH100572 | albifrons | f | Maroantsetra | AMNH |
| AMNH100586 | albifrons | m | Maroantsetra | AMNH |
| AMNH100587 | albifrons | m | Andapa | AMNH |
| AMNH100588 | albifrons | m | Maroantsetra | AMNH |
| AMNH100589 | albifrons | m | Maroantsetra | AMNH |
| AMNH170699 | albifrons | m | Ambatondrama | AMNH |
| AMNH170701 | albifrons | f | Ambatondrama | AMNH |
| AMNH170708 | albifrons | f | Ambatondrama | AMNH |
| AMNH170715 | albifrons | f | Ambatondrama | AMNH |
| AMNH170717 | albifrons | f | Ambatondrama | AMNH |
| AMNH170719 | albifrons | f | Ambatondrama | AMNH |
| AMNH170723 | albifrons | f | Ambatondrama | AMNH |
| AMNH170725 | albifrons | f | Ambatondrama | AMNH |
| AMNH170728 | albifrons | f | Ambatondrama | AMNH |
| AMNH170731 | albifrons | m | Ambatondrama | AMNH |
| ZD19351887 | albifrons | m | Maroantsetra | NHM |
| ZD19351888 | albifrons | m | Maroantsetra | NHM |
| ZD19351890 | albifrons | m | Maroantsetra | NHM |
| ZD19351892 | albifrons | m | Maroantsetra | NHM |
| ZD19351893 | albifrons | f | Maroantsetra | NHM |
| ZD19351894 | albifrons | f | Maroantsetra | NHM |
| ZD19351895 | albifrons | f | Maroantsetra | NHM |
| ZD19351896 | albifrons | f | Maroantsetra | NHM |
| ZD19351897 | albifrons | f | Maroantsetra | NHM |
| ZD19351898 | albifrons | f | Maroantsetra | NHM |

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|---|---|---|---|---|
| ZD19351899 | albifrons | m | Andapa | NHM |
| AMNH100561 | cinereiceps | f | Manombo | AMNH |
| AMNH100562 | cinereiceps | m | Vondrozo | AMNH |
| ZD193518112 | cinereiceps | m | Manombo | NHM |
| AMNH100818 | cinereiceps | f | Vondrozo | AMNH |
| AMNH170749 | collaris | f | Eminiminy | AMNH |
| AMNH170750 | collaris | f | Eminiminy | AMNH |
| AMNH170755 | collaris | f | Eminiminy | AMNH |
| AMNH170759 | collaris | m | Eminiminy | AMNH |
| AMNH170764 | collaris | f | Eminiminy | AMNH |
| AMNH170766 | collaris | m | Eminiminy | AMNH |
| AMNH170770 | collaris | f | Eminiminy | AMNH |
| AMNH170771 | collaris | f | Eminiminy | AMNH |
| AMNH170772 | collaris | m | Eminiminy | AMNH |
| MCZ44887 | collaris | | Manongotry | MCZ |
| MCZ44888 | collaris | | Manongotry | MCZ |
| MCZ44889 | collaris | | Manongotry | MCZ |
| AMNH100520 | coronatus | f | Vohemar | AMNH |
| AMNH100610 | coronatus | m | Vohemar | AMNH |
| AMNH100611 | coronatus | m | Vohemar | AMNH |
| ZD19351859 | coronatus | f | Vohemar | NHM |
| USNM63339 | fulvus | m | Ambatobato near Tamatave | USNM |
| USNM63340 | fulvus | m | Ambatobato near Tamatave | USNM |
| USNM63341 | fulvus | f | Ambatobato near Tamatave | USNM |
| ZD1925833 | fulvus | m | Lakato | NHM |
| ZD1925835 | fulvus | m | Lakato | NHM |
| ZD19351850 | mongoz | m | Ambararatabe | NHM |
| ZD19351852 | mongoz | f | Ambararatabe | NHM |
| MCZ44881 | rubriventer | m | Manonga | MCZ |

