Molecular Systematics of Lemurs

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von

Jennifer Pastorini

von Zürich

Begutachtet von

Prof. Dr. Robert D. Martin

Prof. Dr. Michael R. J. Forstner

Dr. Michael W. Bruford

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Contents

1. Introduction

This study investigates the systematics of the lemurs of Madagascar, which constitute the infraorder Lemuriformes of the primate suborder Strepsirrhini. Evolutionary relationships within this prosimian group remain controversial, despite a long history of previous investigations. In a multi-level approach, the study presented here uses sequence variation in mitochondrial genes to reconstruct phylogenetic relationships among families, genera, species and subspecies of the extant lemurs.

1.1 Systematics and the Comparative Method in Biology

For centuries, naturalists have endeavoured to detect, describe and explain diversity in the biological world. This undertaking is known as systematics, the science of comparative biology. The primary goal of systematists is to describe taxonomic diversity and to reconstruct the natural hierarchy, or phylogenetic relationships, among those taxa. The core concept of phylogenetic systematics is the use of derived (apomorphic) characters to reconstruct common ancestral relationships and to group taxa on the basis of common ancestry. This concept was first formalised for morphological characters by Hennig in 1950. In phylogenetic studies, the inferred evolutionary relationships among a group of organisms are illustrated by means of a phylogenetic tree.

Comparative biology has a long history. Its central goal has been to understand the huge diversity of form and function observed across the world's organisms. Concepts such as taxonomy and homology have been the core intellectual instruments for facilitating this understanding. Thus, comparative biologists have tried to sort the world's organisms into species, largely based on their characteristics of form, and to describe their similarities and differences. For several hundred years, this sustained comparative work has led to the realisation that similarities and differences among species are best ordered in terms of a hierarchy of relationships. The formalisation of a hierarchical system of nomenclature by Linnaeus in 1758 established a framework for describing and categorising biological diversity. This hierarchical system was initially independent of evolutionary theory. Later evolutionists co-opted the system

to produce a classification based on phylogenetic relationships (for review see Li & Graur 1991; Miyamoto & Cracraft 1991; Forey et al. 1992; Hillis et al. 1996).

There are two largely distinct undertakings in systematics. One is 'phylogenetic reconstruction' by inference of ancestral relationships. The other is the generation of a 'classification' that is compatible with the inferred phylogenetic relationships (taxonomy). The primary goal of this thesis is phylogenetic reconstruction. Cladistics is a method of systematics advocated by Hennig (1950) that is used to construct classifications based on strict monophyletic groups. This contrasts with the approach favoured by the more traditional school of evolutionary systematics, which holds that a classification should be compatible with the consensus of opinion regarding phylogenetic relationships among the organisms involved, but should not be based exclusively on the inferred branching pattern in the phylogenetic tree. Such a classification may still contain some taxa that are strictly monophyletic, but not all taxa will be so.

Molecular systematics uses genetic markers to make inferences about population processes and evolutionary relationships among organisms. This area of molecular evolution has generated great interest in the last two decades, largely because in many cases phylogenetic relationships remain difficult to assess by morphological and/or behavioural criteria alone.

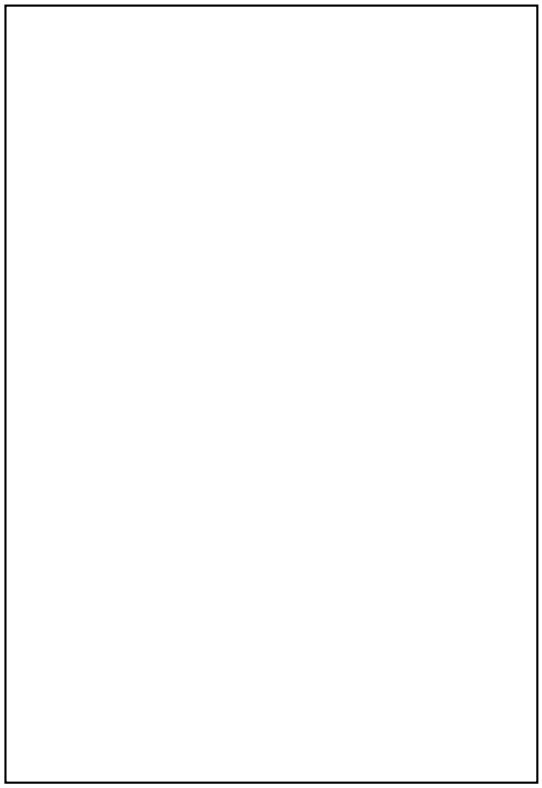

Fig. 1.1 *Eulemur macaco flavifrons* female (left) and male (right) at Apenheul Zoo (August 1999).

1.2 DNA Sequencing

Prior to the 1960s, most systematic studies utilised morphological characters to reconstruct relationships. However, contributions of molecular approaches to phylogenetic research have steadily increased over the last 30 years. Most importantly, the introduction of the polymerase chain reaction (PCR) in 1989 (Litt & Luty 1989; Tautz 1989; Weber & May 1989) has revolutionised the methods for generating nucleotide sequences. In conjunction with the design of broadly applicable sets of primers, gene amplification methods have spawned increasingly larger data sets of DNA sequence variation within and between species. DNA sequences are readily coded as character state data. Thus, molecular sequence analysis provides informative data for the construction of phylogenies because the genome provides a vast number of characters that can be used in phylogenetic analyses. At present, DNA sequences are rapidly becoming the preferred data for molecular systematics (for review see Li & Graur 1991; Miyamoto & Cracraft 1991; Forey et al. 1992; Hillis et al. 1996).

Mitochondrial DNA (mtDNA) analysis has become established as a powerful tool for the study of animal evolution. The mtDNA of animals has many unique properties that make it particularly useful for studies in molecular evolution (for review see Brown 1983; Moritz et al. 1987). The mitochondrial genome is small and simple, in contrast to the large and complex nuclear genome. The animal mitochondrial genome is 25,000 times smaller than the smallest animal nuclear genome. The mtDNA of multicellular animals ranges in size from 15,700 to 19,500 bp. Given the broad taxonomic range represented, the size variation is remarkably small. Among mtDNA from mammals, only that of the domestic rabbit falls outside the extremely narrow range of 16,500 ± 200 bp. Animal mtDNA consists of a single, duplex, closed-circular DNA molecule. Its gene content appears to be uniformly conserved in mammals. There are 2 ribosomal RNA (rRNA) genes, 22 transfer RNA (tRNA) genes and 13 protein genes which code for subunits of enzymes functioning in electron transport or ATP synthesis (Fig. 1.2). A control region is also present. Both spacer sequences between genes and intervening sequences (introns) within transcribed genes are absent from animal mtDNA. Furthermore, the animal mitochondrial genome appears to be much less susceptible to frequent sequence rearrangements, in marked contrast to the very complex and relatively fluid sequence organisation and structure of the nuclear genome. Finally, all of the genetic variables that are a consequence of biparental (sexual) inheritance are absent from the genetics of mtDNA, which is maternally (clonally)

inherited. An important consequence of these nuclear-mitochondrial differences is that many of the complexities and ambiguities that make nuclear data difficult to interpret are reduced or absent from mitochondrial data. Because of the apparently great reduction in the number of mechanisms of variation available to mtDNA, its evolution appears to proceed in a greatly simplified and more straightforward manner than the evolution of nuclear DNA.

The mitochondrial genome has also proved to be particularly useful for phylogenetic studies because it evolves at a higher rate than the mammalian nuclear genome (Brown et al. 1979). Estimates suggest that the rate of base substitutions in mammalian mtDNA is 5–10 times greater than that of single-copy nuclear DNA (Brown et al. 1982). Different parts of mtDNA change at different rates. For example, the control region changes very rapidly, both within and between species. This rapid rate of base substitution quickly leads to the accumulation of parallel and back mutations, particularly among the more distantly related taxa (Brown et al. 1982).

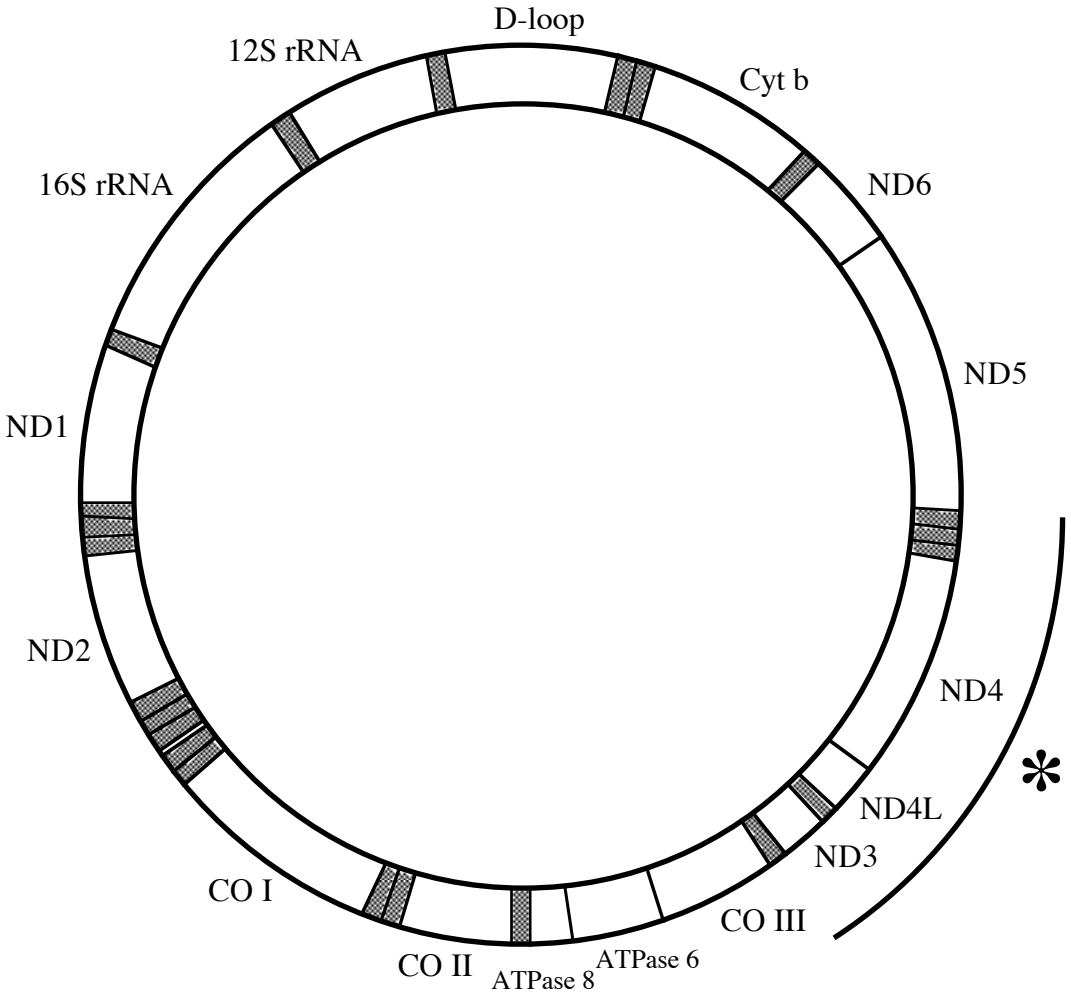

Fig. 1.2 Mammalian mitochondrial genome. Shaded areas are the tRNAs. * Indicates the region sequenced in this study.

1.3 Phylogenetic Reconstruction

The use of molecular data in systematics has dramatically increased over the last two decades. Alongside advances in biotechnology, there have been improvements in the analysis of molecular variation within and among species. There are many different tree-making methods that can be used for molecular data. Each of these methods has both advantages and disadvantages, and the overall relative efficiencies of the methods in recovering the correct phylogenetic tree remain controversial. The major problem in studying the relative efficiencies is that the true tree is usually unknown, so that it is difficult to judge which tree is the correct one. For detailed reviews on phylogenetic analyses of molecular data see Miyamoto & Cracraft 1991, Forey et al. 1992, Hillis et al. 1996 or Nei 1996.

Character Weighting

Character weighting is the process of assigning a 'valuation' factor to characters. Characters are thereby given relative ranks. Some characters will be 'valued' higher than others, and these will figure more prominently in the decision as to which branching diagram is to be preferred.

Nucleotide sites with high substitution rates are not very informative for phylogenetic construction when relatively distantly related sequences are used. The reason is that at these sites multiple substitutions are likely to have occurred, and this will introduce noise into phylogenetic inference.

In nucleotide sequences, adenine (A) and guanine (G) are known as purines and cytosine (C) and thymine (T) as pyrimidines. Substitution events are divided into two kinds. Transitions are changes involving either purines or pyrimidines, of which there are four possible options (A-G, G-A, C-T, T-C). Transversions are changes between purines and pyrimidines, of which there are eight possible options (A-C, C-A, A-T, T-A, G-C, C-G, G-T, T-G). Changes observed in aligned sequences can be thought of as an accumulation of transversional and transitional substitutions, where the frequency of these two classes of changes may be of different orders. Comparisons among closely related mtDNA sequences in vertebrates show that transitions greatly outnumber transversions (Brown et al. 1982).

If transversions occur much less frequently than transitions and the amount of divergence is high, transition differences are likely to approach or reach saturation. When this

happens, transitions will contribute little phylogenetic information. Perhaps the most troublesome aspect is determining how to choose the costs for different kinds of transformations. The appropriate weights can be estimated from the data themselves. For some molecules, it might even be argued that transitions occur so frequently that they quickly degenerate into noise and should therefore be ignored altogether. A disadvantage to the complete rejection of information on transitions is that, while transitions may become saturated over long evolutionary distances, they may nonetheless be highly informative with respect to relationships among closely related taxa.

Silent substitutions in protein-coding genes (i.e. those not leading to a change in amino acid sequence) are much more frequent than replacement substitutions. Thus, the third codon positions tend to become randomised quickly and convey very little information about distant phylogenetic relationships. This may necessitate restriction of analysis to the first two nucleotides of each codon. This strategy is appropriate when a substantial sequence divergence is apparent. The rationale is that the third codon position will be largely randomised and hence phylogenetically uninformative.

Distance Methods

There are a large number of different distance methods for the construction of phylogenetic trees. In distance methods, an evolutionary distance is computed for all pairs of sequences, and a phylogenetic tree is constructed from pairwise distances by using the least squares method, minimum evolution, or some other criterion. The evolutionary distance used for this purpose is usually an estimate of the number of nucleotides or amino acid substitutions per site, but other distance measures may also be used. A pairwise distance estimate is essentially the branch length in an optimal phylogenetic tree of two taxa. The negative aspect of reducing character data to pairwise distances is that information is lost in the transformation.

The distance measure is the degree of dissimilarity between two taxa or two genes. The simplest distance is the number of positions in which the pairwise comparisons differ. This can be expressed as a percentage (uncorrected distance) or a fixed number (absolute distance). The uncorrected distance is simply the total number of differences divided by the total number of available sites. Corrected distances account for substitutions not observed as a result of multiple substitutions at a single nucleotide position.

Because pairwise comparisons of sequences are based entirely on the identity or non-identity of residues at corresponding sequence positions, the first substitution at a site will convert identical residues to non-identical residues. Subsequent changes at the same sequence position cannot further decrease the similarity. In contrast, they might even raise the similarity by converting the compared residues to similar identities (parallelism or reversion). The net effect of this superimposition of substitutions is that dissimilarity does not increase uniformly with the number of events. Instead, it increases rapidly at first and more slowly thereafter. Thus, correction of the distance to account for the unobserved substitutions is necessary for the distances to conform to an additive-tree model, unless all sequences are extremely similar. The corrected distances are then estimates of the true evolutionary distance, which reflects the actual mean number of changes per site that have occurred between a pair of sequences since their divergence from a common ancestor. Many models have been suggested to estimate evolutionary distances between nucleotide sequences (for review see Hillis et al. 1996). In the present study, the distance for Kimura's (1980) two-parameter model, which is calculated from the proportions of transition-type differences and transversion-type differences, has been used.

To construct the phylogenetic tree from pairwise distances, different criteria can be used. In the minimum evolution method, the branch lengths of a tree are estimated by a certain algorithm from pairwise distance data, and the total sum of branch lengths ('S') is computed for each of the possible topologies. The topology that shows the smallest 'S' value will then be chosen as the most likely tree. The neighbor-joining method is a simplified version of the minimum evolution method for inferring a bifurcating tree. In this method, the 'S' value is not computed for all or many of the different topologies, but the examination of different topologies is embedded in the algorithm, so that finally only one tree is produced.

Maximum Parsimony

Parsimony methods attempt to find the tree that requires the least number of changes to explain the observed data. Parsimony methods rely on minimising the number of steps for the transformation of one character to another. This is established by measuring the tree length.

Parsimony criteria as used in tree-building methods attempt to minimise a quantity known as the optimality criterion. The decision as to which optimality criterion is to be used depends upon which underlying model is considered to be most appropriate for the data being analysed. Having selected a particular parsimony criterion as appropriate for the data to be

analysed, it must then be assessed in order to find the optimal trees under this criterion. Methods for finding the maximally parsimonious or minimum length trees fall into two categories. For small data sets of up to about 20 taxa, exact methods can be used that guarantee the discovery of all optimal trees. For larger data sets, heuristic methods must be employed, which need not necessarily find all, or indeed any, of the optimal trees.

Maximum Likelihood

The maximum likelihood approach to phylogeny estimation is simply a method of discovering the tree that gives the highest probability of a data set being derived from it. It is not concerned with the probability of a tree being derived from a data set. Or in other words, maximum likelihood methods evaluate a hypothesis about evolutionary history in terms of the probability that a proposed model of the evolutionary process and the hypothesised history would give rise to the observed data. It is conjectured that a history with a higher probability of giving rise to the current state of affairs is a preferable hypothesis to one with a lower probability of reaching the observed state. The procedure requires one or more trees, a probabilistic model of evolutionary change and a data set. Given a tree and the model, the probability of the data set having resulted from that tree can be calculated. Note that the result is not the probability of the tree being correct. The result can only be as good as the accuracy and assumptions included in the model.

Searching for Optimal Trees

As emphasised above, methods that have explicit optimality criteria (e.g. maximum parsimony or maximum likelihood) separate the problem of evaluating a particular tree under the selected criterion from that of finding the optimal tree. For data sets of small to moderate size (8–20 taxa, depending on the criterion), exact methods that guarantee the discovery of all optimal trees may be used. For larger data sets, exact solutions require a prohibitive amount of computing time. Consequently, approximate methods that do not guarantee optimality must be used.

Conceptually, the simplest approach to the search for optimal trees is to evaluate every possible tree for a given data set. This exact method is known as the 'exhaustive search'.

The 'branch-and-bound search' is an exact method that does not necessarily require all possible trees to be evaluated. Initially, a tree is calculated, the length of which is taken as the

upper bound for trees subsequently generated by the branch-and-bound process. The sequence of tree building and evaluation then proceeds as for exhaustive search, but the length of the tree is calculated as each new taxon is added. As soon as a tree is encountered in which the length exceeds the upper bound, that path is abandoned because the addition of more taxa can only further increase the length. In this way, the number of trees that must actually be evaluated can be significantly reduced. If a tree is found whose length is less than the upper bound, then the length of this tree will replace the original upper bound and the process continues.

When a data set is too large to permit the use of exact methods, optimal trees must be sought via 'heuristic' approaches that sacrifice the guarantee of optimality in favour of reduced computing time. Heuristic tree searches generally operate by hill-climbing methods. An initial tree is used to start the process. This tree is rearranged in a way that improves its score (or decrease its length) under the chosen optimality criterion. When no further improvements can be made, then the process is stopped. However, we can never be sure whether the result thus obtained is the global optimum or merely a local one.

Bootstrap and Jackknife

Most methods for testing the reliability of phylogenetic results concern testing the reliability of the data as a whole or attempt to assign some measure of reliability to each of the internal branches in a tree. Bootstrapping methods are a general set of methods for creating pseudoreplicate data sets in situations where true resampling is impractical or impossible. In phylogenetic analyses, nonparametric bootstrapping is the most commonly used method. Numerical resampling mimics the drawing of new samples from the original sample for each population. Two methods are commonly used: jackknifing and bootstrapping.

For bootstrapping, from the original set of n observations a new sample of the same size is constructed by random sampling with replacement. In other words, each of the original observations is equally likely to be selected to constitute any one of the members of this new sample. The bootstrap sample therefore is likely to have some of the original observations represented many times, and some of them not represented at all.

The jackknife resamples the original data set by randomly dropping k data points (e.g. 50%) at a time and recomputing the estimate from the remaining observations. In contrast to the bootstrap method, each of the original observations can be selected only once.

The parameter is estimated from the new samples, and the process is repeated many times, perhaps 1000 or more. In place of the single estimate from the original samples, bootstrapping or jackknifing provide as many new estimates as desired. The frequency with which a given branch is found in the course of analyses of these pseudoreplicate data sets is recorded as the bootstrap or jackknife proportion. These proportions can be used to assess the reliability of individual branches in the optimal tree.

Outgroup

An outgroup is used for comparative purposes, usually in arguments concerning the relative polarity of a pair of homologous characters. Any group that is not included in the taxa under study (ingroup) can be used as the outgroup. The most suitable outgroup is the sister group of the ingroup. However, a relatively accurate overall phylogeny is necessary in order to be able to identify the appropriate sister group.

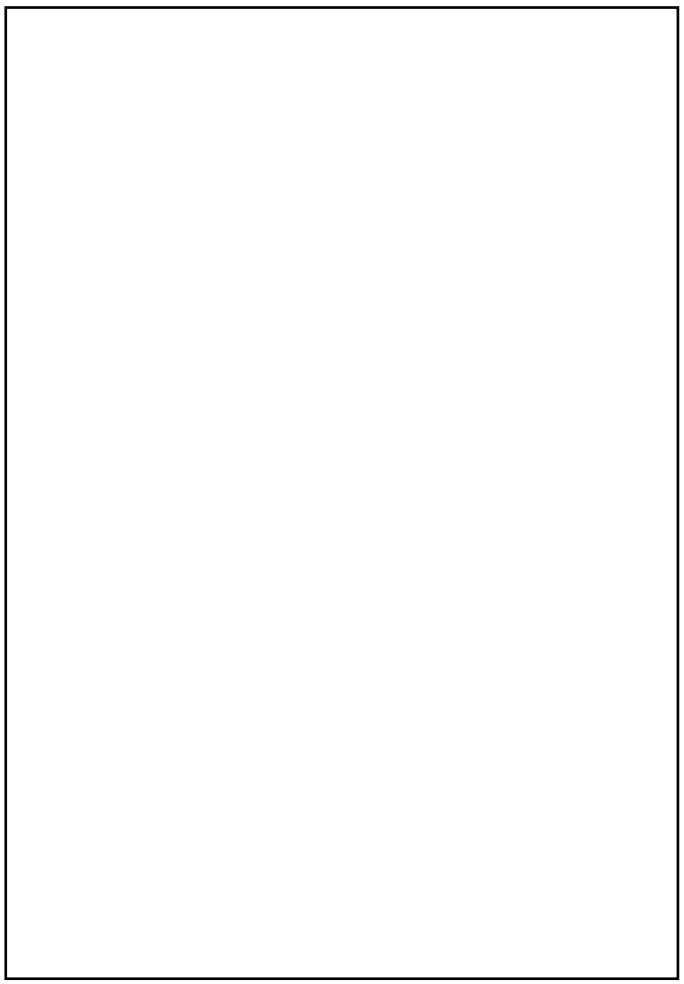

Fig. 1.3 *Eulemur coronatus* female (left) and male (right) in the Ankarana Special Reserve, northern Madagascar (September 1998).

1.4 Lemurs of Madagascar

Madagascar

Madagascar, with a surface area of around 587,000 km^2, is the world's fourth largest island. It lies in the Indian Ocean, separated from the east coast of Africa by a minimum distance of 300 km across the Mozambique Channel. Madagascar has a diverse geology, climate and vegetation, which, in combination with its large size, have led to it being regarded as a microcontinent. Certainly, a substantial part of its flora and fauna is unique to the island.

The origins of Madagascar are still disputed. Geophysical evidence indicates that Madagascar and Africa separated between 165 and 121 million years ago (Rabinowitz et al. 1983). A more recent study suggests that the separation between Madagascar and India did not take place until at about 88 mya, which is more than 30 million years after the separation between Madagascar and Africa (Storey et al. 1995). Recent data from the Indian Ocean support the theory that a link between Antarctica, India and Madagascar existed until about 80 mya (Larsson 1999).

The vegetation of Madagascar is both extremely diverse and unique (for review, see Harcourt & Thornback 1990). The country is divided into two major floral zones, a moister eastern region and a dry western region, and within these a wide range of habitats exists. The central portion of the island consists of an elevated plateau. The highlands fall off sharply to the east, where a strongly eroded escarpment gives way to a narrow coastal strip. To the west, in contrast, the highlands yield more gradually to two major sedimentary basins. A third area of extensive sedimentation lies in the northern portion of the island. In the east, there is coastal evergreen forest with moderately or very high rainfall relatively evenly spread throughout the year. In the north and northwest there is coastal forest with moderately to high annual rainfall and marked seasonality. Madagascar can also be divided into seven major zones of species distribution (Martin 1972, 1995). Each of these zones has distinctive climatic and vegetational characteristics. Major physical barriers can be recognised along all of the boundaries between the present main distribution zones.

Madagascar ranks third highest on the world list of primate species diversity and its 32 currently recognised species and 50 distinct taxa are 100% endemic (Mittermeier et al. 1994). Only *Eulemur fulvus* and *E. mongoz* also live on the nearby Comores, but it is likely that they were introduced there relatively recently from Madagascar. With five primate families and 14

genera, Madagascar's diversity is even more striking at both the generic and family levels. Apart from the lemurs, Madagascar's mammalian fauna is relatively impoverished (Harcourt & Thornback 1990). Similarly, the number of bird species found on Madagascar is low. In contrast to birds and mammals, the reptilian and amphibian fauna is rich compared to that in other African countries.

Madagascar is, without doubt, the world's greatest primate conservation priority, with astounding levels of primate diversity and endemism and more endangered and vulnerable primates than any other country (Harcourt & Thornback 1990). Humans have lived on Madagascar for less than 2000 years and yet, in that comparatively short time, 6 genera and at least 14 species of lemurs have disappeared. Madagascar demonstrates very clearly that primate extinctions are a very real phenomenon. Of the 30 lemur species currently recognised, 10 are considered endangered and another 15 are believed to be in some trouble. Destruction of their habitat is almost certainly the main threat to the lemurs. All lemurs are listed on Appendix 1 of CITES and in Class A of the African Convention, which precludes trade in them or their products except for scientific purposes (Harcourt & Thornback 1990).

Taxonomy of Lemurs

The infraorder Lemuriformes is part of the suborder Strepsirrhini, which also includes the Lorisiformes. Together with the Haplorrhini, the Strepsirrhini belong to the order Primates. Today, most authors recognise five distinct extant lemur families (Lemuridae, Cheirogaleidae, Indridae, Lepilemuridae, Daubentoniidae), all of which are endemic to the island of Madagascar. Classification of the lemurs continues to be a highly controversial topic. Several different taxonomical classifications have been proposed. Tables 1.1 and 1.2 give an overview of the current literature (Hill 1953; Petter et al. 1977; Tattersall 1982; Jenkins 1987; Harcourt & Thornback 1990; Groves 1989; Mittermeier et al. 1994; Rowe 1996).

At present, a tentative consensus accepts four genera (*Eulemur*, *Hapalemur*, *Lemur* and *Varecia*) in the family Lemuridae. Some authors are still not fully convinced whether *Eulemur* or *Varecia* are distinct at the generic level from *Lemur*. The genus *Hapalemur* is classified in the family Lemuridae by most authors, although some consider it to be a member of the family Lepilemuridae. Three partially sympatric species are recognised in the genus *Hapalemur*. *H. griseus* occurs in eastern and western Madagascar and is currently divided into four subspecies (*H.g. griseus*, *H.g. alaotrensis*, *H.g. occidentalis*, *H.g. meridionalis*). *H. simus* and

H. aureus are both extremely rare species, found only in the southeast. The systematic status of *Varecia variegata* from eastern Madagascar remains highly debated. Currently, at least two subspecies are recognised in the single species (*V.v. variegata* and *V.v. rubra*). *Eulemur* is a diverse and widespread genus containing five species. *E. coronatus* is found in the north of Madagascar, and *E. macaco*, which contains two subspecies (*E.m. macaco* and *E.m. flavifrons*), in the northwest. Further south in northwestern Madagascar, *E. mongoz* can be found. The distribution area of *E. rubriventer* covers the eastern coast. *E. fulvus* is found in all forested areas of Madagascar except the south. Subspecies designations within *E. fulvus* remain inadequately defined. At present, at least six subspecies (*E.f. albifrons*, *E.f. albocollaris*, *E.f. collaris*, *E.f. fulvus*, *E.f. rufus*, *E.f. sanfordi*) are recognised. Another member of the Lemuridae is the monotypic *Lemur catta*, which is found in southern Madagascar.

The Cheirogaleidae are currently classified into five genera. *Phaner furcifer* is widely distributed in Madagascar, including at least four different subspecies (*P.f. furcifer*, *P.f. pallescens*, *P.f. parienti*, *P.f. electromontis*). At present, *Allocebus trichotis* occurs in northeastern and eastern Madagascar. The genus *Cheirogaleus* currently contains two species. *C. major* occurs in eastern Madagascar with at least two subspecies (*C.m. major*, *C.m. crossleyi*) and *C. medius* covers the west. The genus *Microcebus* today includes four species. Recently, *M. myoxinus* and *M. ravelobensis* were discovered in central western and northwestern Madagascar, respectively. Both species occur sympatrically with *M. murinus*, which covers the west of Madagascar. Finally, *M. rufus* can be found in the east. For *Cheirogaleus* and *Microcebus* there are studies in progress which will radically expand the number of species. The generic status of the fifth genus, *Mirza coquereli*, is still under discussion.

The family Indridae includes three genera. The monotypic *Indri indri* is found in eastern Madagascar. *Avahi* includes two species, which are sometimes considered as subspecies. *A. laniger* occurs in the east and *A. occidentalis* in the northwest of Madagascar. Three species of *Propithecus* are currently recognised. *P. diadema* is found in eastern Madagascar and *P. verreauxi* inhabits the island's west and south. A maximum of five subspecies has been recognised for *P. diadema* and for *P. verreauxi*, but some of them are subject to doubt. Finally, *P. tattersalli* is an extremely rare species with a small distribution in northern Madagascar.

The family Lepilemuridae includes only one genus. *Lepilemur* is sometimes placed with the extinct genus *Megaladapis* into the family Megaladapidae. *Lepilemur* is a widespread genus

Table 1.1 Summary of taxonomy of the extant lemur families Cheirogaleidae, Indridae and Daubentoniidae.

Family	Genus	Species	Subspecies
Cheirogaleidae	*Microcebus*	*murinus*	-
		rufus	
		myoxinus	-
		ravelobensis	-
	Mirza (*Microcebus*)	*coquereli*	-
	Allocebus	*trichotis*	-
	Cheirogaleus	*major*	- (*major*)
			- (*crossleyi*)
		medius	-
	Phaner	*furcifer*	*furcifer*
			pallescens
			parienti
			electromontis
Indridae	*Avahi*	*laniger*	- (*laniger*)
		occidentalis (*laniger*)	- (*occidentalis*)
	Propithecus	*verreauxi*	*verreauxi*
			verreauxi (*majori*)
			deckeni
			coronatus (*deckeni*)
			coquereli
		diadema	*diadema*
			candidus
			perrieri
			edwardsi
			edwardsi (*holomelas*)
		tattersalli	-
	Indri	*indri*	-
Daubentoniidae	*Daubentonia*	*madagascariensis*	-

with seven different species, which are rarely considered as subspecies. *L. leucopus* occurs in the south, *L. mustelinus* and *L. microdon* in the east, and *L. septentrionalis* in the north of Madagascar. *L. edwardsi* and *L. ruficaudatus* inhabit northwestern and southwestern Madagascar, respectively.

The family Daubentoniidae contains only one extant lemur species. Morphologically, *Daubentonia madagascariensis* is the most peculiar of Madagascar's lemurs. Today, the distribution of *Daubentonia* is wide yet sparse across the forests of the east, north and northwest of Madagascar.

Table 1.2 Summary of taxonomy of extant lemur families Lepilemuridae and Lemuridae.

Family	**Genus**	**Species**	**Subspecies**
Lepilemuridae (Megaladapidae)	*Lepilemur*	*mustelinus*	- (*mustelinus*)
		microdon (*mustelinus*)	- (*microdon*)
		leucopus (*mustelinus*)	- (*leucopus*)
		ruficaudatus (*mustelinus*)	- (*ruficaudatus*)
		edwardsi (*mustelinus*)	- (*edwardsi*)
		dorsalis (*mustelinus*)	- (*dorsalis*)
		septentrionalis (*mustelinus*)	- (*septentrionalis*)
Lemuridae	*Hapalemur*	*griseus*	*griseus*
			occidentalis
			meridionalis
			alaotrensis
		aureus	-
		simus	-
	Lemur	*catta*	-
	Eulemur (*Lemur*)	*fulvus*	*fulvus*
			mayottensis (*fulvus*)
			rufus
			albifrons
			sanfordi
		(*albocollaris*)	*albocollaris*
		(*collaris*)	*collaris*
		macaco	*macaco*
			flavifrons
		coronatus	-
		rubriventer	-
		mongoz	-
	Varecia (*Lemur*)	*variegata*	*variegata*
			rubra

Phylogeny and Evolution of Lemurs

The lemurs of Madagascar provide an excellent model for studies of evolutionary biology. The evolution of the lemurs represents a spectacular example of adaptive radiation among primates (Martin 1972, 1995). Madagascar provided the natural experimental conditions required to produce this outstanding radiation, whose diversity equals that of the anthropoid primates from Asia, Africa or South America.

In addition to the 14 genera or 33 species of extant lemurs, at least 17 species of extinct lemurs have been found all over Madagascar (Godfrey et al. 1999). Locomotor adaptations, differences in body size (Fig. 1.4) or feeding adaptations are highly variable features among lemurs, which highlights the impressive diversity of this group. Equally diverse are the

topography and related climatic and vegetational patterns of Madagascar (e.g. Martin 1972). Climatic and vegetational factors are important in understanding the zoogeographic, evolutionary, and general biology of the Malagasy prosimians.

The phylogeny of the extant lemurs has been a source of controversy for decades and debate continues with respect to the specific relationships among most of these prosimian taxa. Morphological characters, behavioural data (vocalisations), chromosomal banding patterns, fossil evidence and genetical data have been used as taxonomic or phylogenetic criteria in order to better understand the evolution of lemurs. The uncertainty that has characterised the systematics of lemurs stems largely from the lack of a lemur fossil record and the occurrence of a high degree of homoplasy in the evolution of this group. Monophyly of the Lemuridae, Lepilemuridae, Cheirogaleidae or Daubentoniidae has been questioned at one time or another. Dispute centres around the phylogenetic positions of the genera *Lepilemur*, *Daubentonia* and *Hapalemur*. Another problematic issue in primate systematics concerns the phylogenetic position of the family Cheirogaleidae. Some authors have questioned the monophyly of the Malagasy lemurs because they consider the Cheirogaleidae to be a member of the lorisiform clade. Furthermore, the Daubentoniidae have sometimes been placed at the base of all Strepsirrhini. However, currently the monophyly of lemurs is generally accepted.

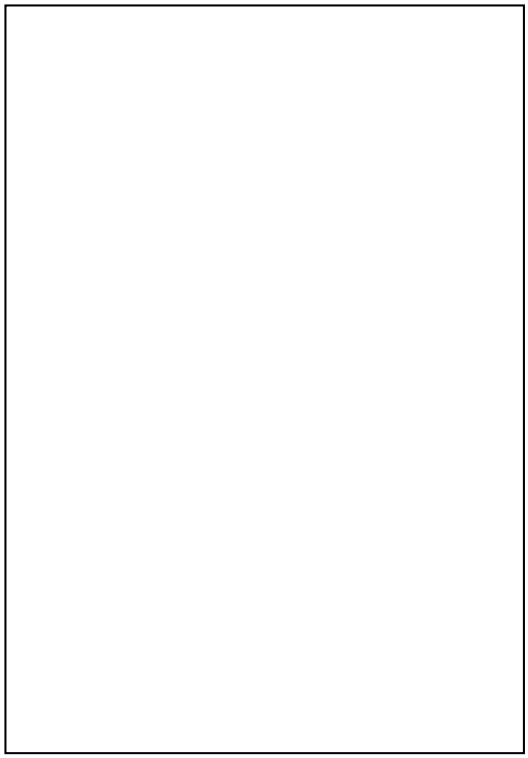

Fig. 1.4 *Lemur catta* sitting beside a life-sized model of the extinct *Megaladapis* at Apenheul Zoo (August 1999).

1.5 Aims

Evolutionary relationships among the lemurs of Madagascar remain controversial, even though many investigations have been carried out. The goal of this study was to generate a mitochondrial DNA sequence data set to reconstruct phylogenetic relationships among lemurs. As many taxa (genera, species and subspecies) as possible have been included in the data set to achieve the best possible taxonomic representation and hence a thorough analysis of lemur evolution. Whenever possible, three individuals per taxon have been sequenced to ensure that variability within each taxon is also examined.

Evolutionary relationships among *Eulemur fulvus* subspecies are still poorly understood and subspecies designations also remain inadequately defined. The present study investigated phylogenetic relationships among *E. fulvus* populations (Chapter 3). Currently, at least six subspecies are recognised. It has recently been suggested that *E.f. albocollaris* and *E.f. collaris* deserve species status. One goal of this study was to test this hypothesis. Some authors recognise *E. fulvus* populations confined to the Comorian island Mayotte as a seventh subspecies, *mayottensis*, while other authors believe that those Comorian populations have been introduced only recently, and therefore not worthy of recognition as distinct taxon. Accordingly, a further aim of this study was to evaluate the taxonomic status of *E.f. mayottensis*. Close attention was also given to genetic variation within *E.f. fulvus* and *E.f. rufus*, as both subspecies are distributed allopatrically along the east and west coasts of Madagascar.

Mitochondrial DNA sequence data should permit clarification of phylogenetic relationships among Lemuridae (Chapter 5). The phylogenetic position of the genus *Hapalemur* among Lemuridae was of special interest, because some authors consider *Hapalemur* as a member of the family Lepilemuridae. Close attention was given to the relative positions of *H. simus*, *L. catta* and *Varecia* in analysing the mtDNA sequence data. The recently suggested close phylogenetic relationship between *Lemur* and *Hapalemur*, as distinct from *Eulemur*, is still doubted by some. A further aim was to assess the validity of generic status for *Eulemur*, *Lemur* and *Varecia*, as some authors are still not convinced that splitting of the genus *Lemur* into three genera is justified. Reconstruction of evolutionary relationships among the five *Eulemur* species has been controversial, and this study aims to provide greater insight into this topic. Few phylogenetic studies have included more than one *Hapalemur* species or subspecies.

Further aims of the present study were to clarify phylogenetic relationships among *Hapalemur* species and subspecies and to assess their taxonomic status (Chapter 4).

Little is known about evolutionary relationships within the family Cheirogaleidae. One goal of the present study was to clarify phylogenetic relationships among cheirogaleid genera and among different species of *Microcebus* (Chapter 6). Close attention was paid to the phylogenetic position of *Allocebus*, from which no DNA sequence data were previously available. Some authors continue to consider *Mirza coquereli* within the genus *Microcebus*. An additional aim was thus to assess the generic status of *Mirza coquereli*. There is little information concerning phylogenetic relationships among the four *Microcebus* species currently recognised. A further goal was to evaluate the taxonomic status of the recently discovered *M. ravelobensis* and to determine its phylogenetic position within the genus *Microcebus*. A final objective with cheirogaleids was to attempt species identification of two captive *Microcebus* of unknown origin.

Evolutionary relationships within the family Indridae remain unclear. Based on the mitochondrial DNA sequenced and the data examined, the phylogenetic relationships among species and subspecies of *Propithecus* have been investigated (Chapter 7). One goal was to confirm the specific status of the recently described *P. tattersalli*. A further aim was to assess the validity of the subspecific status for the four *P. verreauxi* forms from western Madagascar.

The family Lepilemuridae includes only one genus. The genetic data presented here were used to clarify the specific status of the different *Lepilemur* taxa (Chapter 8).

Of special interest were the phylogenetic relationships among the five lemur families. The newly generated DNA sequence data were used to verify the family status of each of the families, especially the Lepilemuridae (Chapter 8). Since 12 of the 14 currently recognised genera have been included into this study, it was possible to verify the affiliation of each genus to its family (e.g. *Hapalemur* to Lemuridae). The phylogenetic position of *Daubentonia* among the Strepsirrhini and the taxonomic status of this very special Malagasy primate was investigated (Chapter 8). Another issue of controversy in primate systematics concerns the monophyly of the Malagasy lemurs. In the past, the Cheirogaleidae or the Daubentoniidae have been placed apart from the other Malagasy lemurs. The monophyly of lemurs was tested by the addition of more distantly related outgroup taxa to the mtDNA sequence data set (Chapter 8).

2. Methods

2.1 Laboratory Methods

DNA was extracted from hair, blood or other tissue samples with PCI (25:24:1 mix of phenol, chloroform, and isoamyl alcohol) and chloroform (Sambrook et al. 1989). Successful extractions were judged visually by staining the DNA with ethidium bromide after electrophoresis in 1% agarose minigels (Fig. 2.1A).

The segment of the mtDNA amplified and sequenced in this study includes a fragment of the COIII gene, complete sequences for the NADH-dehydrogenase subunits 3, 4L and 4 (ND3, ND4L, ND4), as well as the $tRNA^{Gly}$, $tRNA^{Arg}$, $tRNA^{His}$, $tRNA^{Ser}$, and partial $tRNA^{Leu}$ genes (Fig. 1.2). The template DNA was amplified in 100 µl reactions using 0.06 M Tris, 0.015 M $(NH_4)_2SO_4$, 1.5 mM $MgCl_2$, 0.78 M DMSO, 0.025 mM each dNTP, 1 mM each primer, and 2.5 U *Thermophilus aquaticus* (*Taq*) polymerase in a Perkin Elmer Cetus 480 DNA thermal cycler. The amplification primers are listed in Table 2.1 and shown in Figure 2.2. Successful amplifications were obtained using the following protocol: 35 cycles of denaturing at 95°C for 30 seconds, primer annealing at 50°C for 60 seconds, and extension for 60 seconds at 72°C. The PCR products were checked by electrophoresis in 1% agarose minigels against a size standard marker, and visualised using ethidium bromide and UV light (Fig. 2.1B). Negative (no DNA) controls were included.

The PCR products were prepared for sequencing by QIAquick PCR Purification Kit (QIAGEN 1997) using standard protocols. The cleaned products were electrophoresed alongside pGEM®-3Zf(+) sequencing standard (Applied Biosystems 1995; 1998) in 2% agarose to estimate final template concentration (Fig. 2.1C).

The sequencing reactions were carried out with the Dye (#402079) or BigDye (#4303152) Terminator Cycle Sequencing Ready Reaction Kits (Applied Biosystems 1995; 1998), using 3 µl of terminator mix for a 9 µl reaction. The internal sequencing primers used are shown in Table 2.1. Cycling parameters were 25 cycles of 96°C 30 seconds, 50°C 1 minute, and 60°C 4 minutes.

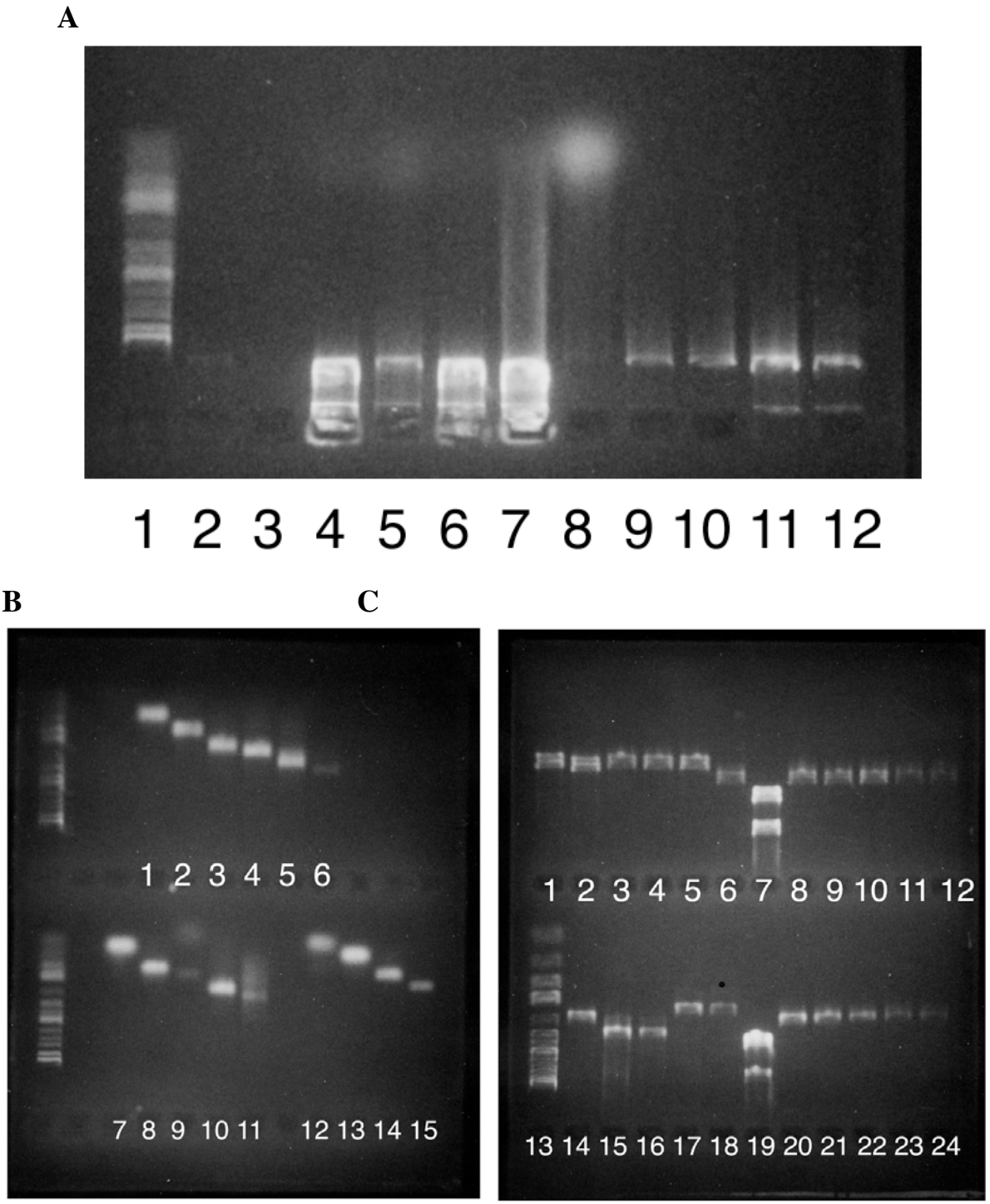

Fig. 2.1 **A** DNA samples loaded on an agarose minigel. Lane 1: marker; Lanes 2 & 3: extraction from hair (as expected only very little DNA); Lanes 4 - 7: extractions from blood; Lane 8: extraction from tissue; Lanes 9 – 11: extractions from blood (using QIAGEN DNA extraction kits instead of PCI as in lanes 1 – 8). **B** PCR products amplified with different primer combinations loaded on an agarose minigel. Lane 1 = LemurND3-LemurR1; 2 = LemurND3-LemurR2; 3 = LemurND3-Nap2M; 4 = LemurND3-LemurR3; 5 = LemurND3-LemurR5; 6 = LemurND3-MLeu; 7 = LemurF1-LemurR2; 8 = LemurF1-Nap2M; 9 = LemurF1-LemurR3; 10 = LemurF1-LemurR5; 11 = LemurF1-MLeu; 12 = LemurHS-Nap2M; 13 = LemurHS-LemurR3; 14 = LemurHS-LemurR5; 15 = LemurHS-MLeu. **C** Clean PCR products amplified with different primer combinations loaded on an agarose minigel alongside pGEM. Lanes 1 = LemurHS-MLeu; 2 = LemurND3-Nap2M; 3-5 = LemurHS-MLeu; 6 = SP1-Nap2M; 7 = pGEM; 8-12 = SP1-Nap2M; 13 = marker; 14 = LemurGly-Nap2M; 15-16 = 282-283; 17-18 = LemurHS-MLeu; 19 = pGEM; 20-24 = LemurGly-Nap2M.

Table 2.1 Forward (F) and reverse (R) primers used for PCR to amplify the fragment (A) and for sequencing reactions (S).