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|---|---|---|---|---|
| MCZ44898 | rubriventer | f | Antsianaka | NHM |
| MCZ44899 | rubriventer | m | Antsianaka | NHM |
| MCZ8045 | rubriventer | | | NHM |
| USNM63335 | rubriventer | f | | USNM |
| ZD18887241 | rubriventer | | Tamatave | NHM |
| ZD18979110 | rubriventer | m | Vinanitelo | NHM |
| ZD1897919 | rubriventer | | Tanala | NHM |
| ZD19351846 | rubriventer | f | Andapa | NHM |
| ZD19351847 | rubriventer | f | Andapa | NHM |
| MCZ16356 | rufifrons | f | 30 miles south of Berevo | MCZ |
| MCZ16394 | rufifrons | m | 30 miles south of Berevo | MCZ |
| ZD18314 | rufifrons | | Fianarantsoa | NHM |
| ZD18314 | rufifrons | | Fianarantsoa | NHM |
| ZD19351876 | rufifrons | | Lokosy | NHM |
| ZD19391268 | rufifrons | m | Manakara | NHM |
| ZD19391269 | rufifrons | f | Manakara | NHM |
| ZD1948149 | rufifrons | f | Beroboka | NHM |
| AMNH100524 | rufifrons | m | Tabiky | AMNH |
| MCZ16353 | rufifrons | m | Upper Siribihina, Bemara Gorges | MCZ |
| MCZ16354 | rufifrons | m | Upper Siribihina Bemara Gorges | MCZ |
| MCZ16357 | rufifrons | f | Upper Siribihina | MCZ |
| MCZ16365 | rufifrons | m | Upper Siribihina | MCZ |
| MCZ16370 | rufifrons | f | Upper Siribihina | MCZ |
| MCZ16393 | rufifrons | f | Upper Siribihina | MCZ |
| MCZ16395 | rufifrons | m | Upper Siribihina | MCZ |
| ZD1913341 | rufus | f | Ambohimanga | NHM |
| ZD19351881 | rufus | f | Tsiandro | NHM |
| ZD19351883 | rufus | m | Namoroka | NHM |

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|---|---|---|---|---|
| AMNH100532 | rufus | f | Bekipany | AMNH |
| AMNH100607 | rufus | m | near Ankoja | AMNH |
| AMNH100614 | rufus | f | Ankoja | AMNH |
| AMNH100819 | rufus | f | Namoroka | AMNH |
| AMNH100521 | sanfordi | m | Tsarakibany | AMNH |
| ZD19351869 | sanfordi | m | MtDambre | NHM |
| ZD19351871 | sanfordi | m | MtDambre | NHM |
| ZD19351872 | sanfordi | f | MtDambre | NHM |
| AMNH100518 | sanfordi | f | MtDambre | AMNH |
| AMNH100577 | sanfordi | f | MtDambre | AMNH |

**Tab. 3: Museum specimen used for pelage color analysis.** AMNH= American Museum of National History, New York; USNM= Smithsonian Institution Washington D.C.; NHM= National History Museum, London; MCZ= Museum of Comparative Zoology, Boston. m= male, f= female

| ID | Species | Locality | Sex | Museum |
|---|---|---|---|---|
| AMNH100486 | albifrons | Maroantsetra | m | AMNH |
| AMNH100558 | albifrons | Maroantsetra | m | AMNH |
| AMNH100587 | albifrons | Andapa | m | AMNH |
| AMNH100588 | albifrons | Maroantsetra | m | AMNH |
| AMNH100589 | albifrons | Maroantsetra | m | AMNH |
| AMNH100590 | albifrons | Maroantsetra | m | AMNH |
| USNM63344 | albifrons | RiverFaraony | m | USNM |
| ZD193518103 | cinereiceps | Vondrozo | m | NHM |
| AMNH100562 | cinereiceps | Manombo | m | AMNH |
| AMNH100573 | cinereiceps | Vondrozo | m | AMNH |
| AMNH100579 | cinereiceps | Vondrozo | m | AMNH |
| AMNH100602 | cinereiceps | Vondrozo | m | AMNH |
| ZD193518100 | cinereiceps | Vondrozo | m | NHM |
| ZD193518101 | cinereiceps | Vondrozo | m | NHM |
| ZD193518102 | cinereiceps | Vondrozo | m | NHM |
| ZD193518104 | cinereiceps | Vondrozo | m | NHM |
| ZD193518105 | cinereiceps | Vondrozo | m | NHM |
| AMNH170751 | collaris | Eminiminy | m | AMNH |
| AMNH170760 | collaris | Eminiminy | m | AMNH |
| AMNH170764 | collaris | Eminiminy | m | AMNH |
| AMNH170765 | collaris | Eminiminy | m | AMNH |
| MCZ44893 | collaris | Fanjahira | m | MCZ |
| MCZ44895 | collaris | Fanjahira | m | MCZ |
| USNM317960 | collaris | Bemangidy | m | USNM |
| USNM317961 | collaris | Bemangidy | m | USNM |
| AMNH100527 | fulvus | Ivohibe | m | AMNH |