Primer	Sequence 5'–3'	Map position [a]	F/R	A	S
SP1	gaagctgcagtctgatactgacattt	9912–9937	F	x	x
LepiP1	ttgatgtagtatgactRttcc	9940–9960	F	x	x
DaubP1	tagacgtagtatgattattcc	9940–9960	F	x	x
LemurGly	ttgacttccaatcaattaacttcgg	10015–10039	F	x	x
LemurND3	ccctttttccataaaatttttYctagtagc	10206–10234	F	x	x
HasiND3	cctttctctatgaagttctttctagtagc	10206–10234	F		x
ProND3	ccattctctataaaattcttcctggtagc	10206–10234	F	x	x
MicND3	ctatttgatctagaRatcgcac	10248–10269	F	x	x
MicLF	ctaggcatactaatatttcg	10518–10537	F	x	x
LemurF1	ctcctagtcttcgcRgcctg	10656–10675	F	x	x
EucoF1	ctcttagttttcgcggcttg	10656–10675	F		x
LepiF1	ctgYtagtacttgcagcctg	10656–10675	F	x	x
AllF1	ctacttgttttttcggcctg	10656–10675	F		x
AvF1	cttctagtattcgcagcgtg	10656–10675	F		x
ProF1	ttactagtgttcgcagcatg	10656–10675	F		x
MmF1	gaagctgctattggtctggc	10677–10696	F		x
MicF1	gaagcYgccatcggcttagc	10677–10696	F	x	x
MmF12	aaataatagcaataacttctcac	10897–10919	F	x	x
LemurHS	ggtaaccaaacagaacgattaaacgc	11168–11193	F	x	x
MzHS	ggcaatcaaacagagcgactaaatgc	11168–11193	F		x
LemurF3	atctgcctacgacaaacagacctaaaatc	11582–11610	F	x	x
LemurF4	gtaactataacatccttYtcatgatc	11900–11925	F	x	x
GalF4	attattatcacaWcattctcatgatc	11900–11925	F		x
MicF4	tacttattactgccctttaYtc	11958–11979	F	x	x
TRLeu	atatttacctcaacacaacgagg	11987–12009	F		x
GalND3R	taagtgtgtgtgtttgcagRgctcatgg	10284–10311	R		x
LemurND3R	tataagttttaggttagttgtttgggatgc	10287–10316	R	x	x
EumaND3R	tataagatttggtttagttgtttgggctgc	10287–10316	R		x
MicND3R	tgtgattttgagattgtttgattgagatgc	10287–10316	R	x	x
LepiND3R	tataagctttagattatttgtYtgggttgc	10287–10316	R	x	x
LedND3R	ttagattatttgtttgggtcgc	10287–10308	R		x
AllND3R	taggtctattgattgggaagc	10287–10307	R	x	x
LemurArgR	gttagtcataatctaatgagtc	10437–10456	R	x	
MicLR	tatRccttctaaRcatagtag	10557–10577	R	x	x
LemurR1	gctaggcctacagctgcttcgcaggctgcRaa	10665–10696	R		x
EuR1	gctaggcctacagcagcttcacaggccgcgaa	10665–10696	R	x	x
LepiR1	gccaaaccgatggctgcttcacaggctgcaag	10665–10696	R	x	x
MicR1	actataactaatagggctaagccgatggcRgc	10680–10711	R	x	x
AllR1	atttgatactataactaggagg	10697–10718	R		x
MmR1	catagatatttgatactgtaac	10704–10725	R		x
LemurR2	gtgatgttggctWgctataat	10988–11008	R	x	x
EumamaR2	gtggtattggcttgccaagat	10988–11008	R		x
EfcoR2	atgatattggctagccatgat	10988–11008	R	x	x
GalR2	ttggttaggtgRtgttggcttgc	10994–11116	R		x
LemurHSR	cctgcgtttagtcgttctgtYtg	11174–11196	R		x
MicHSR	taaattagWgccactaataatgg	11234–11256	R	x	
MicNap	ggcttctacatgtgcYttgg	11409–11428	R	x	x
Nap2M	ttagcttcaacgtgggcttt	11411–11430	R	x	x
LemurR3	agtgattttagRtctgtttgtcg	11591–11613	R	x	x
AvR3	agtgactttaaatctgtctgtcg	11591–11613	R		x
MicR3	Ygctatatggctgactgatga	11624–11644	R	x	x
LemurR5	atggtatgtgagttttcctcgttgtg	12000–12025	R	x	x
LepiR5	gtggtacggggacttgcctcgttggg	12000–12025	R	x	x
MLeu	tacttttatttggagttgcacca	12314–12336	R	x	x

[a] relative to human mtDNA (Anderson et al. 1981)

The completed sequencing reactions were cleaned of excess dyes by centrifugation at 3000 rpm through 0.05 g Sephadex G-50 hydrated in 800 μl of distilled water within CENTRI-SEP Columns (Princeton Separations, Inc.). The final elutants were dried by vacuum centrifugation. The dried samples were stored at –80°C prior to being analysed on an automated DNA sequencer.

The samples were rehydrated in a 1:5 ABI loading buffer:deionised formamide solution and denatured just prior to gel electrophoresis. The reactions were electrophoresed and the sequences were analysed on PE Biosystems Model 373A or 377 DNA Sequencing Systems (ABI PRISM 373A or 377). All templates were sequenced in their entirety for both strands.

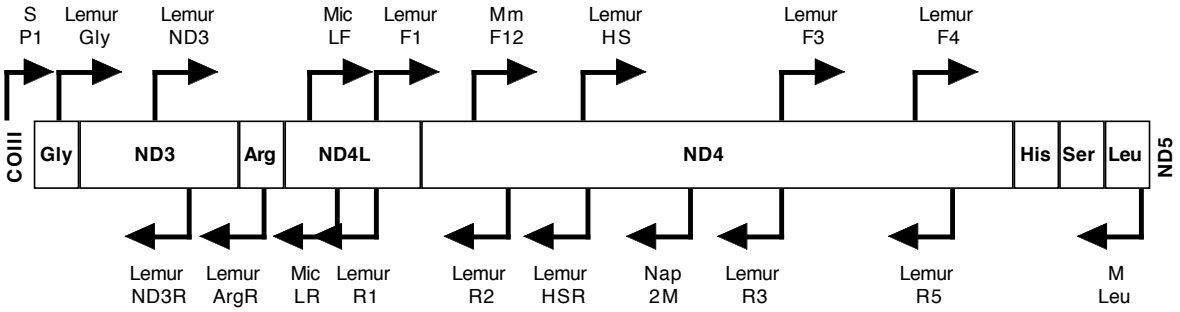

Fig. 2.2 The fragment amplified and sequenced in this study. Arrows indicate the different positions for primers. More than one primer is often used at a position, but only one primer name is indicated on this figure (see Table 2.1 for complete primer list).

2.2 Quantitative Methods

The sequences obtained were entered into the computer programs Phylogenetic Analysis Using Parsimony (PAUP) 3.1.1 (Swofford 1990) or PAUP* 4.0b (Swofford 1999). Sequences were aligned by eye and using CLUSTAL W (Thompson et al. 1994) against the homologous region of human mtDNA sequence (Anderson et al. 1981). Printed electropherograms (Fig. 2.3) were checked to verify accuracy of base-calling by the ABI software. Special attention was paid to structural conformation in the alignments of each of the tRNA genes.

The aligned sequences were analysed using maximum parsimony, neighbor-joining and maximum likelihood methods.

Gaps were considered as a fifth character state in parsimony analyses. The number of taxa examined sometimes precludes the use of the exhaustive or branch-and-bound search option in PAUP* 4.0b. As a result, branch-and-bound or heuristic searches were utilised in parsimony analyses. For partial data sets, parsimony analyses that down-weighted mutations resulting in a transition were performed to examine any topological effects of saturation.

For neighbor-joining analyses, distance measures were employed using corrections for nucleotide sequence data suggested by Kimura (1980). In neighbor-joining analyses gaps were treated as missing data.

For parsimony and neighbor-joining methods, bootstrap (BP) and jackknife (JK) analyses (Felsenstein 1985) of 100 to 2500 replicates were performed to examine the relative support of each relationship in the resultant topologies.

Maximum likelihood trees were calculated via heuristic search by PAUP* 4.0b. For the substitution model, transition/transversion ratios were estimated and a discrete approximation to gamma distribution was estimated for among-site rate variation. For all other options, default settings were maintained, thus yielding the equivalent of the HKY model (Hasegawa et al. 1985).

Tree length, consistency index, retention index, and numbers of transitions/ transversions were obtained from MacClade (Maddison & Maddison 1992) using the topology of the most parsimonious tree.

The compatibility of the nine genes to be included in the analyses was examined using the partition-homogeneity test (Farris et al. 1995) in PAUP* 4.0b (100 replicates, heuristic search).

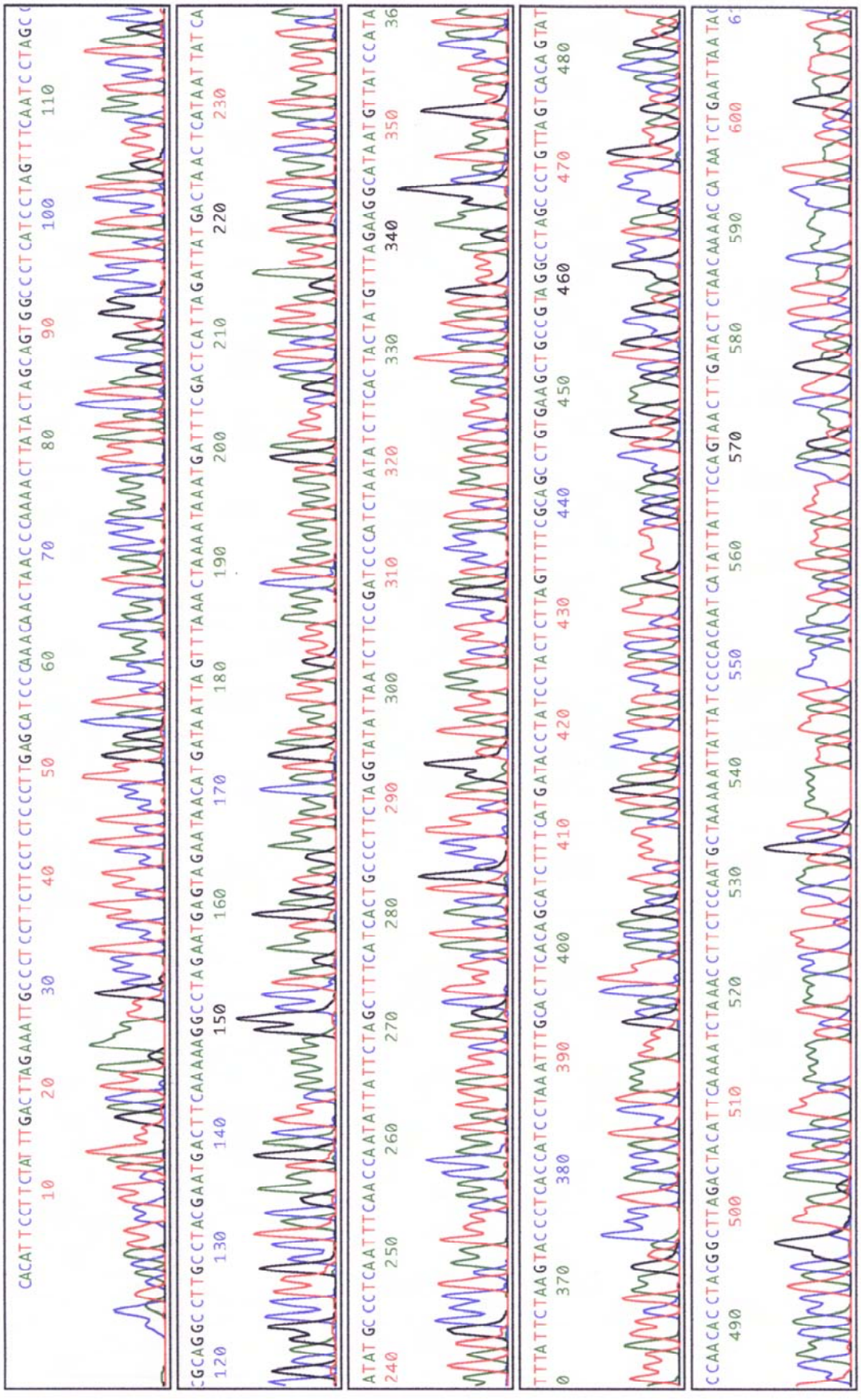

Fig. 2.3 Printed electropherogram (#C275) from *Eulemur fulvus sanfordi* #2 (#JP125). The cycle sequencing reaction was carried out with the primer 'LemurND3' from the PCR product 'SP1-Nap2M'.

3. Subspecies of *Eulemur fulvus*

This chapter has been published in a modified form:
J. Pastorini, M.R.J. Forstner, R.D. Martin (2000)
Relationships among brown lemurs (*Eulemur fulvus*) based on mitochondrial DNA sequences.
Molecular Phylogenetics and Evolution **16**: 418-429.

3.1 Introduction

Eulemur is a diverse, widespread genus containing five recognised species in the endemic Malagasy primate family Lemuridae. The brown lemur *E. fulvus* consists of a series of apparently closely related populations; some are easy to distinguish by pelage coloration, but others are extremely difficult to define. Only sparse field information is available on distribution, geographical variation, or possible secondary contact and hybridisation (Tattersall 1982). *E. fulvus* has the largest distribution among diurnal/cathemeral lemurs, and is found in all forested areas of Madagascar except the south (Harcourt & Thornback 1990) (Fig. 3.1).

Subspecies designations within *E. fulvus* remain inadequately defined. Currently, at least six subspecies (*E.f. albifrons*, *E.f. albocollaris*, *E.f. collaris*, *E.f. fulvus*, *E.f. rufus*, *E.f. sanfordi*) are recognised (Groves 1989; Tattersall 1993). All *Eulemur* taxa show sexual dichromatism, varying in brown lemurs from weak (*E.f. fulvus*) to strong (*E.f. albocollaris*). Differences in pelage coloration between females of different subspecies tend to be slight and it is therefore easier to distinguish subspecies among males than among females (see Mittermeier et al. 1994 for illustrations). *E.f. albifrons* and *E.f. albocollaris* are unique among brown lemur subspecies in that each has an autapomorphic dentition character, and it is possible to identify *E.f. albifrons* and *E.f. sanfordi* from cranial characters (Tattersall 1991).

The evolutionary relationships among *E. fulvus* subspecies are still poorly understood. Analysis of craniodental characters reveals a very high degree of homoplasy among the Lemuridae. As a result, relationships among subspecies of *E. fulvus* remain unresolved (Tattersall 1991; Groves & Trueman 1995). A study on metachromism indicated that *E.f. collaris* may be the most basal *E. fulvus* subspecies (Shedd & Macedonia 1991). Chromosome banding analyses have shown that only the karyotypes of *E.f. collaris* (2N=50, 51, 52) and *E.f. albocollaris* (2N=48) differ from that of all other *E. fulvus* (2N=60) (Rumpler & Dutrillaux 1976; Hamilton & Buettner-Janusch 1977). Consequently, karyotypic analyses do not provide

data that can be used to clarify taxonomic relationships among *E.f. albifrons*, *E.f. fulvus*, *E.f. mayottensis, E.f. rufus*, and *E.f. sanfordi* (Rumpler 1975; Hamilton et al. 1980). Analysis of DNA sequences from a fragment of the D-loop, cytochrome *b*, small ribosomal subunit 12S rRNA and casein kinase II resolved only three brown lemur clades (Wyner et al. 1999). In that study, *E.f. albocollaris* and *E.f. collaris* formed a sister group to the remaining unresolved *E. fulvus*, hence yielding the same tree topology as karyotypic analyses.

It has recently been suggested that *E.f. albocollaris* and *E.f. collaris* deserve species status. When *E.f. albocollaris* and *E.f. collaris* are cross-bred, the resulting offspring are sterile, a finding which favours their division into two distinct species (Djelati et al. 1997). However, despite different chromosome numbers, both can produce fertile offspring with other subspecies of *E. fulvus* in captivity (Rumpler 1975; Hamilton & Buettner-Janusch 1977) and apparently in the wild (*E.f. albocollaris* x *E.f. rufus*, Steig Johnson, personal communication).

Some authors recognise *E. fulvus* populations confined to the Comorian island Mayotte as a seventh subspecies, *mayottensis* (Schwartz 1931; Petter et al. 1977; Tattersall 1982; Harcourt & Thornback 1990). In appearance, the Mayotte brown lemur is very like *E.f. fulvus*, from which it is probably derived (Tattersall 1977). Consequently, some authors believe that the populations on the Comores are nothing more than recently introduced *E.f. fulvus*, and therefore not worthy of recognition as a distinct taxon (Groves 1989; Mittermeier et al. 1994).

In the present study, a large fragment of mitochondrial DNA was sequenced and the data examined in an attempt to resolve the subspecific status of *E.f. albocollaris*, *E.f. collaris* and *E.f. mayottensis*. Close attention was given to genetic variation within *E.f. fulvus* and *E.f. rufus*, as both subspecies are distributed allopatrically along the east and west coasts of Madagascar. Previous successful resolution of problematic taxa using this region of mtDNA (Forstner et al. 1995; Wang et al. 1997; Forstner et al. 1998; Pastorini et al. 1998) indicated that this fragment could resolve phylogenetic relationships among brown lemurs.

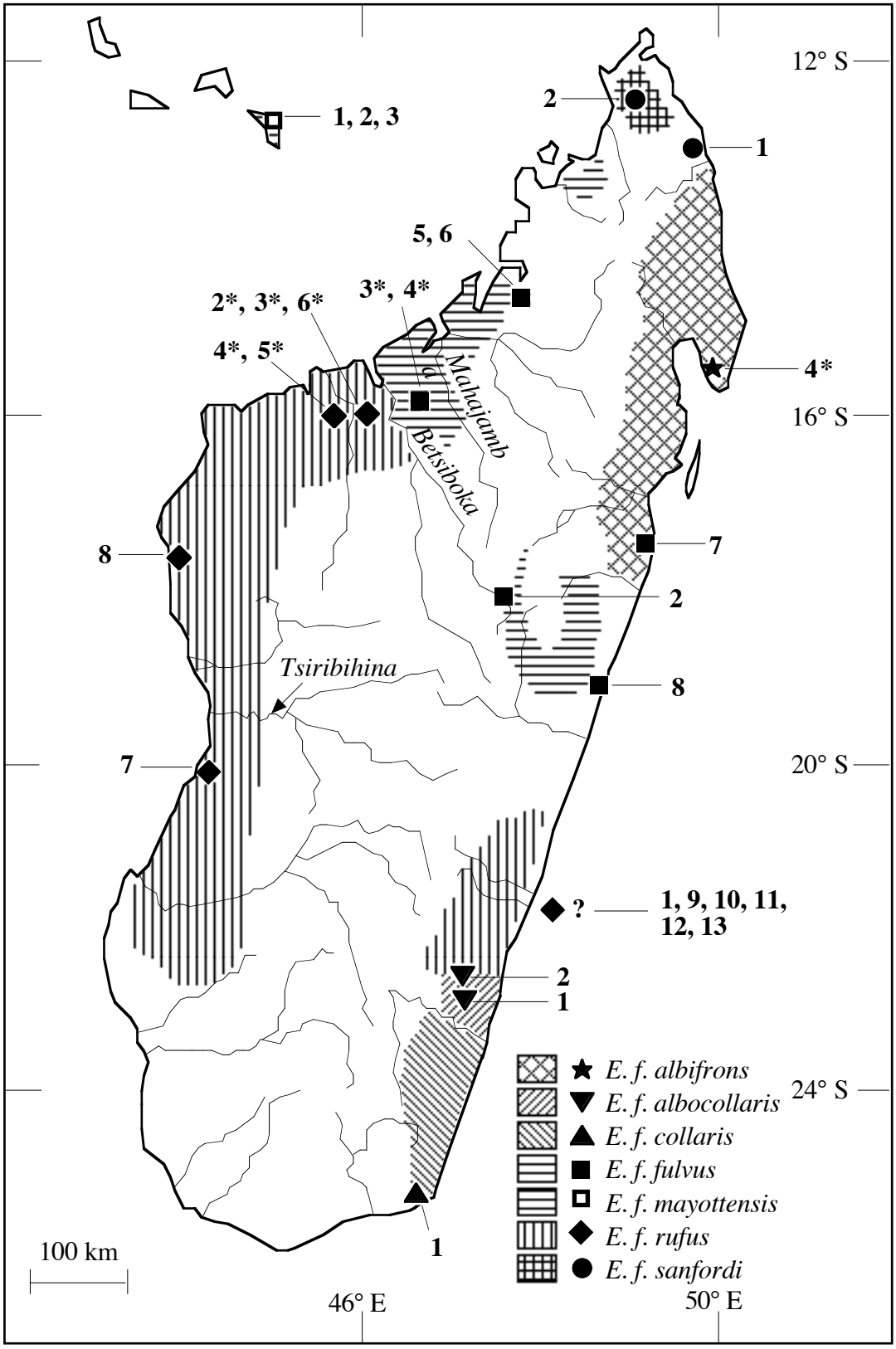

Fig. 3.1 Map of Madagascar showing approximate areas of distribution of the subspecies of *E. fulvus* according to Tattersall (1982). * Indicates individual with exact locality data.

3.2 Material

Samples were collected from all 7 subspecies of *Eulemur fulvus*: 4 *E.f. albifrons*, 2 *E.f. albocollaris*, 2 *E.f. collaris*, 8 *E.f. fulvus*, 3 *E.f. mayottensis*, 13 *E.f. rufus*, and 2 *E.f. sanfordi*. Single samples from *E. macaco macaco* and *E.m. flavifrons* were sequenced for subsequent use as outgroup taxa (Table 3.1).

All *E. fulvus* samples of known origin are depicted on the map shown in Figure 3.1. For each of the four subspecies *E.f. albifrons*, *E.f. fulvus, E.f. mayottensis* and *E.f. rufus*, at least one sample with exact locality data was successfully sequenced. For *E.f. albocollaris*, *E.f. collaris* and *E.f. sanfordi*, all samples are from captivity with only imprecise indications of their origin.

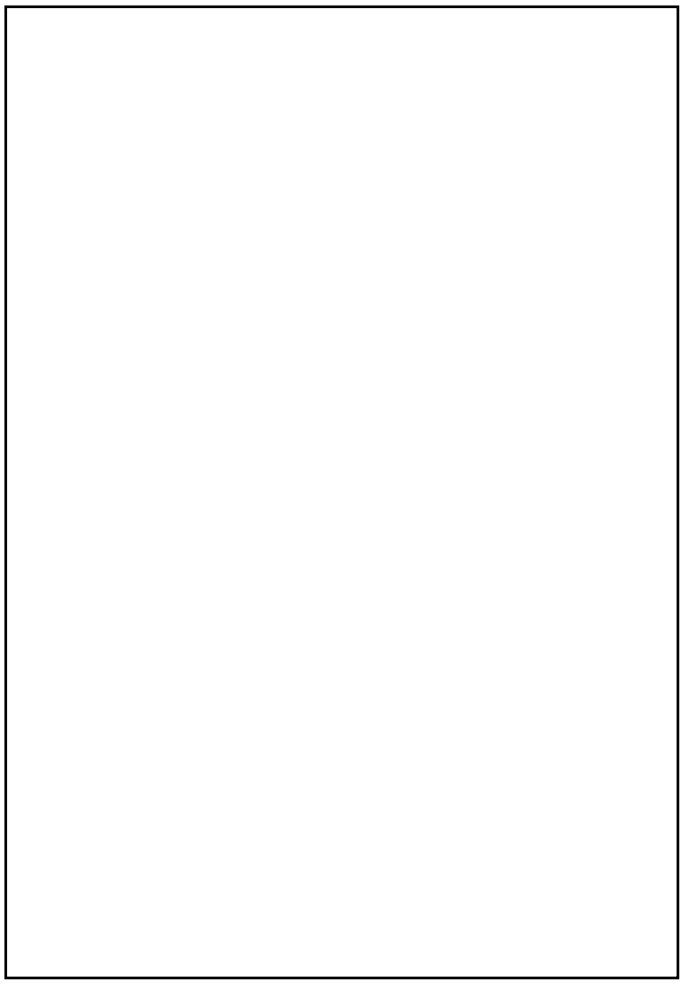

Fig. 3.2 *Eulemur fulvus collaris* pet in Fort Dauphin, southern Madagascar (August 1998).

Table 3.1 Taxa, sex, origin, identification numbers, and GenBank accession numbers for all 36 *Eulemur* individuals sequenced.

Taxon	Sex	Origin	ID #	GenBank #
E. fulvus fulvus 1	female	unknown [a]	JP2	AF224564
E. fulvus fulvus 2	female	pet in Anjozorobe [b]	JP41	AF224534
E. fulvus fulvus 3	female	Ampijoroa (Northwest) [c]	JP215	AF224535
E. fulvus fulvus 4	female	Ampijoroa (Northwest) [c]	JP218	AF224536
E. fulvus fulvus 5	male	pet in Antsohihy (Northwest)	JP330	AF224537
E. fulvus fulvus 6	male	pet in Antsohihy (Northwest)	JP331	AF224538
E. fulvus fulvus 7	male	pet in Foulpointe (East) [b]	JP336	AF224539
E. fulvus fulvus 8	female	pet in Vatomandry (East) [b]	JP337	AF224540
E. fulvus mayottensis 1	male	unknown [d]	JP72	AF224541
E. fulvus mayottensis 2	male	Comoro Islands [e]	JP225	AF224542
E. fulvus mayottensis 3	female	Comoro Islands [e]	JP226	AF224543
E. fulvus rufus 1	female	East [b]	JP123	AF224544
E. fulvus rufus 2	female	Anjamena, east of Mahavavy River (Northwest) [c]	JP161	AF224545
E. fulvus rufus 3	female	Anjamena, east of Mahavavy River (Northwest) [c]	JP171	AF224547
E. fulvus rufus 4	male	Anadabomandry, west of Mahavavy River (Northwest) [c]	JP176	AF224548
E. fulvus rufus 5	male	Anadabomandry, west of Mahavavy River (Northwest) [c]	JP181	AF224549
E. fulvus rufus 6	female	Anjamena, east of Mahavavy River (Northwest) [c]	JP206	AF224550
E. fulvus rufus 7	male	Morondava (West)	JP332	AF224551
E. fulvus rufus 8	male	Maintirano (West)	JP333	AF224552
E. fulvus rufus 9	female	Southeast [b]	JP338	AF224553
E. fulvus rufus 10	female	Southeast [b]	JP339	AF224554
E. fulvus rufus 11	female	Southeast [b]	JP340	AF224555
E. fulvus rufus 12	male	Southeast [b]	JP341	AF224556
E. fulvus rufus 13	male	Southeast [b]	JP342	AF224557
E. fulvus albocollaris 1	female	Vondrozo (Southeast) [e]	JP222	AF224558
E. fulvus albocollaris 2	female	pet, region Mahazoarivo (Southeast) [b]	JP145	AF224562
E. fulvus collaris 1	male	pet in Fort Dauphin (South)	JP304	AF224559
E. fulvus collaris 2	female	unknown [f]	JP307, R390/98	AF224560
E. fulvus sanfordi 1	male	pet, region Vohemar [b]	JP126	AF224561
E. fulvus sanfordi 2	female	pet, region Anivorana-Diego Suarez (North) [b]	JP125	AF224563
E. fulvus albifrons 1	male	unknown [g]	JP25	AF224565
E. fulvus albifrons 2	male	unknown [b]	JP135	AF224566
E. fulvus albifrons 3	female	unknown [b]	JP134	AF224567
E. fulvus albifrons 4	male	Andranobe Forest (Northeast) [c]	JP323	AF224568
E. macaco macaco	female	Ambato (North) [e]	JP83	AF224530
E. macaco flavifrons	male	Maromandia (North) [e]	JP74	AF224531

[a] held at Berlin Zoo, Germany

[b] held at Parc Zoologique et Botanique de Tsimbazaza, Madagascar

[c] wild-caught animals with verified origin, immediately released after capture and sampling

[d] held at Leipzig Zoo, Germany

[e] held at Université Louis Pasteur, Strasbourg, France

[f] specimen held at National Museums of Scotland, U.K. (previously held at Banham Zoo)

[g] held at Parc Zoologique et Botanique de Mulhouse, France

3.3 Results

The new mtDNA sequences generated for the taxa examined have been deposited in GenBank (Table 3.1). The nucleotide sequences span a total of 2389 base positions (bp). The analysed data set consists of the 3' end of the COIII gene (53 bp), the complete NADH-dehydrogenase subunits ND3 (348 bp), ND4L (297 bp) and ND4 (1378 bp), along with the glycine (70 bp), arginine (68 bp), histidine (69 bp), serine (63 bp), and part of leucine (47 bp) tRNA genes. The partition-homogeneity test showed no significant incongruence among these nine genes (P=0.98).

The sequences obtained yielded 266 parsimony-informative characters with a transition:transversion ratio of 10:1. A summary of the frequencies of invariant, parsimony uninformative, and informative characters along the segment sequenced is given in Table 3.2.

In the lemur taxa presented in this study, the ND3 gene is terminated by 'TAA', while in *Homo* only 'T' serves as the stop codon via polyadenylation. Lemurs have an insertion of 2 bp between the ND3 gene and the $tRNA^{Arg}$. The published human mtDNA genome does not contain any untranslated base positions between those genes. Between $tRNA^{Arg}$ and ND4L, lemurs have an additional base position which is not present in *Homo*. In the $tRNA^{Ser}$, insertions or deletions of 3 bp occur, which are all limited to ingroup/outgroup comparisons.

The maximum parsimony heuristic search with all characters weighted equally results in four trees of 2265 steps in length with a consistency index of 0.78 and a retention index of 0.91 (Fig. 3.3). The most parsimonious trees group the 34 *E. fulvus* into six clades. *E.f. albifrons*, *E.f. sanfordi* and 3 *E.f. fulvus* together form a clade which is the sister group to a clade containing the remaining 5 *E.f. fulvus* and all *E.f. mayottensis*. The 13 *E.f. rufus* form two different clades. The earliest offshoot is formed by *E.f. albocollaris* with *E.f. collaris*. These arrangements are strongly supported by bootstrap and jackknife analyses using maximum

Table 3.2 Summary of variation for the sequences across the 36 *Eulemur* individuals examined.

Genes	All	COIII	ND3	ND4L [a]	ND4 [a]	tRNAs	Not translated
characters (nucleotides)	2389	53	348	297	1378	317	3
constant	2038	51	307	250	1148	286	2
parsimony-uninformative	85	0	9	10	58	7	1
parsimony-informative	266	2	32	37	172	24	0
informative proportion	0.11	0.04	0.09	0.12	0.12	0.08	0
insertions/deletions	3	0	0	0	0	3	0

[a] ND4L and ND4 overlap for 7 bp

parsimony heuristic searches (Fig. 3.3).

The distance matrices constructed using the Kimura 2-parameter corrections (Table 3.3) and subsequently analysed by the neighbor-joining methods reconstruct the same topology for the arrangement of the six clades (Fig. 3.4). The support values from bootstrap and jackknife analyses of 2500 replicates are in the same range as for maximum parsimony analyses (Fig. 3.3).

The results of the maximum likelihood analysis are presented in Fig. 3.5. The phylogram

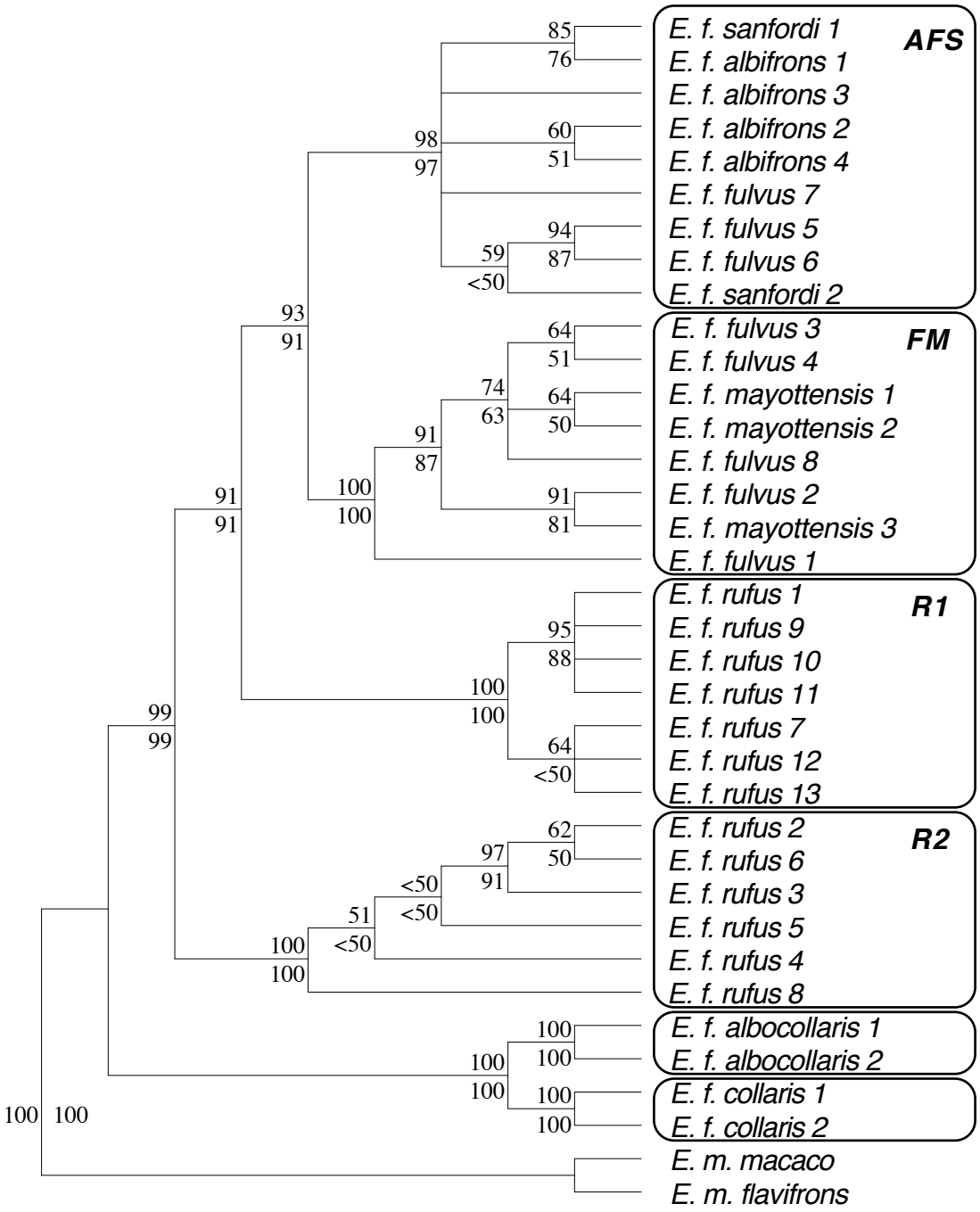

Fig. 3.3 Maximum parsimony tree with bootstrap values (as percentages, above nodes) obtained in 2500 replicates and with jackknife values (below nodes) from 2500 iterations with 50% deletion.

presented maintains branch lengths proportional to the number of changes. The phylogenetic relationships among clades are identical to those from the analyses presented in Figures 3.3 and 3.4. The final maximum likelihood tree (–ln likelihood = 5646.85) was obtained with previously estimated transition/transversion ratio of 13.61 (kappa = 29.01) and gamma shape parameter of 0.05.

The relationships among the clades remain consistent in all analyses. Generally, there is

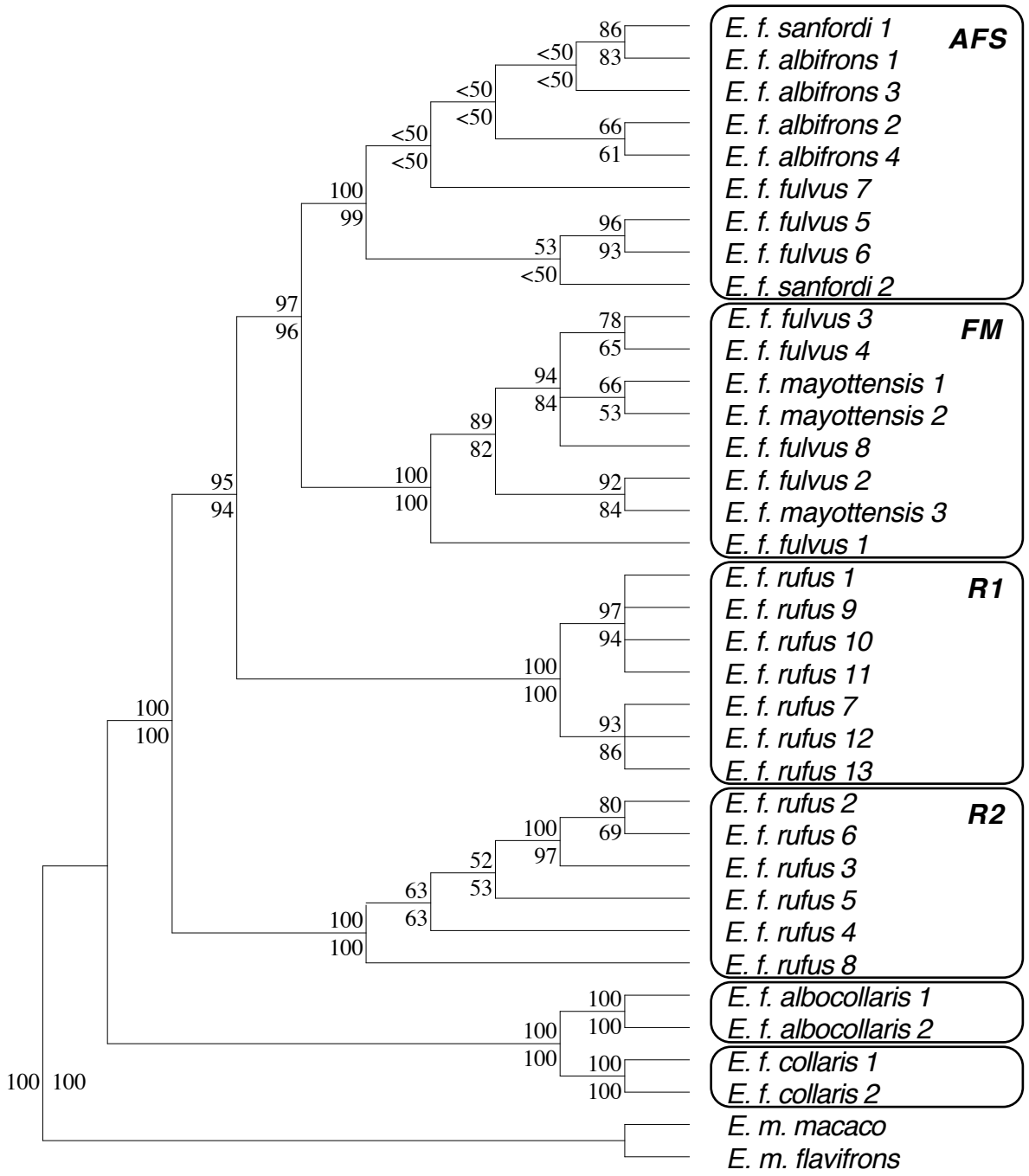

Fig. 3.4 Neighbour-joining tree with bootstrap values (as percentages, above nodes) obtained in 2500 replicates and with jackknife values (below nodes) from 2500 iterations with 50% deletion using Kimura 2-parameter distance correction.

very high bootstrap and jackknife support in maximum parsimony or neighbor-joining analyses for the branching order of the six clades (Figs. 3.3 and 3.4).

The clade containing 4 *E.f. fulvus* and all 3 *E.f. mayottensis* individuals (=*FM*) has strongest BP or JK support (100%), as do all of the four clades containing the 2 *E.f. albocollaris*, the 2 *E.f. collaris*, the first 7 *E.f. rufus* (=*R1*), or the remaining 6 *E.f. rufus* (=*R2*). *AFS* is the only clade which is not consistently supported with 100% BP or JK values

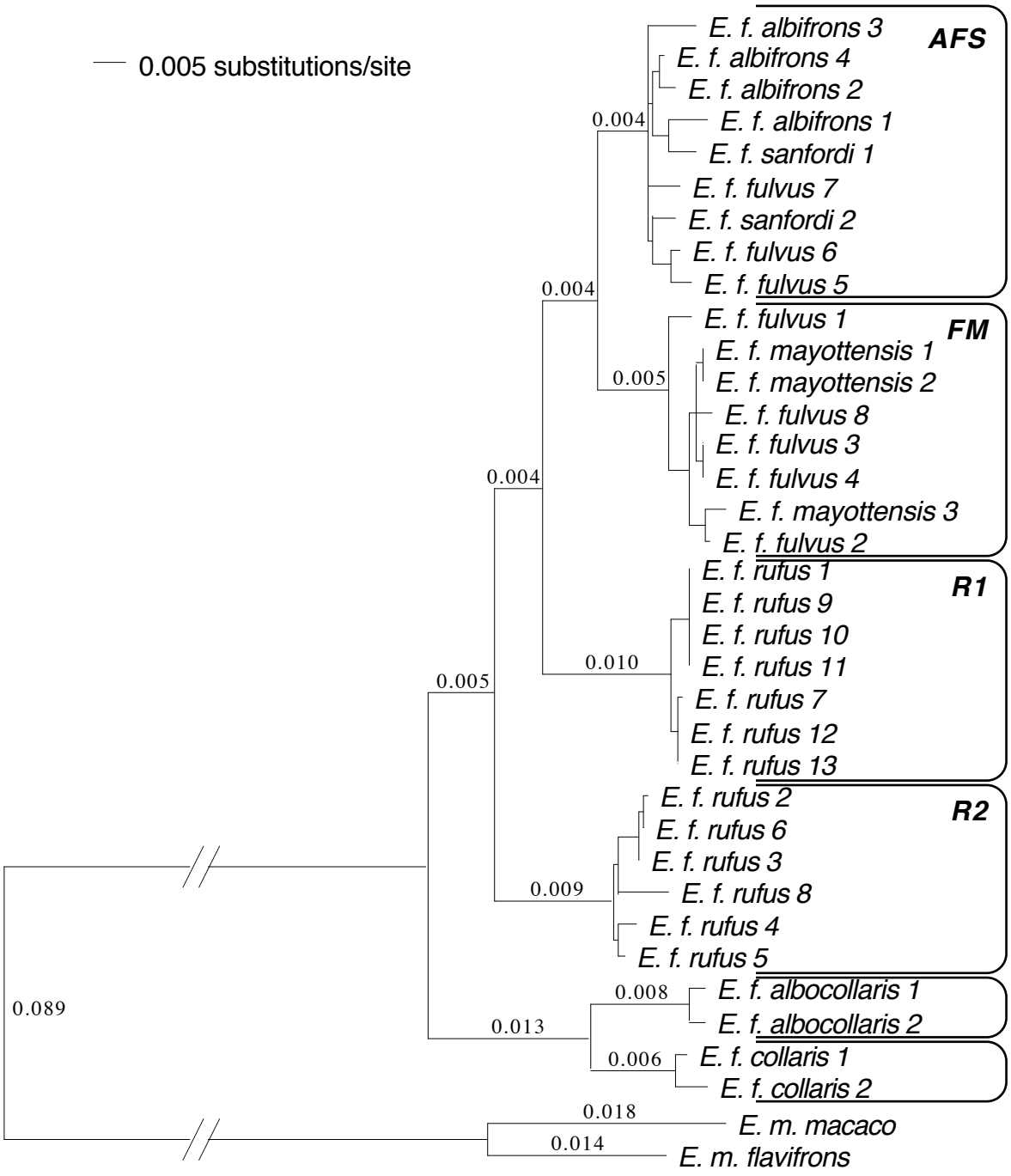

Fig. 3.5 Maximum likelihood phylogram with proportional branch lengths (values provided on each branch). // Indicates artificially shortened branch to fit page format.

(87–100%). All analyses fail to clearly resolve the branching order within the *E.f. albifrons/fulvus/sanfordi* clade (=*AFS*). *E.f. albocollaris* and *E.f. collaris* form a subclade with 100% BP or JK support. The maximum parsimony or neighbor-joining analyses unambiguously link *AFS* and *FM* with 91–97% BP or JK support. The sister-group relationship of *R1* with *AFS/FM* is supported with BP or JK values of 91–95%. *R2* groups next with *AFS/FM/R1* with BP or JK values of 99–100%, making *E.f. albocollaris* with *E.f. collaris* the most basal clade of the species *E. fulvus*.

Absolute pairwise distances (Table 3.3) range from a maximum of 196 bp between *E. macaco* and the ingroup to between 0 and 90 bp within the species *E. fulvus*. Examination of absolute pairwise distances within the species *E. fulvus* reveals three levels of differentiation (Fig. 3.6, Table 3.3): The divergences between *E.f. albocollaris* or *E.f. collaris* and other clades of *E. fulvus* are higher (73–90 bp) than between *E.f. albocollaris* and *E.f. collaris*, or between *AFS*, *FM*, *R1* and *R2* (29–61 bp). Pairwise comparisons of the individuals within each clade give values in the range of 0 to 17 bp.

Branch lengths confirm the results of pairwise distance comparisons or BP/JK supports in maximum parsimony or neighbor-joining analyses. The six clades within *E. fulvus* are separated from each other by relatively long branches (Fig. 3.5).

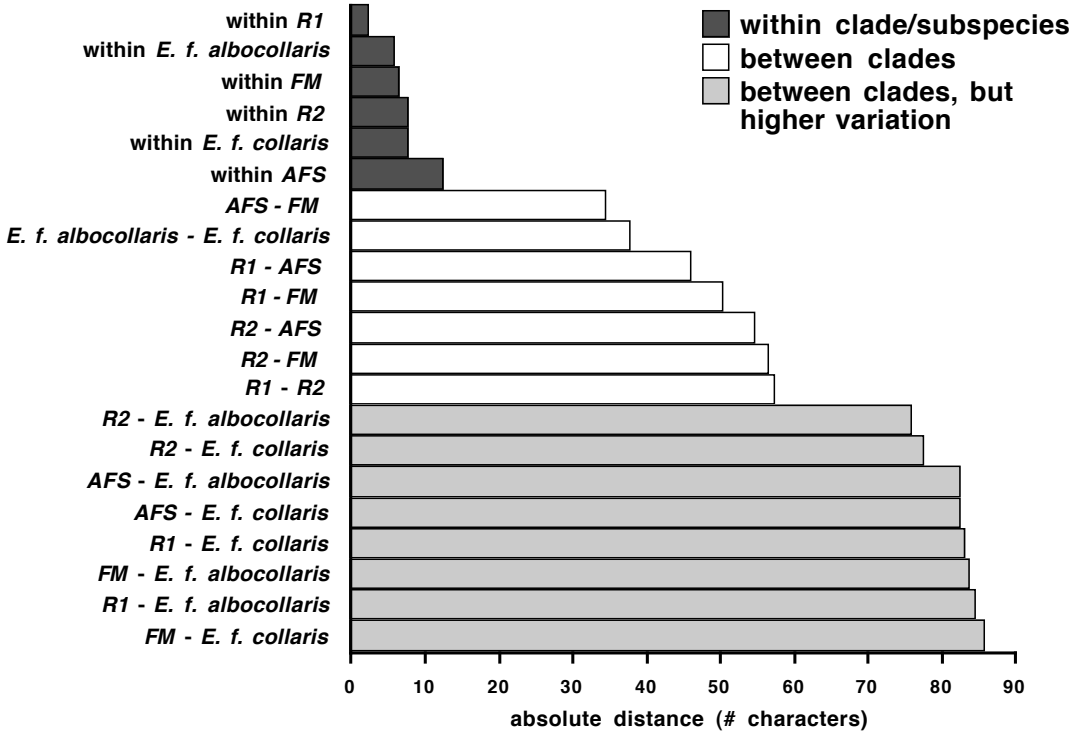

Fig. 3.6 Absolute pairwise distances over three defined taxonomic levels. Each bar represents the average of all possible comparisons between individuals of the two taxa. Single values can be seen in Table 3.3. Abbreviations for clades are indicated in Figure 3.3.

Table 3.3 Kimura 2-parameter distance (above the diagonal) and absolute distance (under the diagonal) matrices derived from the 2389 bp mitochondrial DNA sequence data set, with gaps treated as missing data.