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|---|---|---|---|---|
| AMNH100528 | fulvus | Ivohibe | m | AMNH |
| USNM63339 | fulvus | Ambatobato | m | AMNH |
| USNM63340 | fulvus | Ambatobato | m | AMNH |
| ZD18823123 | rufifrons | Fianarantsoa | m | NHM |
| ZD19351879 | rufifrons | Tabiky | m | NHM |
| ZD19351882 | rufus | Ankoja | m | NHM |
| ZD19351886 | rufifrons | Ivohibe | m | NHM |
| AMNH100519 | rufifrons | Tabiky | m | AMNH |
| AMNH100524 | rufifrons | Tabiky | m | AMNH |
| MCZ16394 | rufifrons | 30msouthofBerevo | m | MCZ |
| MCZ16365 | rufifrons | UpperSiribihinariver | m | MCZ |
| MCZ16395 | rufifrons | UpperSiribihinariver | m | MCZ |
| MCZ16354 | rufifrons | UpperSiribihinariver | m | MCZ |
| MCZ16353 | rufifrons | UpperSiribihinariver | m | MCZ |
| MCZ16355 | rufifrons | UpperSiribihinariver | m | MCZ |
| USNM63338 | rufifrons | Fianarantsoa | m | AMNH |
| AMNH100517 | rufus | Soalala | m | AMNH |
| AMNH100522 | rufus | Namoroka | m | AMNH |
| AMNH100523 | rufus | Namoroka | m | AMNH |
| AMNH100525 | rufus | Soalala | m | AMNH |
| AMNH100569 | rufus | Tsitampiky | m | AMNH |
| AMNH100607 | rufus | Ankoja | m | AMNH |
| AMNH100521 | sanfordi | Tsarakibany | m | AMNH |
| AMNH100585 | sanfordi | MtDambre | m | AMNH |
| ZD18705527 | sanfordi | Vohemar | m | NHM |
| ZD19351869 | sanfordi | MtDambre | m | NHM |
| ZD19351870 | sanfordi | MtDambre | m | NHM |
| ZD19351871 | sanfordi | MtDambre | m | NHM |
| AMMH170725 | albifrons | Ambatondrandama | f | AMNH |
| AMNH100559 | albifrons | | f | AMNH |
| AMNH100560 | albifrons | Maroantsetra | f | AMNH |