	E.m.m.	*E.m.f.*	*E.f.fu 2*	*E.f.fu 3*	*E.f.fu 4*	*E.f.fu 5*	*E.f.fu 6*	*E.f.fu 7*	*E.f.fu 8*	*E.f.ma 1*	*E.f.ma 2*	*E.f.ma 3*	*E.f.ru 1*	*E.f.ru 2*	*E.f.ru 3*	*E.f.ru 4*	*E.f.ru 5*	*E.f.ru 6*
E. m. macaco	-	0.029	0.086	0.088	0.088	0.083	0.083	0.085	0.088	0.087	0.087	0.088	0.087	0.087	0.086	0.087	0.085	0.086
E. m. flavifrons	68	-	0.087	0.089	0.089	0.085	0.086	0.087	0.090	0.088	0.088	0.088	0.086	0.085	0.084	0.086	0.085	0.085
E. f. fulvus 2	190	191	-	0.003	0.003	0.014	0.014	0.014	0.003	0.003	0.003	0.002	0.022	0.024	0.024	0.024	0.024	0.024
E. f. fulvus 3	194	195	6	-	0	0.015	0.014	0.014	0.002	0.001	0.001	0.004	0.022	0.024	0.024	0.024	0.024	0.024
E. f. fulvus 4	194	195	6	0	-	0.015	0.014	0.014	0.002	0.001	0.001	0.004	0.022	0.024	0.024	0.024	0.024	0.024
E. f. fulvus 5	184	189	34	36	36	-	0.003	0.006	0.016	0.015	0.015	0.017	0.021	0.025	0.024	0.023	0.023	0.025
E. f. fulvus 6	184	189	32	34	34	6	-	0.005	0.015	0.014	0.014	0.016	0.020	0.024	0.024	0.022	0.022	0.024
E. f. fulvus 7	188	191	34	34	34	14	12	-	0.015	0.014	0.014	0.016	0.020	0.025	0.024	0.023	0.023	0.025
E. f. fulvus 8	194	197	8	4	4	38	36	36	-	0.002	0.002	0.005	0.023	0.024	0.024	0.024	0.024	0.024
E. f. mayottensis 1	192	193	6	2	2	36	34	34	4	-	0	0.004	0.022	0.024	0.024	0.024	0.024	0.024
E. f. mayottensis 2	192	193	6	2	2	36	34	34	4	0	-	0.004	0.022	0.024	0.024	0.024	0.024	0.024
E. f. mayottensis 3	193	194	5	9	9	39	37	37	11	9	9	-	0.023	0.026	0.025	0.026	0.025	0.025
E. f. rufus 1	192	189	51	51	51	49	47	47	53	51	51	54	-	0.026	0.025	0.024	0.024	0.026
E. f. rufus 2	191	188	57	57	57	59	57	59	57	57	57	60	61	-	0.001	0.004	0.003	0.000
E. f. rufus 3	189	186	55	55	55	57	55	57	55	55	55	58	59	2	-	0.003	0.002	0.000
E. f. rufus 4	191	190	57	57	57	53	51	53	57	57	57	60	55	10	8	-	0.002	0.004
E. f. rufus 5	188	187	56	56	56	54	52	54	56	56	56	59	56	7	5	4	-	0.003
E. f. rufus 6	190	187	56	56	56	58	56	58	56	56	56	59	60	1	1	9	6	-
E. f. rufus 7	193	188	50	50	50	48	46	46	52	50	50	53	5	60	58	54	55	59
E. f. rufus 8	196	195	59	59	59	59	57	59	59	59	59	60	61	15	13	13	12	14
E. f. rufus 9	192	189	51	51	51	49	47	47	53	51	51	54	0	61	59	55	56	60
E. f. rufus 10	192	189	51	51	51	49	47	47	53	51	51	54	0	61	59	55	56	60
E. f. rufus 11	192	189	51	51	51	49	47	47	53	51	51	54	0	61	59	55	56	60
E. f. rufus 12	192	187	49	49	49	47	45	45	51	49	49	52	4	59	57	53	54	58
E. f. rufus 13	192	187	49	49	49	47	45	45	51	49	49	52	4	59	57	53	54	58
E. f. albocollaris 1	184	182	82	86	86	84	82	86	86	84	84	85	86	75	73	77	74	74
E. f. collaris 1	187	187	83	85	85	83	81	81	85	83	83	84	81	76	74	74	73	75
E. f. collaris 2	187	187	87	89	89	89	87	87	89	87	87	88	87	80	78	80	77	79
E. f. sanfordi 1	189	194	35	35	35	17	15	15	37	35	35	38	46	56	54	52	51	55
E. f. albocollaris 2	190	184	80	84	84	84	82	84	84	82	82	83	85	77	75	79	76	76
E. f. sanfordi 2	184	189	33	33	33	11	9	11	35	33	33	36	43	53	51	47	48	52
E. f. fulvus 1	193	194	12	10	10	34	32	32	12	10	10	15	49	55	53	55	54	54
E. f. albifrons 1	191	196	38	38	38	17	17	17	40	38	38	41	52	60	58	56	55	59
E. f. albifrons 2	186	192	31	31	31	13	11	11	33	31	31	34	46	56	54	50	51	55
E. f. albifrons 3	188	196	37	37	37	17	15	15	39	37	37	40	48	60	58	54	55	59
E. f. albifrons 4	185	190	31	31	31	11	9	9	33	31	31	34	44	54	52	48	49	53

	E.f.ru 7	*E.f.ru 8*	*E.f.ru 9*	*E.f.ru 10*	*E.f.ru 11*	*E.f.ru 12*	*E.f.ru 13*	*E.f.ac 1*	*E.f.co 1*	*E.f.co 2*	*E.f.sa 1*	*E.f.ac 2*	*E.f.sa 2*	*E.f.fu 1*	*E.f.af 1*	*E.f.af 2*	*E.f.af 3*	*E.f.af 4*
E. m. macaco	0.087	0.089	0.087	0.087	0.087	0.087	0.087	0.083	0.085	0.084	0.086	0.086	0.083	0.088	0.086	0.084	0.085	0.084
E. m. flavifrons	0.085	0.089	0.086	0.086	0.086	0.085	0.085	0.082	0.085	0.084	0.088	0.083	0.086	0.088	0.089	0.087	0.089	0.086
E. f. fulvus 2	0.021	0.025	0.022	0.022	0.022	0.021	0.021	0.035	0.036	0.038	0.015	0.035	0.014	0.005	0.016	0.013	0.016	0.013
E. f. fulvus 3	0.021	0.025	0.022	0.022	0.022	0.021	0.021	0.037	0.037	0.039	0.015	0.036	0.014	0.004	0.016	0.013	0.016	0.013
E. f. fulvus 4	0.021	0.025	0.022	0.022	0.022	0.021	0.021	0.037	0.037	0.039	0.015	0.036	0.014	0.004	0.016	0.013	0.016	0.013
E. f. fulvus 5	0.020	0.025	0.021	0.021	0.021	0.020	0.020	0.036	0.036	0.039	0.007	0.036	0.005	0.014	0.007	0.005	0.007	0.005
E. f. fulvus 6	0.020	0.024	0.020	0.020	0.020	0.019	0.019	0.035	0.035	0.038	0.006	0.035	0.004	0.014	0.007	0.005	0.006	0.004
E. f. fulvus 7	0.020	0.025	0.020	0.020	0.020	0.019	0.019	0.037	0.035	0.038	0.006	0.036	0.005	0.014	0.007	0.005	0.006	0.004
E. f. fulvus 8	0.022	0.025	0.023	0.023	0.023	0.022	0.022	0.037	0.037	0.039	0.016	0.036	0.015	0.005	0.017	0.014	0.017	0.014
E. f. mayottensis 1	0.021	0.025	0.022	0.022	0.022	0.021	0.021	0.036	0.036	0.038	0.015	0.036	0.014	0.004	0.016	0.013	0.016	0.013
E. f. mayottensis 2	0.021	0.025	0.022	0.022	0.022	0.021	0.021	0.036	0.036	0.038	0.015	0.036	0.014	0.004	0.016	0.013	0.016	0.013
E. f. mayottensis 3	0.023	0.026	0.023	0.023	0.023	0.022	0.022	0.037	0.036	0.038	0.016	0.036	0.015	0.006	0.017	0.014	0.017	0.014
E. f. rufus 1	0.002	0.026	0	0	0	0.002	0.002	0.037	0.035	0.038	0.020	0.037	0.018	0.021	0.022	0.020	0.020	0.019
E. f. rufus 2	0.026	0.006	0.026	0.026	0.026	0.025	0.025	0.032	0.033	0.035	0.024	0.033	0.023	0.024	0.026	0.024	0.026	0.023
E. f. rufus 3	0.025	0.005	0.025	0.025	0.025	0.024	0.024	0.031	0.032	0.034	0.023	0.032	0.022	0.023	0.025	0.023	0.025	0.022
E. f. rufus 4	0.023	0.005	0.024	0.024	0.024	0.023	0.023	0.033	0.032	0.035	0.022	0.034	0.020	0.024	0.024	0.021	0.023	0.020
E. f. rufus 5	0.024	0.005	0.024	0.024	0.024	0.023	0.023	0.032	0.031	0.033	0.022	0.033	0.020	0.023	0.024	0.022	0.024	0.021
E. f. rufus 6	0.025	0.006	0.026	0.026	0.026	0.025	0.025	0.032	0.032	0.034	0.024	0.033	0.022	0.023	0.025	0.024	0.025	0.023
E. f. rufus 7	-	0.026	0.002	0.002	0.002	0.000	0.000	0.037	0.035	0.037	0.020	0.036	0.018	0.020	0.022	0.019	0.020	0.018
E. f. rufus 8	60	-	0.026	0.026	0.026	0.025	0.025	0.034	0.035	0.037	0.025	0.035	0.022	0.024	0.027	0.024	0.026	0.023
E. f. rufus 9	5	61	-	0	0	0.002	0.002	0.037	0.035	0.038	0.020	0.037	0.018	0.021	0.022	0.020	0.020	0.019
E. f. rufus 10	5	61	0	-	0	0.002	0.002	0.037	0.035	0.038	0.020	0.037	0.018	0.021	0.022	0.020	0.020	0.019
E. f. rufus 11	5	61	0	0	-	0.002	0.002	0.037	0.035	0.038	0.020	0.037	0.018	0.021	0.022	0.020	0.020	0.019
E. f. rufus 12	1	59	4	4	4	-	0	0.036	0.034	0.037	0.020	0.036	0.017	0.020	0.021	0.019	0.020	0.018
E. f. rufus 13	1	59	4	4	4	0	-	0.036	0.034	0.037	0.020	0.036	0.017	0.020	0.021	0.019	0.020	0.018
E. f. albocollaris 1	85	78	86	86	86	84	84	-	0.016	0.017	0.036	0.003	0.034	0.037	0.039	0.035	0.038	0.035
E. f. collaris 1	80	81	81	81	81	79	79	37	-	0.003	0.035	0.016	0.032	0.037	0.036	0.033	0.035	0.033
E. f. collaris 2	86	85	87	87	87	85	85	39	8	-	0.037	0.017	0.035	0.038	0.039	0.035	0.038	0.035
E. f. sanfordi 1	47	58	46	46	46	46	46	83	80	86	-	0.035	0.006	0.014	0.005	0.005	0.007	0.004
E. f. albocollaris 2	84	80	85	85	85	83	83	6	37	39	81	-	0.034	0.036	0.038	0.034	0.037	0.034
E. f. sanfordi 2	42	52	43	43	43	41	41	78	75	81	14	78	-	0.013	0.007	0.004	0.006	0.003
E. f. fulvus 1	48	57	49	49	49	47	47	86	85	87	33	84	31	-	0.015	0.012	0.015	0.012
E. f. albifrons 1	51	62	52	52	52	50	50	89	84	90	12	87	16	36	-	0.006	0.008	0.005
E. f. albifrons 2	45	56	46	46	46	44	44	81	76	82	12	79	10	29	14	-	0.005	0.002
E. f. albifrons 3	47	60	48	48	48	46	46	87	82	88	16	85	14	35	18	12	-	0.005
E. f. albifrons 4	43	54	44	44	44	42	42	81	76	82	10	79	8	29	12	4	12	-

3.4 Discussion

Subspecies Status

Analyses of the sequence data resolve six clades which are strongly supported by both bootstrap and jackknife values (Figs. 3.3 and 3.4). Pairwise distances and branch lengths clearly support these six clades (Figs. 3.5 and 3.6). The only surprising fact is simply the lack of correspondence between the six clades and the designation of the seven subspecies currently accepted.

One clade contains 4 *E.f. fulvus* and all 3 *E.f. mayottensis* individuals (=*FM*). The 3 *E.f. mayottensis* individuals do not form a monophyletic subclade (Figs. 3.3 - 3.5). Such paraphyly indicates that *E.f. mayottensis* does not deserve subspecies status and may actually represent multiple introductions of *E.f. fulvus* to the island. Furthermore, genetic distances between the Malagasy *E.f. fulvus* and Comorian *E.f. mayottensis* populations are not equivalent to those between subspecies (Table 3.3). As the karyotype of *E.f. mayottensis* cannot be distinguished from the karyotypes of *E.f. albifrons*, *E.f. fulvus*, *E.f. rufus* or *E.f. sanfordi* (Hamilton et al. 1980), and as Comorian and Malagasy brown lemurs are extremely similar in appearance (Tattersall 1977), the conclusion is supported that Comorian brown lemur populations on the Comores are recently derived from *E.f. fulvus* in north-west Madagascar. It has been suggested that the brown lemur population on the Comores might be a mixture of *E.f. fulvus* and *E.f. rufus* (Petter et al. 1977). However, in the small sample analysed here there was no evidence of a genetic contribution from *E.f. rufus*.

E.f. fulvus individuals #3 and #4 in the clade *FM* are wild-caught animals with verified origin at Ampijoroa in north-western Madagascar (Fig. 3.1). Based on the records from 'Parc Zoologique et Botanique de Tsimbazaza', *E.f. fulvus* #2 and #8 originate from Anjozorobe and Vatomandry on the eastern coast (Fig. 3.1). If these localities are correct, it would indicate that the central plateau does not serve as an effective barrier between eastern and western populations of *E.f. fulvus*.

Neighbor-joining and maximum parsimony methods fail to resolve the branching order within a clade (=*AFS*) containing 4 *E.f. albifrons*, 3 *E.f. fulvus* and 2 *E.f. sanfordi* individuals (Figs. 3.3 and 3.4). None of the three taxa involved in this clade forms a monophyletic subclade. Furthermore, pairwise genetic distances between individuals within this clade never reach the level typical of between-clade or subspecies comparisons (Fig. 3.6). Therefore, this sequencing

data set does not permit distinction between *E.f. albifrons* and *E.f. sanfordi*. However, most animals in this clade are from captivity, the only wild-caught animal with verified origin being *E.f. albifrons* #4. There is therefore a possibility that localities of captive animals were incorrectly assigned. A mistake in identifying males is not very likely, because it is quite easy to distinguish among males from *E.f. albifrons*, *E.f. fulvus* and *E.f. sanfordi*. Only 1 *E.f. albifrons* and 1 *E.f. sanfordi* of the 9 brown lemurs in this clade *AFS* are females, for which subspecies designations are quite difficult. Even if those two females were removed, the three taxa would remain paraphyletic. Of special interest are the three *E.f. fulvus* males in this clade *AFS*. Based on zoo records, individual #7 originated from Foulpointe on the eastern coast, which already lies in the distribution range of *E.f. albifrons* (Fig. 3.1). Individuals #5 and #6 are pets from Antsohihy near the north-western coast (Fig. 3.1). Even if the localities attributed to these three *E.f. fulvus* are incorrect, this does not explain why the three males with the unmistakable pelage of *E.f. fulvus* cluster in a clade with *E.f. albifrons* and *E.f. sanfordi*. It would appear that the genetic data indicate different (potentially historical) barriers to gene flow which do not reflect current morphologically diagnosed units. These data also suggest that the river Mahajamba is acting as a potential isolating barrier between northern and southern populations of brown lemurs in north-western Madagascar.

Of particular interest are the two genetically distinct clades of *E.f. rufus* (=*R1* and *R2*). Genetic distances between individuals of those two clades clearly lie in the range of distinctions between subspecies (Fig. 3.6). Clade *R1* contains six individuals from 'Parc Zoologique et Botanique de Tsimbazaza', whose origin is reported to be in the south-east or east of Madagascar, along with a seventh individual #7 from Morondava on the western coast (Fig. 3.1). The second clade *R2* includes five wild-caught animals with verified origin from north-west Madagascar. The sixth individual #8 in clade *R2* originates from Maintirano, which is further south than the other five individuals of this clade, but further north than individual #7 of clade *R1* (Fig. 3.1). The genetic analyses indicate that there are two distinct forms of *E.f. rufus*. If the locality data of the captive individuals are correct, it is not the central plateau that separates between populations in the east and west but the river Tsiribihina that acts as a barrier to gene flow between *E.f. rufus* from northern and southern parts of the range in west Madagascar. On the eastern coast, only one form of *E.f. rufus* is detectable in the sample.

The two individuals of *E.f. albocollaris* form a clade with strong bootstrap support, as do the two individuals of *E.f. collaris*. Those two subspecies also show a close physical resemblance (Tattersall 1993). It has been suggested that *E.f albocollaris* and *E.f. collaris* not only differ in chromosome number from the other brown lemur subspecies but are also reproductively isolated from each other because crosses between *E.f. albocollaris* and *E.f. collaris* result in sterile offspring (Djelati et al. 1997). This parallels the results of artificial crosses between two well-separated species such as *E. macaco* and *E. coronatus*, or *E. macaco* and *E. fulvus*, which regularly give rise to sterile hybrids (Djelati et al. 1997). However, captive matings of *E.f. collaris* or *E.f. albocollaris* with the various 2N=60 subspecies of *E. fulvus* consistently yield fertile offspring (Rumpler 1975; Hamilton & Buettner-Janusch 1977). Indeed, the different subspecies of *E. fulvus* with 2N=60 regularly produce fertile hybrids (Rumpler 1975). In the wild, hybrids between *E.f. albocollaris* and *E.f. rufus* have been observed (Steig Johnson, personal communication). Absolute pairwise distances in this study throw new light on the picture provided by karyotype studies. Pairwise distances between *E.f. albocollaris* and *E.f. collaris* are among the lowest of all comparisons between subspecies. In contrast, the divergences between *E.f. albocollaris* or *E.f. collaris* and other clades of *E. fulvus* are higher than between *AFS*, *FM*, *R1* and *R2* (Fig. 3.6). However, none of those pairwise distances comes close to the values observed between *E. macaco* and *E. fulvus*. Tattersall (1993) infers a recent divergence that has not as yet reached the level of separate species and the current data would support this conclusion. However, the information of Djelati et al. (1997) cannot be discounted. The consensus phylogeny indicates that a relevant and potentially very recent karyotypic event has occurred. However, as *E.f. albocollaris* and *E.f. collaris* fail to meet the widely specified criterion, namely reproductive isolation from all other actually or potentially interbreeding *E. fulvus*, recognition of two species is questionable. Until reproductive data which detail the nature and extent of hybridisation between either of these taxa and the remaining subspecies becomes available the recognition of either *E.f. albocollaris* or *E.f. collaris* as full species is premature.

Phylogenetic Relationships

Tattersall (1991) analysed 37 craniodental characters in Lemuridae, including specimens from all *E. fulvus* subspecies. Despite the existence of substantial amount of craniodental variation among the taxa, it was virtually impossible to obtain a single most parsimonious

cladogram because of the high level of homoplasy. Previously published DNA sequence data from the D-loop, cytochrome *b*, 12S rRNA and casein kinase (Wyner et al. 1999) resolved the same 3 clades as early karyotypic studies (Rumpler & Dutrillaux 1976): The two clades *E.f. albocollaris* and *E.f. collaris* form a sister group to the clade constituted by all other brown lemurs. However, karyotypic and previous DNA sequence analyses did not provide data that can be used to clarify the taxonomic relationships among *E.f. albifrons*, *E.f. fulvus*, *E.f. mayottensis*, *E.f. rufus*, and *E.f. sanfordi* (Rumpler 1975; Hamilton et al. 1980; Wyner et al. 1999). In contrast to a study on metachromism, which came to the conclusion that *E.f. collaris* may be the most primitive brown lemur subspecies (Shedd & Macedonia 1991), a basal position of *E.f. albocollaris* and *E.f. collaris* among brown lemurs has been confirmed by the current study. The current analyses provide the first clear resolution of six clades from the 34 *E. fulvus* sequenced (Figs. 3.3 - 3.5). *E.f. albifrons*, *E.f. sanfordi* and 3 *E.f. fulvus* together form a clade (=*AFS*) which is sister group to a clade containing the remaining 5 *E.f. fulvus* and all *E.f. mayottensis* (=*FM*). The 13 *E.f. rufus* form two different clades (=*R1* and *R2*). *R1* is sister group to *AFS/FM* and *R2* groups next with *AFS/FM/R1*, making *E.f. albocollaris* with *E.f. collaris* the first clade to diverge among brown lemurs. Those arrangements are strongly supported by bootstrap and jackknife analyses using maximum parsimony or neighbor-joining searches.

Concluding Remarks

Based on karyotypes alone, *E.f. collaris* and *E.f. albocollaris* can be distinguished from *E.f. albifrons*, *E.f. fulvus*, *E.f. mayottensis*, *E.f. rufus*, or *E.f. sanfordi* (Rumpler 1975; Hamilton et al. 1980). *E.f. albifrons*, *E.f. albocollaris* and *E.f. sanfordi* can be identified by craniodental characters (Tattersall 1991). Based on the mitochondrial DNA sequences presented in this study, it is possible to distinguish six brown lemur clades. Apart from pelage, this is hence the most decisive feature for diagnosis of subspecies designations in brown lemurs thus far reported.

Of course there are concerns about the use of zoo specimens in systematics, yet these very same zoo samples are extremely valuable due to the difficulty in getting any samples at all. This study could be enhanced by the inclusion of additional *E.f. albifrons* and *E.f. sanfordi* specimens with a wider array of localities, and the same could be said for *E.f. fulvus* samples. Dense locality sampling of *E.f. fulvus* will be required to obtain a better interpretation of the

current contrast between diagnosable genetic and phenotypic units. Finally, future studies may want to examine *E.f. rufus* samples from the eastern and western coasts to corroborate the diagnosis of a distinct lineage of brown lemurs that is currently undescribed. Based on the data presented here, it is suggested that *E.f. mayottensis* does not deserve subspecies status. Another conclusion is that *E.f. albocollaris* and *E.f. collaris* diverged relatively recently from each other and that they should continue to be identified as subspecies of *E. fulvus.*

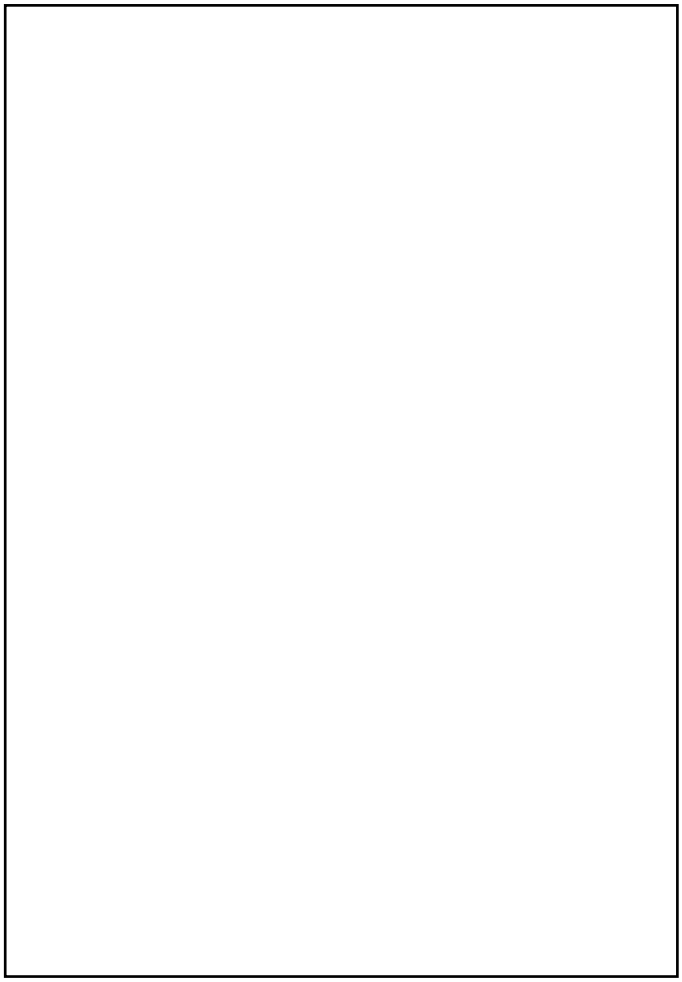

Fig. 3.7 *Eulemur fulvus rufus* in the Berenty Private Reserve, southern Madagascar (August 1998).

4. Genus *Hapalemur*

This chapter is to be published in a modified form:
J. Pastorini, M.R.J. Forstner, R.D. Martin (submitted)
Phylogenetic relationships of the gentle lemurs (*Hapalemur*).

4.1 Introduction

The genus *Hapalemur* (Geoffroy 1851) is classified in the endemic Malagasy primate family Lemuridae by most authors (Hill 1953; Petter et al. 1977; Groves 1989; Harcourt & Thornback 1990), although some consider it to be a member of the family Lepilemuridae (Tattersall 1982; Jenkins 1987). Three partially sympatric species are recognised: *H. aureus*, *H. griseus* and *H. simus*.

The grey gentle lemur *H. griseus* (Link 1795) is currently divided into four subspecies. *H.g. griseus* occurs in the humid eastern forests of Madagascar (Fig. 4.1). *H.g. olivaceus*, long recognised as a distinct subspecies, is now included in *H.g. griseus* (Tattersall 1982). A larger form is confined to lake Alaotra in the east (*H.g. alaotrensis*), while a smaller-bodied form occurs in the deciduous forests of the west (*H.g. occidentalis*). A fourth subspecies *H.g. meridionalis*, whose coat is darker than that of the other gentle lemurs, has recently been discovered near Fort Dauphin in southern Madagascar (Warter et al. 1987).

The broad-nosed gentle lemur *H. simus* (Gray 1870) is substantially larger than *H. griseus* and its ears are noticeably tufted with white hairs (Tattersall 1982). In the past, *H. simus* was regarded as sufficiently distinct to warrant generic separation under the name *Prolemur* (Gray 1871) (Pocock 1917; Tattersall & Schwartz 1974). Today, *H. simus* is extremely rare, found only in the southeast (Fig. 4.1). However, subfossil representatives of this species, including some formerly referred to as *H. gallieni*, have been found in central (Ampasambazimba), northern (Ankarana, Montagne des Français), and northwestern (Anjohibe) Madagascar (Vuillaume-Randriamanantena et al. 1985; Godfrey et al. 1999) (Fig. 4.1). Furthermore, records from individuals in museum collections confirm that less than a century ago *H. simus* occurred throughout the eastern rainforest (Vuillaume-Randriamanantena et al. 1985).

The golden bamboo lemur *H. aureus* (Meier et al. 1987), named after its unique golden facial colouring, was recently discovered near Ranomafana in southeast Madagascar (Meier et al. 1987). *H. aureus* is somewhat larger than *H. griseus* but much smaller than *H. simus*. *H. aureus* is an extremely rare species with a very patchy distribution (Fig. 4.1).

Few phylogenetic studies have included more than one *Hapalemur* species. All previously published data sets that examined *H. aureus* and *H. griseus* support a sister-group relationship for these (Rumpler et al. 1991; Crovella & Rumpler 1992; Macedonia & Stanger 1994; Crovella et al. 1995; Vezuli et al. 1997). The only phylogenetic studies to include more then one *H. griseus* subspecies are based on chromosomes (Rumpler & Dutrillaux 1978; Warter et al. 1987; Rumpler et al. 1991). The karyotypes of *H.g. alaotrensis* and *H.g. griseus* are indistinguishable (2N=54), while that of *H.g. meridionalis* (2N=54) differs in one metacentric chromosome. Cytogenetic comparisons indicate that *H.g. meridionalis* is the sister group to a clade containing *H.g. alaotrensis* and *H.g. griseus*, while *H.g. occidentalis* (2N=58) was the first subspecies to diverge.

In the present study, a large fragment of mitochondrial DNA was sequenced and the data examined in an attempt to clarify phylogenetic relationships among gentle lemur species and subspecies and to assess their taxonomic status.

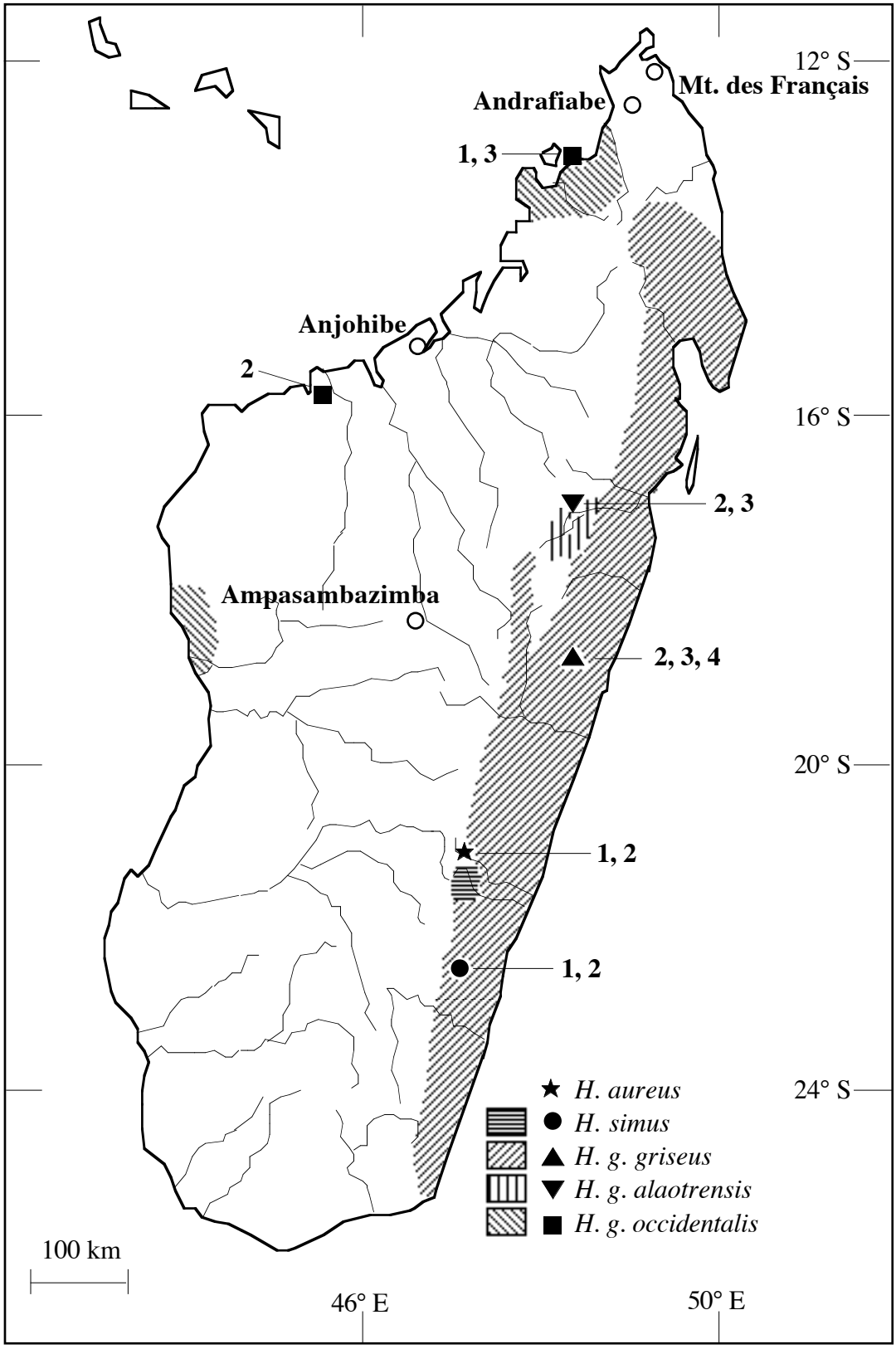

Fig. 4.1 Map of Madagascar showing approximate distribution of *Hapalemur* species and subspecies (Tattersall 1982; Meier et al. 1987; Warter et al. 1987). Symbols indicate individuals with exact locality data that were included in the present study. Subfossil *H. simus* are indicated with open circles.

4.2 Material

Hair, blood or tissue samples were collected from 2 *Hapalemur aureus*, 2 *H. simus*, 4 *H. griseus griseus*, 3 *H.g. alaotrensis* and 3 *H.g. occidentalis*. Unfortunately, no sample was available from *H.g. meridionalis*. Single individuals of *Varecia variegata variegata* and *V.v. rubra* were sequenced for subsequent use as outgroup taxa. Taxa, origins and identification numbers for the individuals sequenced are listed in Table 4.1.

Based on the records from 'Parc Zoologique et Botanique de Tsimbazaza', the 2 *H. simus* were captured at Karianga, the 2 *H. aureus* at Reserve Ranomafana, and the 2 *H. griseus alaotrensis* at Lac Alaotra (Belempona and Anorohoro). Three of the *H.g. griseus* samples used are from Maromiza. The captive population of *H.g. occidentalis* at 'Parc Zoologique et Botanique de Mulhouse' was founded by individuals from Ambato. One *H.g. occidentalis* sample was collected from Tsiombikibo forest (Curtis et al. 1995). No locality data is available for one *H.g. alaotrensis* and one *H.g. griseus*. All samples of known origin are depicted on the map shown in Fig. 4.1.

Table 4.1 Taxa, origin, identification numbers, and GenBank accession numbers for the 16 individuals sequenced.

Taxon	Origin	ID #	GenBank #
Hapalemur aureus 1	Ranomafana (Southeast) [a]	JP143, 931202	AF224581
Hapalemur aureus 2	Ranomafana (Southeast) [a]	JP144, 931203	AF224582
Hapalemur griseus alaotrensis 1	unknown [b]	JP4, 017	AF224575
Hapalemur griseus alaotrensis 2	Belempona (East) [a]	JP139	AF224576
Hapalemur griseus alaotrensis 3	Anororo (East) [a]	JP140	AF224577
Hapalemur griseus griseus 1	unknown	JP234	AF224571
Hapalemur griseus griseus 2	Maromiza (East)	JP346	AF224572
Hapalemur griseus griseus 3	Maromiza (East)	JP347	AF224573
Hapalemur griseus griseus 4	Maromiza (East)	JP348	AF224574
Hapalemur griseus occidentalis 1	Ambato (North) [c]	JP31, 950084	AF224578
Hapalemur griseus cf. *occidentalis 2*	Forêt de Tsiombikibo (Northwest)	JP241	AF224579
Hapalemur griseus occidentalis 3	Ambato (North) [c]	JP275, 920031	AF224580
Hapalemur simus 1	Karianga (Southeast) [a]	JP127	AF224583
Hapalemur simus 2	Karianga (Southeast) [a]	JP128	AF224584
Varecia variegata rubra	unknown [d]	JP5	AF224588
Varecia variegata variegata	unknown [a]	JP132	AF224587

[a] held at Parc Zoologique et Botanique de Tsimbazaza, Madagascar

[b] held at Duke University Primate Center, U.S.A.

[c] held at Parc Zoologique et Botanique de Mulhouse, France

[d] held at Zoo Zürich, Switzerland

4.3 Results

The new mtDNA sequences generated for the taxa examined have been deposited in GenBank (Table 4.1). The nucleotide sequences span a total of 2388 base positions (bp). The sequences obtained provided 599 parsimony-informative characters with a transition:transversion ratio of 6.2:1. A summary of the frequencies of invariant, parsimony-uninformative and informative characters along the segment sequenced is given in Table 4.2. Absolute pairwise distances are presented in Table 4.3 and range from a maximum of 403–423 bp between *Varecia* and the ingroup to 0–291 bp within the genus *Hapalemur*.

The maximum parsimony branch-and-bound search results in one tree 851 steps in length with a consistency index of 0.81 and a retention index of 0.88 (Fig. 4.2). The final maximum likelihood tree (–ln likelihood = 6897.59) was obtained with an estimated transition/transversion ratio of 20.76 (kappa = 44.90) and gamma shape parameter of 0.12 (Fig. 4.3). Neighbor-joining methods (Fig. 4.4) reconstruct the same topology as maximum parsimony and maximum likelihood methods. All arrangements are generally strongly supported by bootstrap and jackknife analyses with both maximum parsimony and neighbor-joining methods.

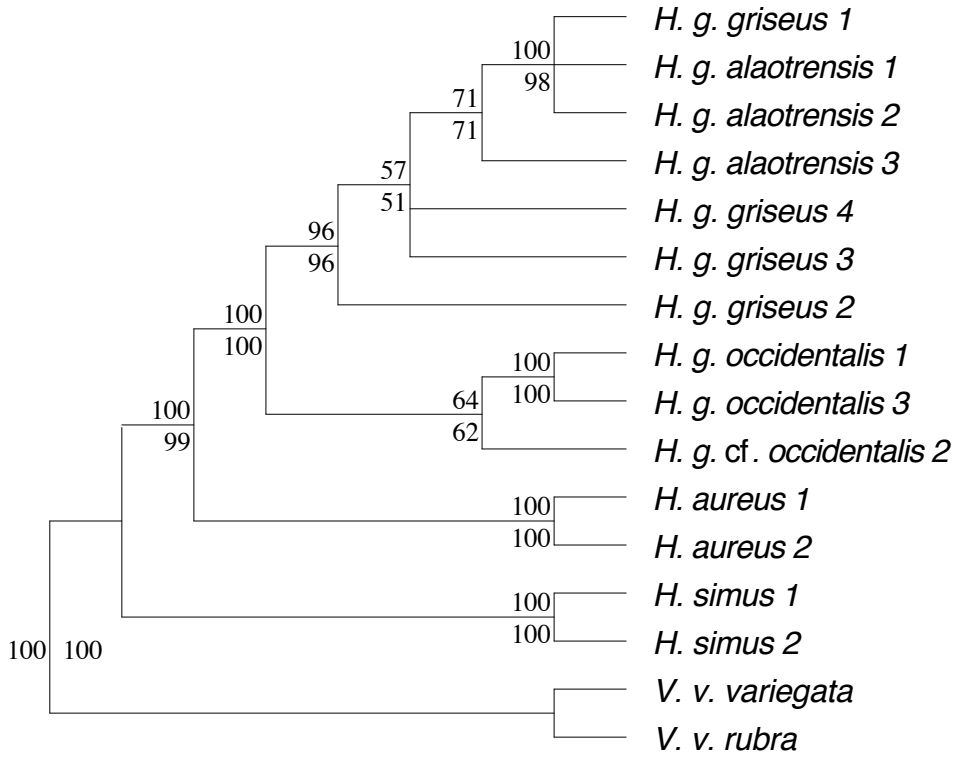

Fig. 4.2 Maximum parsimony tree with bootstrap (as percentages, above nodes) and jackknife (below nodes) values.

Table 4.2 Summary of variation for the sequences across the 16 lemurs examined.

Genes	All	COIII	ND3	ND4L [a]	ND4 [a]	tRNAs	Not translated
characters (nucleotides)	2388	53	348	297	1378	316	3
constant	1741	43	245	218	977	265	0
parsimony-uninformative	48	1	8	5	32	2	0
parsimony-informative	599	9	95	74	369	49	3
informative proportion	0.25	0.17	0.27	0.25	0.27	0.16	1.00
insertions/deletions	8	0	0	0	3	4	1

[a] ND4L and ND4 overlap for 7 bp

All analyses group *H. griseus* with *H. aureus* as a sister group to *H. simus*. *H.g. griseus* and *H.g. alaotrensis* fail to resolve two monophyletic lineages. The clades containing individuals of the species *H. simus*, *H. aureus* or *H. griseus* always have complete bootstrap or jackknife support (100%). While the *H. griseus*/*H.g. alaotrensis* clade is supported with 96–100% bootstrap or jackknife values, the three *H.g. occidentalis* individuals are linked with only 62–82% bootstrap of jackknife support. The subclade of *H. aureus* and *H. griseus* has strongest bootstrap or jackknife support (100%).

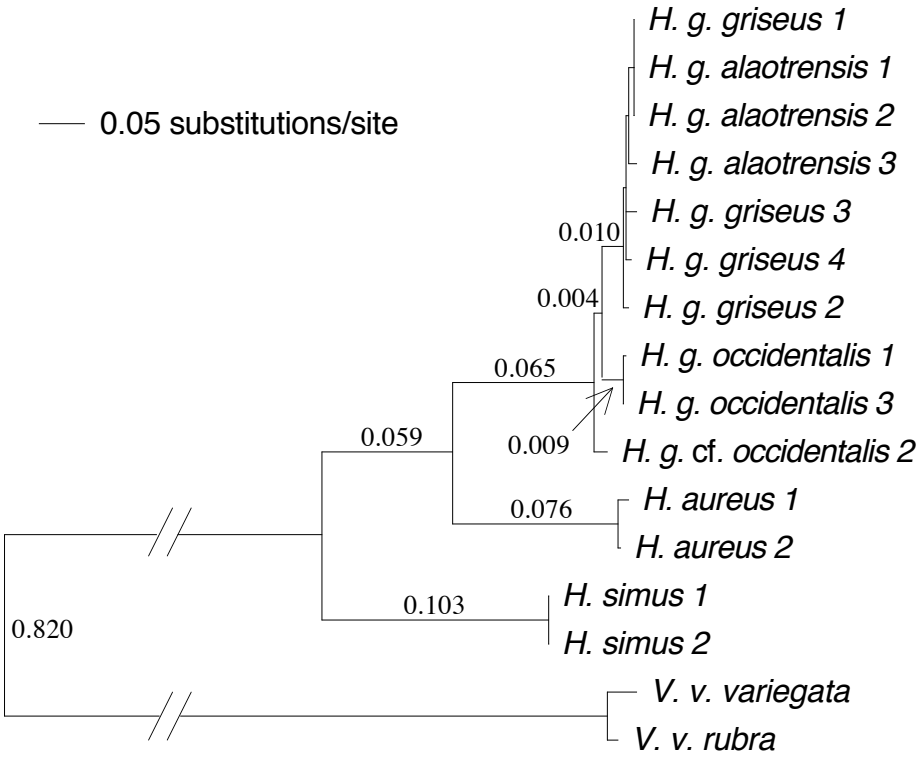

Fig. 4.3 Maximum likelihood phylogram with branch lengths proportional to the number of changes (values provided on each branch). // Indicates artificially shortened branch to fit page format.

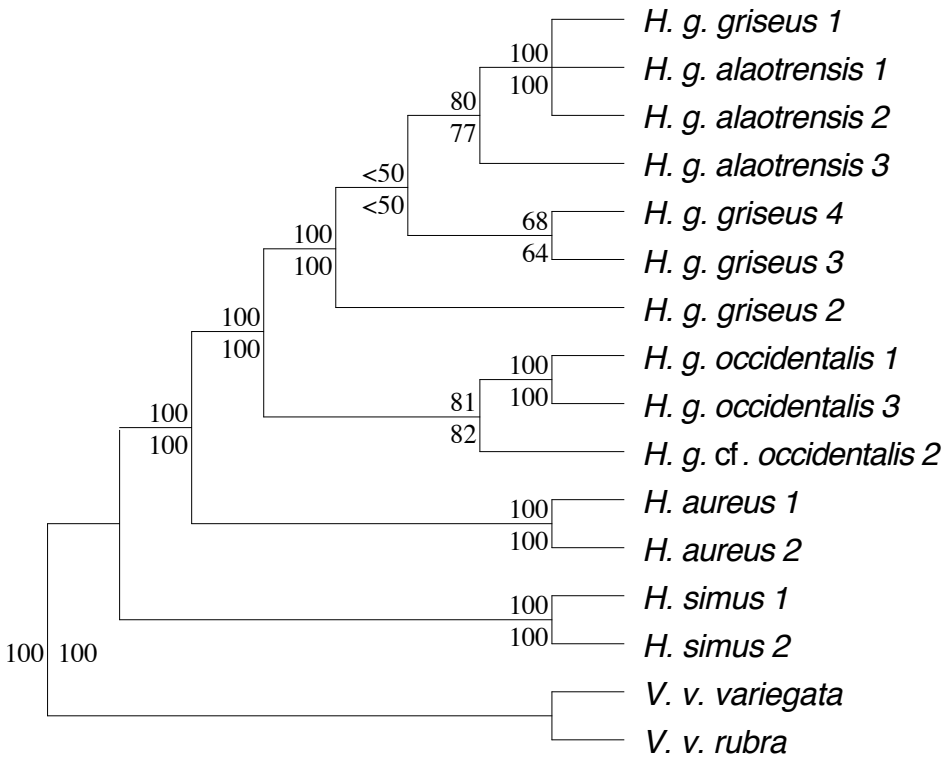

Fig. 4.4 Neighbor-joining tree with bootstrap (as percentages, above nodes) and jackknife (below nodes) values.

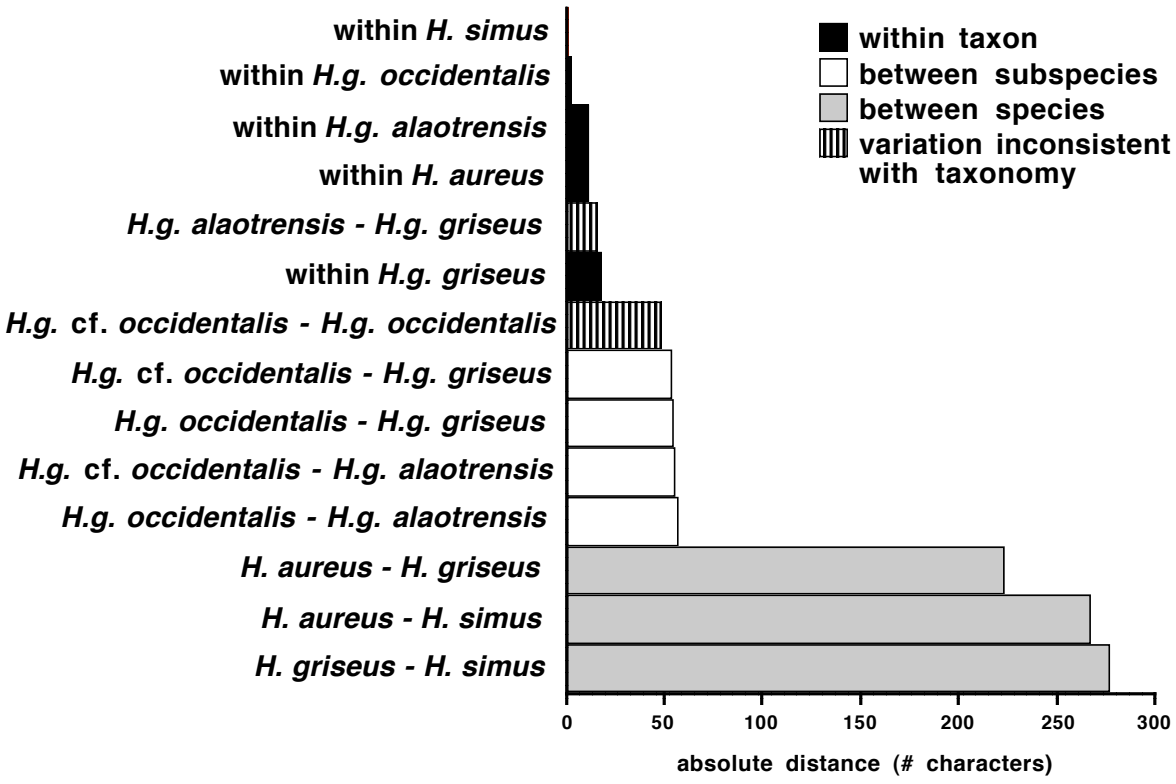

Fig. 4.5 Absolute pairwise distances over three defined taxonomic levels. Each bar represents the average of all possible comparisons between individuals of the two taxa. Single values can be seen in Table 4.3.

Absolute pairwise distances are presented in Table 4.3 and range from a maximum of 403–423 bp between *Varecia* and the ingroup to 0–291 bp within the genus *Hapalemur*. Examination of absolute pairwise distances within the genus *Hapalemur* reveals three levels of differentiation (Fig. 4.5, Table 4.3). Divergences between the three *Hapalemur* species are 218–291 bp. Pairwise distances between *H.g. griseus* and *H.g. occidentalis* or *H.g. alaotrensis* and *H.g. occidentalis* range from 50–61 bp, whereas distances between *H.g. griseus* and *H.g. alaotrensis* range from 0–26 bp. Within *H.g. occidentalis* two subclades can be separated that differ from each other on the order of 47–48 bp. Within *H. aureus*, *H. simus*, *H.g. griseus*, *H.g. alaotrensis* and one subclade of *H.g. occidentalis* distances range from 1–22 bp.