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|---|---|---|---|---|
| AMNH100566 | albifrons | | f | AMNH |
| AMNH100572 | albifrons | Maroantsetra | f | AMNH |
| AMNH170705 | albifrons | Ambatondrandama | f | AMNH |
| AMNH170708 | albifrons | Ambatondrandama | f | AMNH |
| AMNH170715 | albifrons | Ambatondrandama | f | AMNH |
| AMNH170717 | albifrons | Ambatondrandama | f | AMNH |
| AMNH170720 | albifrons | Ambatondrandama | f | AMNH |
| AMNH170723 | albifrons | Ambatondrandama | f | AMNH |
| AMNH170728 | albifrons | Ambatondrandama | f | AMNH |
| AMNH100561 | cinereiceps | | f | AMNH |
| AMNH100564 | cinereiceps | | f | AMNH |
| AMNH100565 | cinereiceps | | f | AMNH |
| AMNH100568 | cinereiceps | | f | AMNH |
| AMNH100570 | cinereiceps | | f | AMNH |
| AMNH100575 | cinereiceps | | f | AMNH |
| AMNH100576 | cinereiceps | Vondrozo | f | AMNH |
| AMNH100580 | cinereiceps | | f | AMNH |
| AMNH100581 | cinereiceps | | f | AMNH |
| ZD193518106 | cinereiceps | | f | NHM |
| ZD193518107 | cinereiceps | | f | NHM |
| ZD193518108 | cinereiceps | Vondrozo | f | London |
| ZD193518109 | cinereiceps | | f | NHM |
| ZD193518110 | cinereiceps | | f | NHM |
| ZD193518111 | cinereiceps | | f | NHM |
| ZD193518113 | cinereiceps | | f | NHM |
| AMNH170750 | collaris | | f | AMNH |
| AMNH170755 | collaris | | f | AMNH |
| AMNH170771 | collaris | | f | AMNH |
| AMNH100529 | fulvus | | f | AMNH |
| MCZ16371 | fulvus | | f | AMNH |
| USNM63341 | fulvus | | f | AMNH |

| | | | | |
|---|---|---|---|---|
| USNM63342 | fulvus | | f | AMNH |
| USNM63343 | fulvus | | f | AMNH |
| ZD1913341 | fulvus | Ambohimanga | f | NHM |
| AMNH100526 | rufifrons | Ivohibe | f | AMNH |
| AMNH100571 | rufifrons | Tabiky/Ankazoabo | f | NHM |
| AMNH100582 | rufifrons | Tabiky/Ankazoabo | f | AMNH |
| MCZ16356 | rufifrons | 30m south of Berevo | f | MCZ |
| ZD1882311 | rufifrons | Fianarantsoa | f | NHM |
| ZD19351877 | rufifrons | Tabiky/Ankazoabo | f | NHM |
| ZD19351878 | rufifrons | Tabiky/Ankazoabo | f | NHM |
| ZD19391269 | rufifrons | Manakara | f | NHM |
| ZD1948149 | rufifrons | Beroboka | f | NHM |
| AMNH100532 | rufus | Bekipany | f | AMNH |
| AMNH100614 | rufus | Ankoja | f | AMNH |
| MCZ18630 | rufus | Upper Siribihina river | f | MCZ |
| MCZ18630 | rufus | 80km south of Majunga | f | MCZ |
| ZD18911225 | rufus | near Majunga | f | NHM |
| ZD19351881 | rufus | Tsiandro | f | NHM |
| AMNH100518 | sanfordi | MtDambre | f | AMNH |
| AMNH100577 | sanfordi | MtDambre | f | AMNH |
| AMNH100578 | sanfordi | MtDambre | f | AMNH |
| ZD19351872 | sanfordi | MtDambre | f | NHM |
| ZD19351873 | sanfordi | MtDambre | f | NHM |

**Tab. 4: Primer and annealing temperatures used in this study.** MID= Multiplexidentfier, °C= Annealing temperature.

| Primer | 454-Adaptor | MID | Universal tail | Template specific | Comment | °C |
|---|---|---|---|---|---|---|
| vWF forward | | | GTAAAACG ACGGCCA GT | CTTCTCCA TTGTCATT GAGAC | Template specific primer with universal tale for 1st PCR | 60 |
| vWF reverse | | | AACAGCTA TGACCATG | AGCTTCRC ACAGCAGA GACT | Template specific primer with universal tale for 1st PCR | 60 |
| NRAMP forward | | | GTAAAACG ACGGCCA GT | TAATCCTG CTGTCTCC TGAC | Template specific primer with universal tale for 1st PCR | 60 |
| NRAMP revers | | | AACAGCTA TGACCATG | GGGATTCT GAAACCAG AGTG | Template specific primer with universal tale for 1st PCR | 60 |
| ENO forward | | | GTAAAACG ACGGCCA GT | GCGCCACA CTAAATGA CTTG | Template specific primer with universal tale for 1st PCR | 60 |
| ENO reverse | | | AACAGCTA TGACCATG | CTGCCTCA TGCCACTC TCA | Template specific primer with universal tale for 1st PCR | 60 |
| For MID 1 | CGTATCGC CTCCCTCG CGCCATCA G | ACGAGTGC GT | GTAAAACG ACGGCCA GT | | 454 Primer for 2nd PCR | 60 |
| For MID 2 | CGTATCGC CTCCCTCG CGCCATCA G | ACGCTCGA CA | GTAAAACG ACGGCCA GT | | 454 Primer for 2nd PCR | 60 |
| For MID 3 | CGTATCGC CTCCCTCG CGCCATCA G | AGACGCAC TC | GTAAAACG ACGGCCA GT | | 454 Primer for 2nd PCR | 60 |
| For MID 4 | CGTATCGC CTCCCTCG CGCCATCA G | AGCACTGT AG | GTAAAACG ACGGCCA GT | | 454 Primer for 2nd PCR | 60 |
| For MID 5 | CGTATCGC CTCCCTCG CGCCATCA G | ATCAGACA CG | GTAAAACG ACGGCCA GT | | 454 Primer for 2nd PCR | 60 |
| For MID 6 | CGTATCGC CTCCCTCG CGCCATCA G | ATATCGCG AG | GTAAAACG ACGGCCA GT | | 454 Primer for 2nd PCR | 60 |

| | | | | | | |
|---|---|---|---|---|---|---|
| For MID 7 | CGTATCGCCTCCCTCGCGCCATCAG | CGTGTCTCTA | GTAAAACGACGGCCAGT | | 454 Primer for 2nd PCR | 60 |
| For MID 8 | CGTATCGCCTCCCTCGCGCCATCAG | CTCGCGTGTC | GTAAAACGACGGCCAGT | | 454 Primer for 2nd PCR | 60 |
| For MID 9 | CGTATCGCCTCCCTCGCGCCATCAG | TAGTATCAGC | GTAAAACGACGGCCAGT | | 454 Primer for 2nd PCR | 60 |
| For MID 10 | CGTATCGCCTCCCTCGCGCCATCAG | TCTCTATGCG | GTAAAACGACGGCCAGT | | 454 Primer for 2nd PCR | 60 |
| Rev MID 1 | CTATGCGCCTTGCCAGCCCGCTCAG | ACGAGTGCGT | AACAGCTATGACCATG | | 454 Primer for 2nd PCR | 60 |
| Rev MID 2 | CTATGCGCCTTGCCAGCCCGCTCAG | ACGCTCGACA | AACAGCTATGACCATG | | 454 Primer for 2nd PCR | 60 |
| Rev MID 3 | CTATGCGCCTTGCCAGCCCGCTCAG | AGACGCACTC | AACAGCTATGACCATG | | 454 Primer for 2nd PCR | 60 |
| Rev MID 4 | CTATGCGCCTTGCCAGCCCGCTCAG | AGCACTGTAG | AACAGCTATGACCATG | | 454 Primer for 2nd PCR | 60 |
| Rev MID 5 | CTATGCGCCTTGCCAGCCCGCTCAG | ATCAGACACG | AACAGCTATGACCATG | | 454 Primer for 2nd PCR | 60 |
| Rev MID 6 | CTATGCGCCTTGCCAGCCCGCTCAG | ATATCGCGAG | AACAGCTATGACCATG | | 454 Primer for 2nd PCR | 60 |
| Rev MID 7 | CTATGCGCCTTGCCAGCCCGCTCAG | CGTGTCTCTA | AACAGCTATGACCATG | | 454 Primer for 2nd PCR | 60 |
| Rev MID 8 | CTATGCGCCTTGCCAGCCCGCTCAG | CTCGCGTGTC | AACAGCTATGACCATG | | 454 Primer for 2nd PCR | 60 |