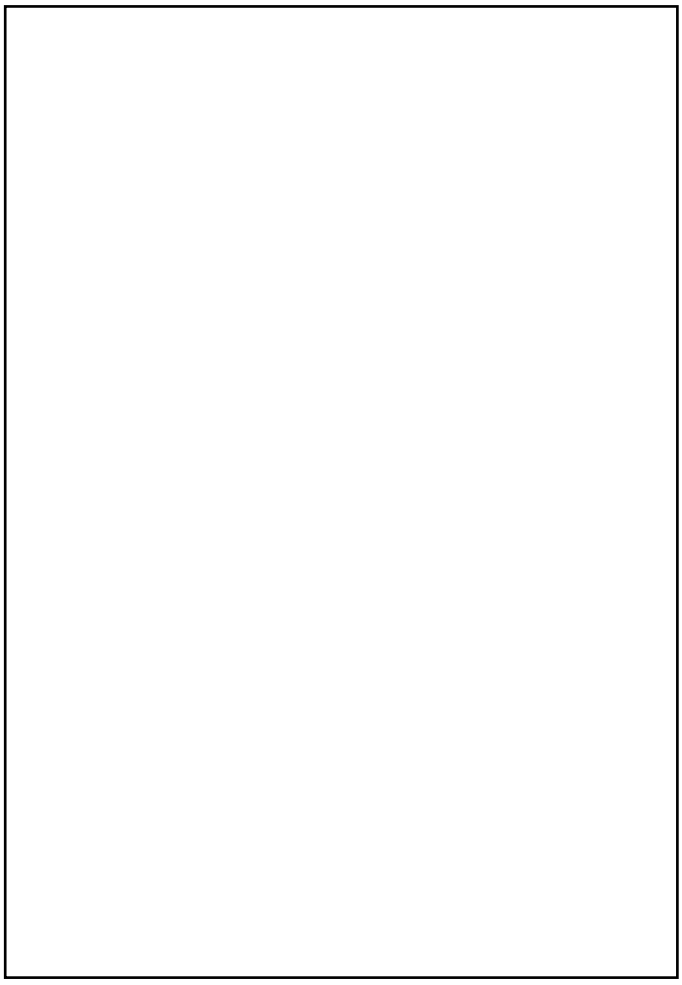

Fig. 4.6 *Hapalemur simus* (left), *Hapalemur griseus* (middle) and *Hapalemur aureus* (right) from Parc Zoologique et Botanique de Tsimbazaza (July 1997).

Table 4.3 Kimura 2-parameter distance (above the diagonal) and absolute distance (under the diagonal) matrices derived from the 2388 bp mitochondrial DNA sequence data set, with gaps treated as missing data.

	H.g.g. 1	*H.g.g. 2*	*H.g.g. 3*	*H.g.g. 4*	*H.g.a. 1*	*H.g.a. 2*	*H.g.a. 3*	*H.g.o. 1*	*H.g.o. 2*	*H.g.o. 3*	*H.a. 1*	*H.a. 2*	*H.s. 1*	*H.s. 2*	*V.v.v.*	*V.v.r.*
H. g. griseus 1	-	0.007	0.009	0.007	0	0.000	0.007	0.024	0.024	0.024	0.102	0.102	0.129	0.129	0.204	0.204
H. g. griseus 2	16	-	0.008	0.006	0.007	0.007	0.008	0.022	0.021	0.022	0.100	0.100	0.127	0.127	0.206	0.207
H. g. griseus 3	22	20	-	0.008	0.009	0.010	0.011	0.026	0.024	0.026	0.105	0.105	0.130	0.131	0.209	0.209
H. g. griseus 4	16	14	20	-	0.007	0.007	0.008	0.024	0.024	0.023	0.102	0.102	0.131	0.131	0.204	0.204
H. g. alaotrensis 1	0	16	22	16	-	0.000	0.007	0.024	0.024	0.024	0.102	0.102	0.129	0.129	0.204	0.204
H. g. alaotrensis 2	1	17	23	17	1	-	0.007	0.025	0.024	0.024	0.103	0.103	0.129	0.130	0.204	0.204
H. g. alaotrensis 3	16	20	26	20	16	17	-	0.026	0.024	0.026	0.102	0.102	0.127	0.128	0.204	0.204
H. g. occidentalis 1	57	52	61	55	57	58	61	-	0.021	0.001	0.105	0.104	0.137	0.138	0.209	0.210
H. g. cf. occidentalis 2	55	50	57	55	55	56	57	48	-	0.020	0.101	0.101	0.127	0.127	0.207	0.207
H. g. occidentalis 3	56	51	60	54	56	57	60	3	47	-	0.106	0.105	0.137	0.137	0.209	0.211
H. aureus 1	222	218	227	222	222	223	222	228	220	229	-	0.005	0.126	0.126	0.205	0.202
H. aureus 2	222	218	227	222	222	223	222	226	220	227	12	-	0.125	0.125	0.204	0.201
H. simus 1	274	270	277	278	274	275	271	290	271	289	268	266	-	0.000	0.198	0.199
H. simus 2	275	271	278	279	275	276	272	291	272	290	269	267	1	-	0.199	0.199
V. v. variegata	411	416	420	412	411	412	411	420	417	421	413	412	403	404	-	0.019
V. v. rubra	411	416	420	412	411	412	411	422	417	423	408	407	403	404	44	-

4.4 Discussion

Hapalemur *Species*

The relationships among *Hapalemur* species remain consistent in all analyses. The clades containing individuals of the species *H. simus*, *H. aureus* or *H. griseus* always have complete bootstrap or jackknife support (100%). The resulting topologies indicate that *H. simus* is deeply separated from other gentle lemurs (Figs. 4.2 - 4.4). The subclade of *H. aureus* and *H. griseus* has strongest bootstrap and jackknife support (100%), and pairwise distances between *H. simus* and other species of *Hapalemur* are the highest among within-genus comparisons (Table 4.3). This study therefore suggests a close relationship between *H. aureus* and *H. griseus*, agreeing with results from analyses of morphological (Meier et al. 1987), chromosomal (Rumpler et al. 1991; Vezuli et al. 1997), genetical (Crovella & Rumpler 1992; Crovella et al. 1995), and communication (Macedonia & Stanger 1994) characters.

Divergences between *H. aureus* and *H. griseus* (218–229 bp) are slightly smaller than divergences between *H. simus* and *H. aureus* (266–269 bp) or *H. simus* and *H. griseus* (270–291 bp, Table 4.3). However, among all *Hapalemur* species the pairwise distances are higher than among subspecies (50–61 bp) and even exceed all between-species comparisons in *Eulemur* (140–199 bp, see Chapter 5). Branch lengths in the maximum likelihood phylogram confirm the deep divergence of *H. aureus* from other gentle lemurs (Fig. 4.3). These molecular data thus strongly support specific status for *H. aureus*. This is in agreement with previous studies on restriction genomic DNA banding patterns (Crovella & Rumpler 1992; Crovella et al. 1995).

H. griseus alaotrensis

The two taxa *H.g. griseus* and *H.g. alaotrensis* consistently fail to resolve into two monophyletic lineages (Figs. 4.2 - 4.4). Additionally, average genetic distances between *H.g. griseus* and *H.g. alaotrensis* (0–26 bp) clearly lie in the range of within-taxon comparisons (1–22 bp, Table 4.3). Based on tree topology and pairwise distances, the molecular data presented in this study thus do not support two monophyletic lineages for *H.g. griseus* and *H.g. alaotrensis*. The possibility that this is due to the genes chosen for sequencing can be excluded. Phylogenetic studies on other lemurs using the same genes clearly resolved the subspecies (see Chapters 3 and 5). If *H.g. alaotrensis* is, indeed, nested within the *H.g. griseus* clade, a cladistic

approach to classification would require combination of *H.g. griseus* and *H.g. alaotrensis* into a single subspecies. Groves (1989) had already noted that it is unclear whether one taxon (*H.g. alaotrensis*) whose range is entirely surrounded by that of another (*H.g. griseus*) can justifiably be ranked as a subspecies. He was therefore tempted to recognise the former as a full species *H. alaotrensis*. The karyotype of *H.g. alaotrensis* is indistinguishable from that of *H.g. griseus* (Rumpler & Dutrillaux 1978) and hybridisation between the two taxa has been possible in captivity (Petter et al. 1977). *H.g. alaotrensis* is larger than the other two subspecies; it also has a shorter tail, a more pointed snout and a less-defined facial pattern (Groves 1989). However, karyotypes, distribution and mtDNA sequences would all favour subsuming *H.g. alaotrensis* within *H.g. griseus*.

Evidence for a Further* H. griseus *subspecies

Pairwise distances, being used as a gross measure of divergence, show that one individual of *H.g. occidentalis* (#2) is very different from the other two *H.g. occidentalis* examined (Table 4.3). This high degree of divergence (47–48 bp) lies in the range of comparisons between other subspecies of *Hapalemur* (50–61 bp), *Eulemur* (29–90 bp, see Chapters 3 and 5) or *Varecia* (42–65 bp, Chapter 5) and is much higher than the highest value (22 bp) for within-taxon comparisons (Table 4.3). The relatively low bootstrap or jackknife support linking the 3 *H.g. occidentalis* individuals (62–82%) and the long branch length of the maximum likelihood phylogram separating the two *H.g. occidentalis* individuals from the third confirm this result (Fig. 4.3).

The captive population of *H.g. occidentalis* at 'Parc Zoologique et Botanique de Mulhouse' which yielded two of the individuals studied, is derived from founders from Ambato in northern Madagascar (Fig. 4.1). The third *H.g. occidentalis* sample (#2) analysed in this study was collected further south at Tsiombikibo forest in northwest Madagascar. Interestingly, a large river drainage system, the Betsiboka, lies between those two localities. This river is a known isolating barrier for other lemur subspecies (e. g. separating *Eulemur fulvus fulvus* from *E.f. rufus* and *Propithecus verreauxi coronatus* from *P.v. coquereli*). The relatively high level of genetic differentiation among *H.g. occidentalis* individuals, combined with geographic distribution, suggests that more phylogenetic structure may exist within this taxon than is indicated by current taxonomy.

Conclusions

Molecular data strongly support the specific status of *H. aureus*, a sister-group relationship between *H. aureus* and *H. griseus*, and a basal position of *H. simus* among gentle lemurs. The sequence data do not yield clear resolution of *H.g. griseus* from *H.g. alaotrensis*. There is a potential taxonomic problem regarding paraphyly of these two subspecies that requires further investigation. Considerable genetic differentiation exists among the small sample of *H.g. occidentalis* individuals examined here. A more detailed examination of the western gentle lemurs would be valuable in an attempt to explore the possibility that more than one subspecies exists along the western coast.

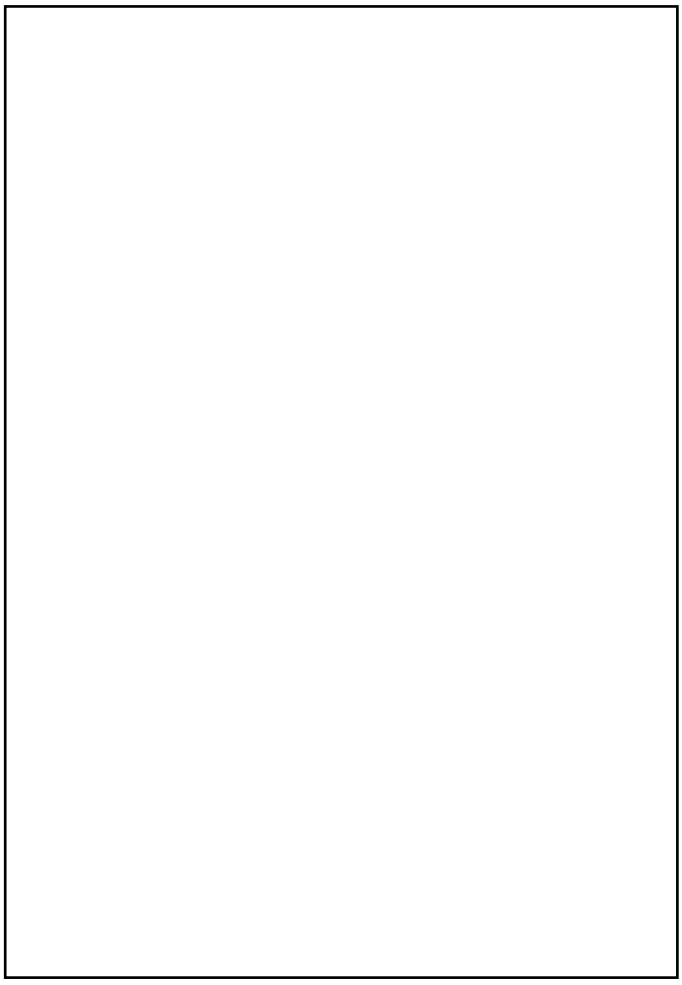

Fig. 4.7 *Hapalemur griseus occidentalis* from Parc Zoologique et Botanique de Mulhouse (December 1997).

5. Family Lemuridae

This chapter is to be published in a modified form:
J. Pastorini, M.R.J. Forstner, R.D. Martin (submitted)
Mitochondrial sequence phylogeny of the family Lemuridae (Primates).

5.1 Introduction

The Lemuridae is one of five extant lemur families endemic to the island of Madagascar. Systematics within the Lemuridae have constituted one of the most controversial subjects in lemur biology. Traditionally this family included three genera: *Lemur*, *Hapalemur*, and *Lepilemur* (Schwartz 1931; Hill 1953). However, some species previously included in *Lemur* are now commonly separated in the genera *Varecia* (Gray 1863) and *Eulemur* (Simons & Rumpler 1988). Furthermore, Petter et al. (1977) proposed separation of *Lepilemur* at the family level. At present, therefore, a tentative consensus accepts four genera (*Eulemur*, *Hapalemur*, *Lemur* and *Varecia*) in the family Lemuridae. These genera include 10 recognised species and at least 13 subspecies. Even thus restricted, the family Lemuridae is difficult to define. All members lack the ascending pharyngeal artery of the Cheirogaleidae, and they have the full dental complement, unlike the Indridae or Lepilemuridae (Groves 1989), but these are generally regarded as primitive features. Despite many investigations, the phylogenetic relationships among lemurid taxa are still not well understood.

The genus *Hapalemur* (Geoffroy 1851) is classified in the family Lemuridae by most authors (Hill 1953; Petter et al. 1977; Groves 1989; Harcourt & Thornback 1990). Only Tattersall (1982) considered it to be a member of the family Lepilemuridae, although this allocation was later followed by Jenkins (1987). Phylogenetic studies generally group *Hapalemur* within the family Lemuridae, but its phylogenetic position remains uncertain (Fig. 5.1). Three species are universally recognised: *H. aureus*, *H. griseus*, and *H. simus*. Whereas all previously published data sets support a sister-group relationship of *H. aureus* with *H. griseus*, they often fail to resolve *H. simus* into a monophyletic clade with these two species (Fig. 5.1). Thus far, however, no phylogenetic study based on DNA sequence data has included either *H. simus* or *H. aureus*.

The systematic status of the largest extant lemurid, *Varecia variegata* (Kerr 1792), remains a highly debated issue. Originally recognised as a separate genus (Gray 1863), this

taxon was subsequently included in the genus *Lemur* (Schwartz 1931; Hill 1953). More recently, the ruffed lemur has been reclassified as *Varecia* (Petter 1962), and this is now commonly accepted (Tattersall 1982; Jenkins 1987; Groves 1989; Harcourt & Thornback 1990). There is nevertheless a divergence of opinion: Whereas Macedonia and Stanger (1994) question the grounds on which *Varecia* has been assigned to the family Lemuridae, Tattersall (1991) states that the least misleading move under present circumstances would be to return to a taxonomy which includes *Varecia* within the genus *Lemur*. The phylogenetic relationship between *Varecia* and other lemurids remains controversial with many studies unable to resolve the position of *Varecia* within the Lemuridae (Fig. 5.1) or even placing it outside of the Lemuridae completely (Adkins & Honeycutt 1994; Stanger-Hall 1997).

In 1918, Pocock suggested separating *L. catta* (Linnaeus 1758) at the generic level from other members of the genus *Lemur*, and Hill (1953) also noted that the species *L. catta* deserved at least subgeneric separation. In 1988, three papers independently recommended new genus-level taxonomy for *Lemur* species other than *L. catta* (Simons & Rumpler 1988; Groves & Eaglen 1988; Tattersall 1988a). However, the purported close relationship of *L. catta* to *Hapalemur* underlying this move has not been confirmed by some analyses (Fig. 5.1).

The genus *Eulemur* (Simons & Rumpler 1988) is the most diverse and widespread genus of the Lemuridae, with five currently recognised species. *E. fulvus* has the largest distribution of those five species and contains at least six subspecies. For *E. macaco*, two subspecies have been described. Reconstruction of the evolutionary relationships among *Eulemur* species have also been controversial (Fig. 5.1). Of the 11 studies shown in Figure 5.1, only two character sets (Tattersall 1991; Groves & Trueman 1995) yield the same tree topology among *Eulemur* species. All other phylogenetic arrangements show unique patterns. On the basis of chromosomal studies (Rumpler et al. 1989) and two DNA-sequencing studies (Stanger-Hall & Cunningham 1998; Yoder & Irwin 1999), little resolution has yet been achieved. However, all of these studies do support a monophyletic genus *Eulemur*.

In the present study, a large fragment of mitochondrial DNA was sequenced and the data examined in an attempt to clarify phylogenetic relationships among Lemuridae. Close attention was given to the relative positions of *H. simus*, *L. catta* and *Varecia*. A further aim was to assess the validity of generic status for *Eulemur*, *Lemur* and *Varecia*. All ten currently recognised lemurid species have been included in this study. Previous successful resolution of problematic

taxa using this region of mtDNA (Forstner et al. 1995; Wang et al. 1997; Forstner et al. 1998; Pastorini et al. 1998) indicated that this fragment could potentially resolve phylogenetic relationships among the genera and species of the family Lemuridae.

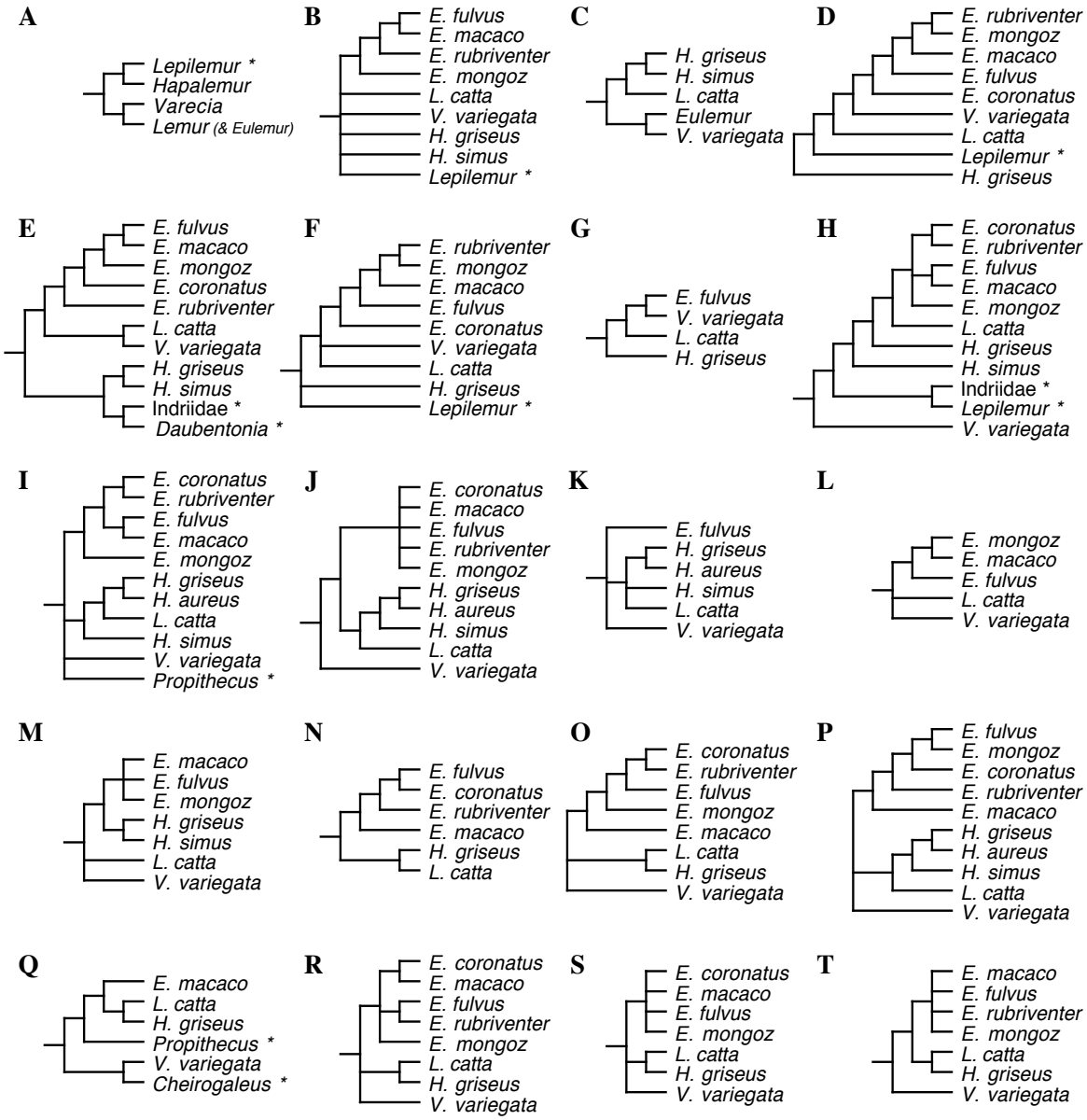

Fig. 5.1 Alternative phylogenetic trees based on different lines of evidence: **A** craniodental morphology (Tattersall & Schwartz 1974); **B** 30 morphological and behavioural characters (Eaglen 1983); **C** 32 mainly morphological characters (Groves & Eaglen 1988); **D** 37 craniodental characters (Tattersall 1991); **E** 86 morphological characters (Yoder 1994); **F** 53 mainly morphological characters (Groves & Trueman 1995); **G** 125 morphological and behavioural characters (Yoder et al. 1996); **H** 25 morphological and behavioural characters (Stanger-Hall 1997); **I** 19 communication signal characters (Macedonia & Stanger 1994); **J** karyotypes (Rumpler et al. 1989 and 1991); **K** chromosome painting (Vezuli et al. 1997); **L** immunodiffusion data (Dene et al. 1980); **M** albumin and transferrin (Sarich & Cronin 1976); **N** restriction genomic DNA banding patterns (Jung et al. 1992); **O** highly repeated DNA sequences (Crovella et al. 1993); **P** highly repeated DNA sequences (Crovella et al. 1995); **Q** 682 bp of the cytochrome *c* oxidase subunit II (COII) gene (Adkins & Honeycutt 1994); **R** 290 bp of the cytochrome *b* gene (Del Pero et al. 1995); **S** 461 bp of the large ribosomal subunit (16S) gene (Stanger-Hall & Cunningham 1998); **T** a total of 3303 bp from cytochrome *b*, D-loop, IRBP, and COII genes (Yoder & Irwin 1999).

5.2 Material

Samples were collected from all 10 species among the four lemurid genera: 3 *Eulemur coronatus*, 6 *E. fulvus* (6 ssp.), 6 *E. macaco* (2 ssp.), 3 *E. mongoz*, 3 *E. rubriventer*, 2 *Hapalemur aureus*, 3 *H. griseus* (3 ssp.), 2 *H. simus*, 3 *Lemur catta*, and 8 *Varecia variegata* (2 ssp.). Three samples from *Daubentonia madagascariensis* were sequenced for subsequent use as outgroup taxa (Table 5.1).

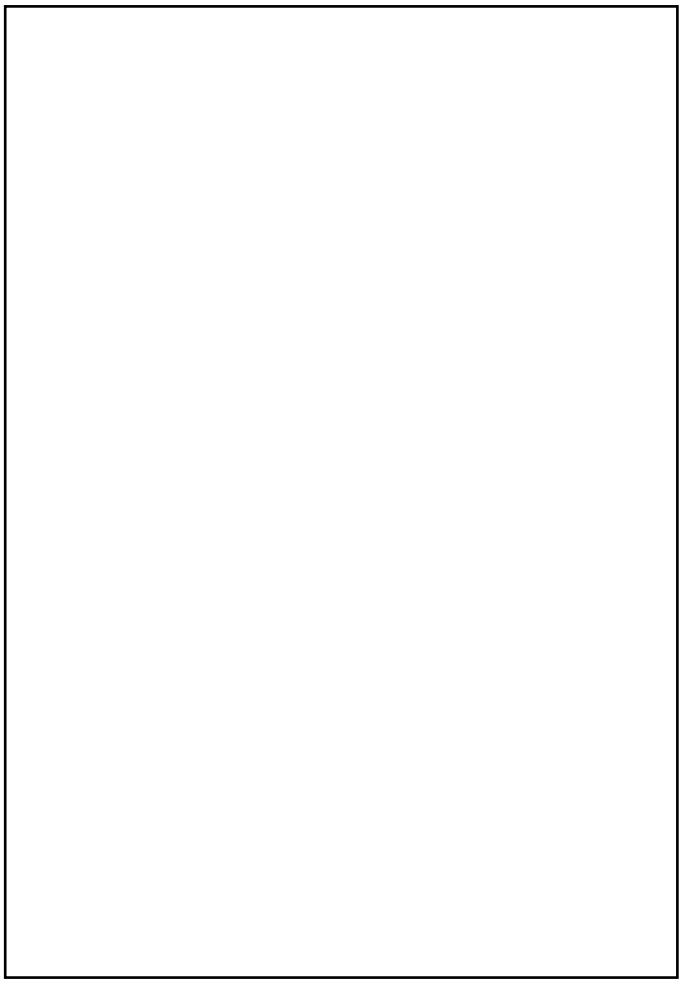

Fig. 5.2 *Eulemur rubriventer* at Apenheul Zoo (August 1999).