| | | | | | | |
|---|---|---|---|---|---|---|
| Rev MID 9 | CTATGCGCCTTGCCAGCCCGCTCAG | TAGTATCAGC | AACAGCTATGACCATG | | 454 Primer for 2nd PCR | 60 |
| Rev MID 10 | CTATGCGCCTTGCCAGCCCGCTCAG | TCTCTATGCG | AACAGCTATGACCATG | | 454 Primer for 2nd PCR | 60 |
| CytB_fulvus_group_rev1 | | | | CCTCATGGAAGGACATATCC | CytB- museum samples Ealb, Ecol, Esan, Eful, Ecin | 58 |
| CytB_fulvus_group_rev2 | | | | TCCTCATGGAAGGACATACC | CytB- museum samples Erufi, Erufu, Eful | 58 |
| CytB_outgroup_for | | | | AYAGCAGACACAACAACCGC | CytB- museum samples Emac, Erub, Ecor, Emon | 58 |
| CytB_outgroup_rev | | | | TCCTCATGGGAGRACRTACC | CytB- museum samples Emac, Erub, Ecor, Emon | 58 |
| CytB_fulvus_group_for | | | | ACAGCAGACACAACAACAGC | CytB- museum + fecal samples | 60 |
| CytB_for_94 | | | | AAYTTCGGTTCCCTCCTAGG | CytB- fecal samples | 60 |
| CytB_rev_173 | | | | TCTGCTGTGTAGTGTATTGC | CytB- fecal samples | 60 |
| CytB_rev_413 | | | | ATTTGTCCYCATGGAAGGAC | CytB- fecal samples | 60 |
| CytB_for_436 | | | | ATTACAAAYCTCCTCTCAGC | CytB- fecal samples | 60 |
| CytB_rev_507 | | | | GGAGAAACCDCCTCAGATTC | CytB- fecal samples | 60 |
| CytB_for_610 | | | | GGATCTAAYAACCCACTAGG | CytB- fecal samples | 60 |
| CytB_rev_642 | | | | GTCTGATGARGTTCCTAGTGG | CytB- fecal samples | 60 |
| CytB_for_757 | | | | CCCGACAACTACACACCAGC | CytB- fecal samples | 60 |

| | | | | | | |
|---|---|---|---|---|---|---|
| CytB_rev_895 | | | | GGATAGAGARGATTAGGGC | CytB- fecal samples | 60 |
| CytB_for_937 | | | | CGAAGCATATTATTCCGACCCC | CytB- fecal samples | 60 |
| CytB_rev_1034 | | | | TATTCGACGGGTTGGCCTCC | CytB- fecal samples | 60 |

**Tab. 5: PCR reaction mixtures.**

| | Cytochrome B | | 454 (nuclear) | |
|---|---|---|---|---|
| | ancient | feces | 1st PCR | 2nd PCR |
| Ingredient | µl | | | |
| Water | 7,3 | 13,9 | 13,9 | 22,9 |
| Hifi Buffer/ MasterMixPlus | 12,5 | 3,0 | 3,0 | 3,0 |
| DMSO | | 0,6 | 0,6 | 0,6 |
| MgCl2 (15mM) | | 1,3 | 1,3 | 1,3 |
| dNTPs | 0,6 | 0,6 | 0,6 | 0,6 |
| Primer A | 0,1 | 0,15 | 0,15 | 0,15 |
| Primer B | 0,1 | 0,15 | 0,15 | 0,15 |
| Taq | | 0,3 | 0,3 | 0,3 |
| Template | 5,0 | 10,0 | 10,0 | 1,0 |
| Total | 25,0 | 30,0 | 30,0 | 30,0 |

**Tab. 6: fdr- corrected p- values for pairwise comparisons after permutational MANOVA of loud calls.** n.s.= not significant.

| **chucks** | albifrons | cinereiceps | collaris | fulvus | rufifrons | rufus | sanfordi |
|---|---|---|---|---|---|---|---|
| cinereiceps | < 0,05 | | | | | | |
| collaris | < 0,01 | < 0,05 | | | | | |
| fulvus | n.s. | n.s. | < 0,001 | | | | |
| rufifrons | n.s. | < 0,01 | < 0,001 | < 0,001 | | | |
| rufus | n.s. | n.s. | < 0,001 | < 0,05 | < 0,001 | | |
| sanfordi | n.s. | < 0,01 | < 0,001 | n.s. | < 0,001 | < 0,01 | |

| **croaks** | albifrons | cinereiceps | collaris | fulvus | rufifrons | rufus |
|---|---|---|---|---|---|---|
| cinereiceps | n.s. | | | | | |
| collaris | n.s. | n.s. | | | | |
| fulvus | n.s. | n.s. | < 0,01 | | | |
| rufifrons | n.s. | n.s. | < 0,01 | n.s. | | |
| rufus | n.s. | n.s. | n.s. | n.s. | n.s. | |
| sanfordi | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. |

**Tab 7: FDR- corrected p-values for pairwise comparison of shapes.** n.s.= not significant.

| | albifrons | cinereiceps | collaris | coronatus | fulvus | mongoz | rubriventer | rufifrons | rufus |
|---|---|---|---|---|---|---|---|---|---|
| cinereiceps | < 0,05 | | | | | | | | |
| collaris | < 0,01 | n.s. | | | | | | | |
| coronatus | < 0,001 | < 0,001 | < 0,001 | | | | | | |
| fulvus | < 0,05 | n.s. | < 0,05 | < 0,001 | | | | | |
| mongoz | < 0,001 | < 0,001 | < 0,001 | < 0,001 | < 0,001 | | | | |
| rubriventer | < 0,001 | < 0,01 | < 0,001 | < 0,001 | < 0,001 | < 0,001 | | | |
| rufifrons | < 0,001 | n.s. | < 0,01 | < 0,01 | < 0,05 | < 0,001 | < 0,001 | | |
| rufus | < 0,05 | n.s. | < 0,05 | < 0,001 | n.s. | < 0,001 | < 0,001 | n.s. | |
| sanfordi | 0,068 | n.s. | n.s. | < 0,001 | 0,050 | < 0,001 | < 0,01 | < 0,05 | n.s. |

**Tab. 8: FDR-corrected p-values for pairwise comparisons of permutational MANOVA for pelage coloration.** n.s.= not significant.

| **females** | albifrons | cinereiceps | collaris | fulvus | rufifrons | rufus |
|---|---|---|---|---|---|---|
| cinereiceps | n.s. | | | | | |
| collaris | n.s. | n.s. | | | | |
| fulvus | < 0,05 | < 0,01 | n.s. | | | |
| rufifrons | < 0,001 | < 0,001 | < 0,05 | < 0,01 | | |
| rufus | < 0,001 | < 0,001 | n.s. | < 0,05 | n.s. | |
| sanfordi | < 0,01 | < 0,001 | n.s. | < 0,05 | n.s. | n.s. |

| **males** | albifrons | cinereiceps | collaris | fulvus | rufifrons | rufus |
|---|---|---|---|---|---|---|
| cinereiceps | < 0,001 | | | | | |
| collaris | < 0,001 | n.s. | | | | |
| fulvus | < 0,01 | n.s. | n.s. | | | |
| rufifrons | < 0,001 | < 0,001 | < 0,01 | < 0,05 | | |
| rufus | < 0,01 | < 0,001 | < 0,001 | < 0,01 | < 0,05 | |
| sanfordi | < 0,01 | < 0,001 | < 0,01 | < 0,01 | n.s. | < 0,05 |

**Tab. 9: Parameter estimates of Θ (Theta= $N_e\mu$) and M (M= mμ) for each migration model comparison over all loci.** Effective population size expressed as $N_e\mu$ (Θ) (μ= mutation rate) and migration rate expressed as mμ. Values give mean values and the 2.5-97.5% percentiles in brackets for each parameter. Note that for this analysis the heritability of the nDNA loci were scaled down by a factor of four so that the parameter values over all loci are interpreted the same as mtDNA.

| Pop/ species | Θ 1 | Θ 2 | Θ 3 | M2>1 | M3>1 | M1>2 | M3>2 | M1>3 | M2>3 |
|---|---|---|---|---|---|---|---|---|---|
| *E. rufifrons* west (1)- east (2) | 0.01062 (0.0- 0.018) | 0.00265 (0.0- 0.00467) | - | 1566.0 (1016.7- 2111.7) | - | 1384.0 (936.7-1 978.3) | - | - | - |
| *E. fulvus* (1)- *E. rufifrons* (2)- *E. rufus* (3) | 0.00231 (0.00013 - 0.00412) | 0.00253 (0.00027 - 0.00467 | 0.00381 (0.0-0.00 473 | 341.9 (32.7- 270) | 150.3 (14-210. 7) | 196.2 (52- 363.3) | 308.6 (25.3- 84.7) | 631.6 (496. 7- 960) | 624 (535.3- 858.7) |
| *E. albifrons* (1)- *E. fulvus* (2)- *E. sanfordi* (3) | 0.00953 (0.0- 0.0273) | 0.01139 (0.0- 0.0287) | 0.00596 (0.0- 0.0227) | 194.2 (72.7- 332.7) | 801.2 (568.7- 1000) | 156.1 (59.3- 266.7) | 89.1 (22- 158.7) | 227.5 (71.3- 408.7) | 99.3 (12- 192) |

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# Curriculum vitae

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| 2000 | High school diploma, Rhenanus school Bad Sooden- Allendorf |

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| 2000- 2001 | Evangelic nursery Rossbach |

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| 2001- 2003 | Basic study period in Biology |
| 2003 | Intermediate diploma in Zoology, Microbiology, Chemistry and Physics |
| 2003- 2006 | Scientific assistant in the Department of Virology of the |

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| 2004- 2005 | Scientific assistant in the research group Voice and Development of Senses of the Department of Phoniatrics und Pedaudiology of the University of Göttingen |
| 2005 | Mammalian Biodiversity Research Expedition Mongolia and field internship in Madagascar |
| 2006 | Master thesis: Genetic population structure on different spatial scales in a strepsirrhine primate, *Mirza coquereli* |
| 2006-2008 | Scientific assistant in the Department of Behavioral Ecology and Sociobiology of the German Primate Center Göttingen |
| 2007 | Diploma degree in Biology,<br>Project in Madagascar: Assessment of the distribution and the conservation status of *Mirza zaza* |
| 2008 | Participant at the BBSRC Summer School in Molecular Biology, Evolution and Diversity, University of Nottingham |
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Rode EJ, Nekaris KA, **Markolf M**, Schliehe-Diecks S, Seiler M, Radespiel U, Schwitzer C, 2013, "Social organization of northern giant mouse lemurs *Mirza zaza* in Sahamalaza, north western Madagascar, inferred from nest group composition and genetic relatedness", Contributions to Zoology, 82(2): 71-83

**Markolf M** & Rakotonirina HM 2012, "Wie viele Lemurenarten gibt es eigentlich?", In Madagaskar - Von Makis und Menschen, Editor: Lennart W. Pyritz, Springer Spektrum Verlag

Rainer Dolch, Rita Ratsisetraina, **Matthias Markolf**, Tiana Ratolojanahary, Hanitriniaina Rakotonirina, Edward Louis Jr., Evrard Wendenbaum 2012, Assessment of lemur diversity of the Makay massif", Lemur News 16

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**Markolf** et al. 2011, "On species delimitation: Yet another lemur species or just genetic variation", BMC Evolutionary Biology, 11:216

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Excel, Word, Power point, Adobe Photoshop/Lightroom/Illustrator/ Premiere Pro, basic HTML and CSS coding, GIS

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Ich versichere, dass ich die vorliegende Arbeit „Biodiversity of true lemurs (*Eulemur* spp.):- Species delimitation and phylogeography in the brown lemur complex“ selbstständig verfasst und keine anderen als die angegebenen Hilfsmittel verwendet habe. Die Stellen, die anderen Werken wörtlich oder sinngemäß entnommen sind, sind als solche kenntlich gemacht. Eigene Beiträge im Verhältnis zu denen von Koautoren bei bereits publizierten oder zur Publikation einzureichenden Teilen dieser Arbeit sind wie folgt:

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Göttingen, Juni, 2013