Table 5.1 Taxa, origin, identification numbers, and GenBank accession numbers for the 42 individuals sequenced.

Taxon	Origin	ID #	GenBank #
Eulemur mongoz 1	Anjamena (Northwest) [a]	JP169	AF224514
Eulemur mongoz 2	Anadabomandry (Northwest) [a]	JP177	AF224515
Eulemur mongoz 3	Ampijoroa (Northwest) [a]	JP220	AF224519
Eulemur coronatus 1	unknown [b]	JP33, 830043	AF224522
Eulemur coronatus 2	unknown [b]	JP34, 830041	AF224523
Eulemur coronatus 3	unknown [c]	JP121, 930209	AF224524
Eulemur rubriventer 1	unknown [c]	JP129	AF224525
Eulemur rubriventer 2	unknown [c]	JP130, 880605	AF224526
Eulemur rubriventer 3	Andasibe (East) [d]	JP229	AF224527
Eulemur macaco macaco 1	unknown (North) [d]	JP80	AF224528
Eulemur macaco macaco 2	Ambato (North) [d]	JP82	AF224529
Eulemur macaco macaco 3	Ambato (North) [d]	JP83	AF224530
Eulemur macaco flavifrons 1	Maromandia (North) [d]	JP74	AF224531
Eulemur macaco flavifrons 2	Maromandia (North) [d]	JP75	AF224532
Eulemur macaco flavifrons 3	Maromandia (North) [d]	JP77	AF224533
Eulemur fulvus fulvus	Ampijoroa (Northwest) [a]	JP218	AF224536
Eulemur fulvus rufus 1	Anjamena (Northwest) [a]	JP161	AF224545
Eulemur fulvus rufus 2	Morondava (West)	JP332	AF224551
Eulemur fulvus albocollaris	Vondrozo (Southeast) [d]	JP222	AF224558
Eulemur fulvus collaris	unknown [e]	JP307, R390/98	AF224560
Eulemur fulvus albifrons	Andranobe Forest (Northeast) [a]	JP323	AF224568
Lemur catta 1	unknown [f]	JP3, AIMUZ8535	AF053684
Lemur catta 2	unknown [b]	JP27, 900052	AF224569
Lemur catta 3	unknown [f]	JP52, AIMUZ10118	AF224570
Hapalemur griseus griseus	Maromiza (East) [a]	JP346	AF224572
Hapalemur griseus occidentalis 1	Ambato (North) [b]	JP31, 950084	AF224578
Hapalemur griseus occidentalis 2	Forêt de Tsiombikibo (Northwest) [a]	JP241	AF224579
Hapalemur aureus 1	Ranomafana (Southeast) [c]	JP143, 931202	AF224581
Hapalemur aureus 2	Ranomafana (Southeast) [c]	JP144, 931203	AF224582
Hapalemur simus 1	Karianga (Southeast) [c]	JP127	AF224583
Hapalemur simus 2	Karianga (Southeast) [c]	JP128	AF224584
Varecia variegata variegata 1	unknown [g]	JP30	AF224585
Varecia variegata variegata 2	unknown [c]	JP131	AF224586
Varecia variegata variegata 3	unknown [c]	JP132, 931706	AF224587
Varecia variegata rubra 1	unknown [h]	JP5	AF224588
Varecia variegata rubra 2	unknown [b]	JP32, 920065	AF224589
Varecia variegata rubra 3	unknown [g]	JP236	AF224590
Varecia variegata rubra 4	Andranobe Forest (Northeast) [a]	JP324	AF224591
Varecia variegata rubra 5	Andranobe Forest (Northeast) [a]	JP325	AF224592
Daubentonia madagascariensis 1	Andratamarina (Northeast) [f]	JP7, AIMUZ 11902	AF224640
Daubentonia madagascariensis 2	Anjiamangirana (Northwest) [c]	JP119	AF224641
Daubentonia madagascariensis 3	Anjiamangirana (Northwest) [c]	JP120	AF224642

[a] wild-caught animals with verified origin

[b] held at Parc Zoologique et Botanique de Mulhouse, France

[c] held at Parc Zoologique et Botanique de Tsimbazaza, Madagascar

[d] held at Université Louis Pasteur, Strasbourg, France

[e] specimen held at National Museums of Scotland, U.K. (previously held at Banham Zoo)

[f] specimen held at Anthropological Institute and Museum of the University of Zürich (AIMUZ), Switzerland

[g] held at Tierpark Berlin-Friedrichsfelde, Germany

[h] held at Zürich Zoo, Switzerland

5.3 Results

The new mtDNA sequences generated for the taxa examined have been deposited in GenBank (Table 5.1). The nucleotide sequences span a total of 2393 base positions (bp). The analysed dataset consists of 3' end of the COIII gene (53 bp), the complete NADH-dehydrogenase subunits ND3 (348 bp), ND4L (297 bp) and ND4 (1378 bp), along with the glycine (72 bp), arginine (68 bp), histidine (70 bp), serine (64 bp), and part of leucine (47 bp) tRNA genes. Two of the 3 *Daubentonia* were not sequenced for the first 23 bp of the COIII fragment.

The sequences obtained yielded 973 parsimony-informative characters with a transition:transversion ratio of 6:1. A summary of the frequencies of invariant, parsimony uninformative, and informative characters along the segment sequenced is given in Table 5.2. The partition-homogeneity test was carried out for the nine genes (COIII, ND3, ND4L, ND4, 5 tRNAs) sequenced. The genes were not found to be significantly incongruent (P=0.96) and were hence combined in all analyses.

In the family Lemuridae, the ND3 gene is terminated by 'TAA', whereas in *Daubentonia* and in *Homo*, only 'TA' and 'T', respectively, serve as the stop codons via polyadenylation. Lemurids have an insertion of 2 bp between the ND3 gene and the $tRNA^{Arg}$. The mtDNA genomes of *Homo* or *Daubentonia* do not contain any untranslated bp between these genes. Between $tRNA^{Arg}$ and ND4L, lemurs (including *H. simus*) have an additional base position that is not present in *Homo*, *H. aureus* and *H. griseus*. In the ND4 gene of *H.g. griseus* , *H.g. alaotrensis* and *H. aureus*, a deletion of 3 bp, coding for the 48th amino acid (Asn in the human genome), has occurred. In the ND4 gene of *Daubentonia*, a deletion of 6 bp, coding for two amino acids, has occurred (positions 49 and 50, Leu and Phe based on human genome). All other indels are limited to loops of tRNA genes.

Table 5.2 Summary of variation for the sequences across the 42 lemurs examined.

Genes	All	COIII	ND3	ND4L[a]	ND4[a]	tRNAs	Not translated
characters (nucleotides)	2393	53	348	297	1378	321	3
constant	1391	41	199	166	777	214	0
parsimony-uninformative	29	0	5	4	16	4	0
parsimony-informative	973	12	144	127	585	103	3
informative proportion	0.41	0.23	0.41	0.43	0.42	0.32	1.00
insertions/deletions	24	0	1	0	9	11	3

[a] ND4L and ND4 overlap for 7 bp

The maximum parsimony heuristic search with all characters weighted equally results in one tree 2345 steps in length with a consistency index of 0.54 and a retention index of 0.87 (Fig. 5.3). The distance matrices (Table 5.3) constructed using Kimura 2-parameter corrections, and subsequently analysed by neighbor-joining methods, reconstruct the tree shown in Figure 5.4. The support values from bootstrap and jackknife analyses of 2500 replicates are in the same range for maximum parsimony and neighbor-joining analyses.

In maximum parsimony (MP) and neighbor-joining (NJ) analyses, the five *Eulemur* species form one clade. *Lemur* and *Hapalemur* together form a clade which is the sister group

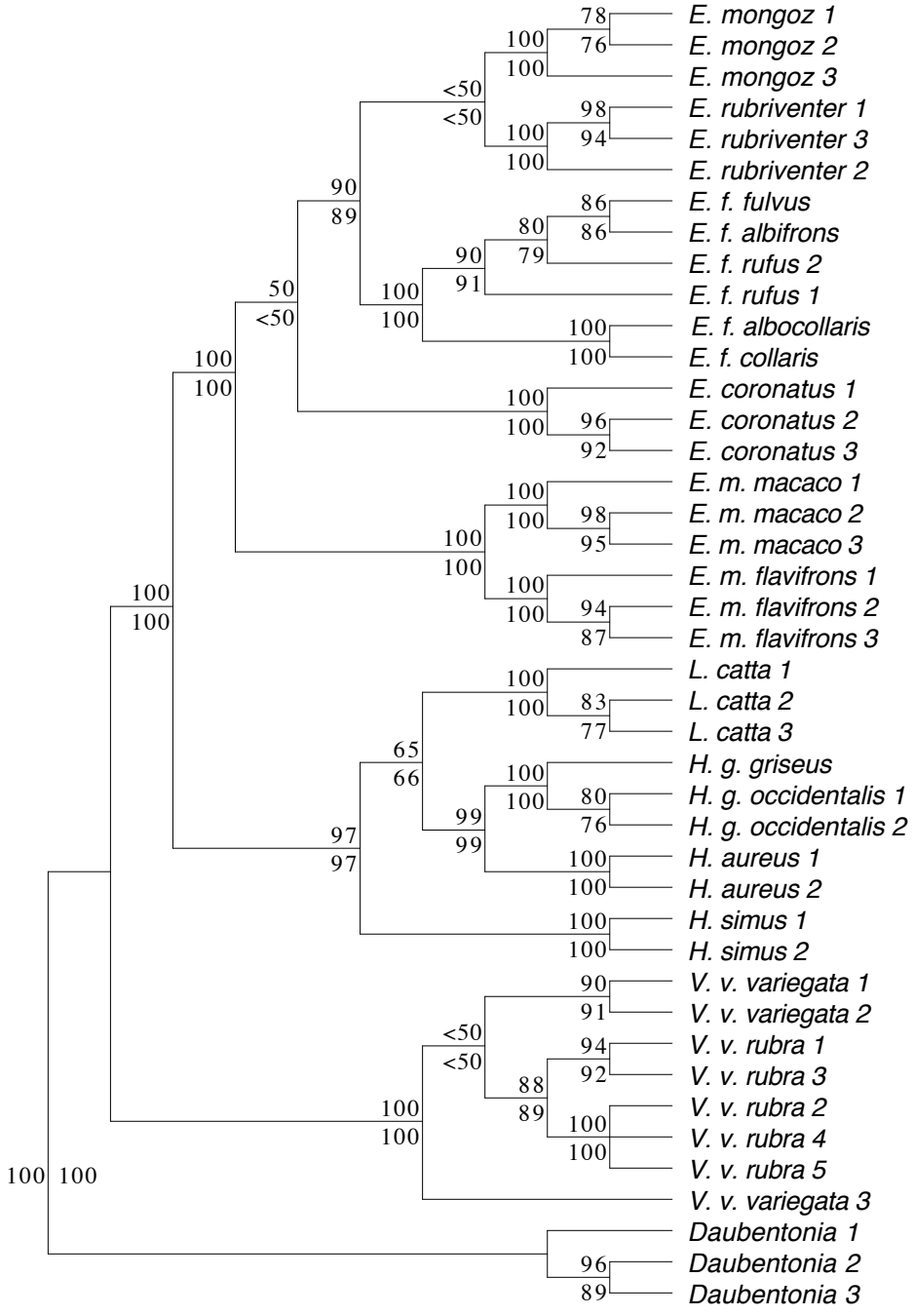

Fig. 5.3 Maximum parsimony tree with bootstrap values (as percentages, above nodes) obtained in 2500 replicates and with jackknife values (below nodes) from 2500

to *Eulemur*. *Varecia* is the first genus to diverge from the family Lemuridae. The arrangements among those four clades are strongly supported by bootstrap (BP) and jackknife (JK) analyses (97–100%) using MP or NJ searches. The clades containing individuals of one species always have complete BP or JK support (100%).

The *E.m. macaco* clade and that of *E.m. flavifrons* is supported in all BP or JK replicates at 100%. In contrast, clades containing *V.v. variegata* or *V.v. rubra* have weaker BP

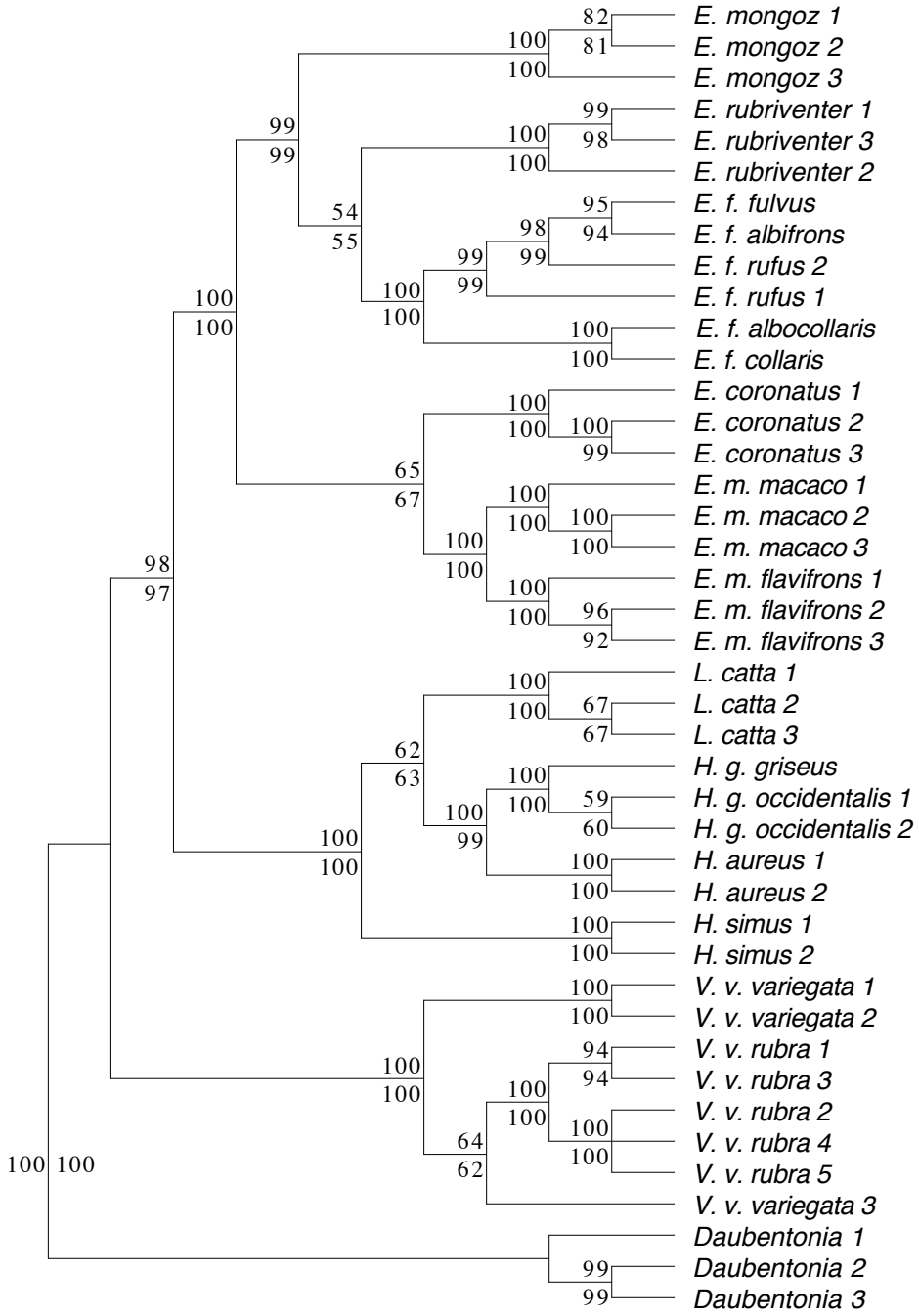

Fig. 5.4 Neighbor-joining tree with bootstrap values (as percentages, above nodes) obtained in 2500 replicates and jackknife values (below nodes) from 2500 iterations with 50% deletion using Kimura 2-parameter distance correction.

or JK support. In all MP and NJ analyses, the 3 *V.v. variegata* do not form a single clade. The branching arrangement of the *V.v. rubra* clade, 1 *V.v. variegata* individual and a clade containing the remaining 2 *V.v. variegata* individuals differs in MP and NJ analyses. However, both topologies have only poor BP or JK support (<64%). In MP and NJ analyses, *L. catta* groups within the genus *Hapalemur*. The sister-group relationship of *L. catta* with *H. griseus*/*H. aureus* is weakly supported with BP or JK values between 62 and 66%, making *H. simus* the first offshoot of the *Lemur*/*Hapalemur* clade.

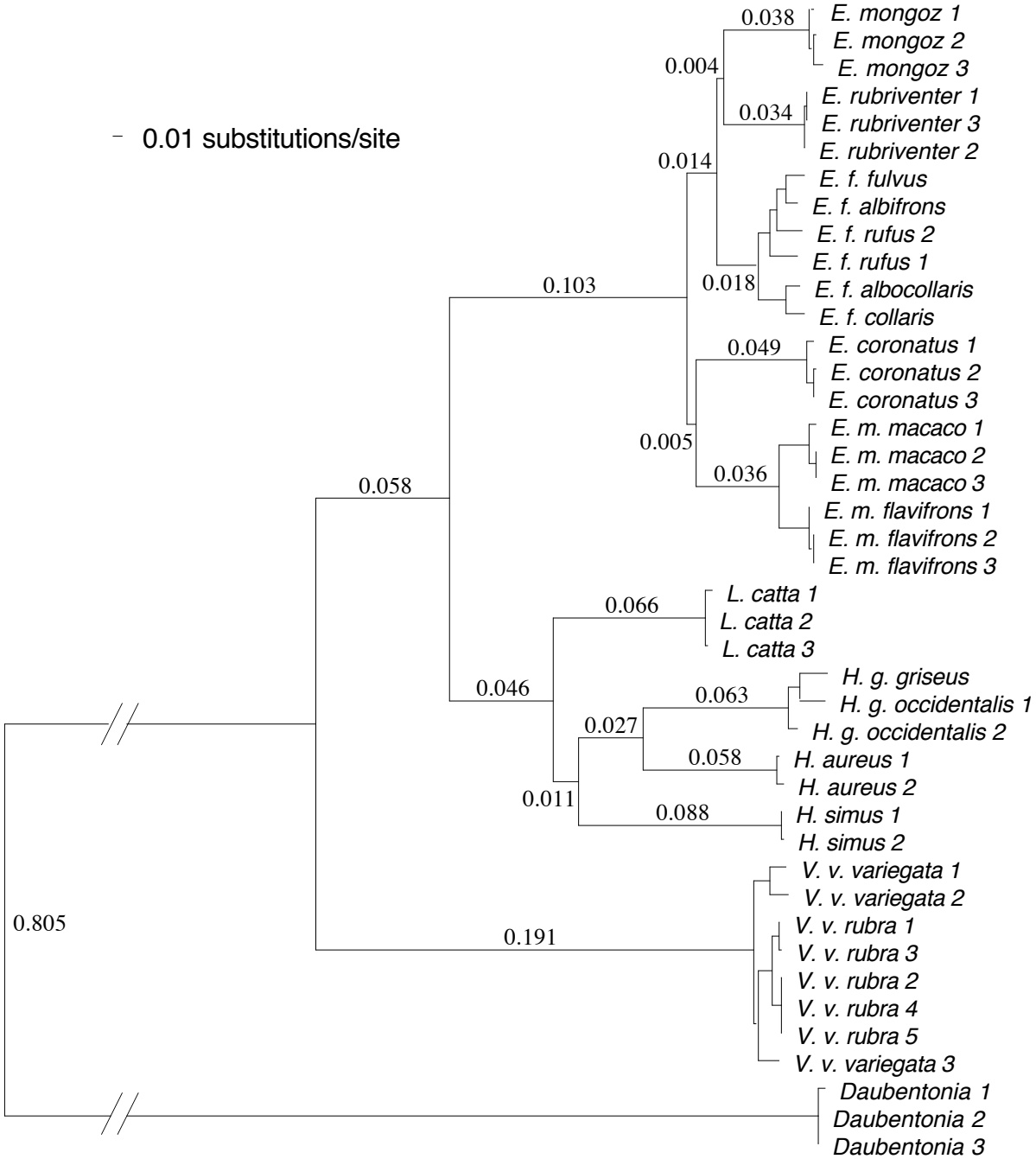

Fig. 5.5 Maximum likelihood phylogram with proportional branch lengths (values provided on each branch). // Indicates artificially shortened branch to fit page format.

The branching order of the five *Eulemur* species differs in MP and NJ analyses: Both analyses unambiguously linked *E. fulvus*, *E. mongoz*, and *E. rubriventer* with BP or JK supports of 89–99%. However, in NJ analyses, *E. fulvus* and *E. rubriventer* form a subclade, whereas in the MP tree, *E. mongoz* and *E. rubriventer* group together. However, in MP and NJ analyses there is virtually no BP or JK support for those arrangements (<55%). In NJ analyses, *E. coronatus* and *E. macaco* form a subclade which is sister to a second subclade containing the other three *Eulemur* species (BP/JK=65/67%). In contrast, MP analyses place *E. coronatus* as sister to the *E. fulvus*/*E. mongoz*/*E. rubriventer* clade, leaving *E. macaco* as the first species of *Eulemur* to branch away (BP/JK=50/<50%).

A final maximum likelihood tree score (–ln likelihood = 13237.77) was obtained with a transition/transversion ratio of 9.10 (kappa = 19.54) and gamma shape parameter of 0.23. The phylogram presented in Figure 5.5 maintains branch lengths proportional to the number of changes. The phylogenetic relationships among clades are very similar to those obtained from the analyses presented in Figures 5.3 and 5.4. In contrast to maximum parsimony and neighbor-joining analyses, *L. catta* is sister to a clade formed by all three *Hapalemur* species. However, the branch separating *L. catta* from the monophyletic *Hapalemur* remains minimal relative to the branch length which defines the *Hapalemur*/*Lemur* clade.

Absolute pairwise distances range from a maximum of 539–562 bp between *Daubentonia* and the ingroup to between 0 and 427 bp within the family Lemuridae (Table 5.3). Examination of absolute pairwise distances within the family Lemuridae reveals five levels of differentiation (Fig. 5.6, Table 5.3). The divergences between the three *Hapalemur* species are higher (218–290 bp) than between the five *Eulemur* species (140–199 bp). Pairwise distances between *L. catta* and each of the three *Hapalemur* species (241–274 bp) clearly lie in the range of comparisons between *Hapalemur* species. Between other genera of the family Lemuridae, genetic distances are in the range of 318–427 bp. As expected, pairwise comparisons between subspecies (39–89 bp) are generally higher than within a monotypic clade (0–17 bp), excepting the relatively high values for comparisons within *V.v. variegata* (35–57 bp).

The potential necessity for *a posteriori* weighting of the data to obtain better phylogenetic resolution was examined. One commonly applied method is differential weighting of substitutions resulting in transversions (TV) over those resulting in transitions (TI) (Miyamoto & Cracraft 1991; Hillis et al. 1996). Figure 5.6 gives the absolute numbers of TI

and TV for pairwise comparisons between different lemur taxa. The TV show no saturation within the Lemuridae. Pairwise distances considering TI only between the genera *Eulemur*, *Varecia* and *Lemur/Hapalemur* (260–348 bp) reach values of between-family comparisons (332–353 bp). However, TI do not show saturation within the genus *Eulemur* (128–180 bp) or within the *Lemur/Hapalemur* clade (205–231 bp). This indicates that the TI should give valuable information for questions within the genus *Eulemur* or within the *Lemur/Hapalemur* clade. The TV were compensatorily weighted by 6 and the TI by 1 according to the TI:TV ratio of the dataset (Hillis et al. 1996). In contrast to the unweighted analyses, in the weighted maximum parsimony analyses, *Hapalemur* is monophyletic with *L. catta* as a basal member of this clade (BP/JK=72/73%, data not depicted). *E. fulvus*, *E. mongoz*, and *E. rubriventer* form a clade which is supported by BP/JK values of 71/69%. However, the arrangement of *E. rubriventer* as sister to a clade containing *E. fulvus* and *E. mongoz* has no BP or JK support (<50%). The grouping of *E. coronatus* and *E. macaco* as a sister clade to the other three *Eulemur* species is supported with 54 or 58% BP or JK.

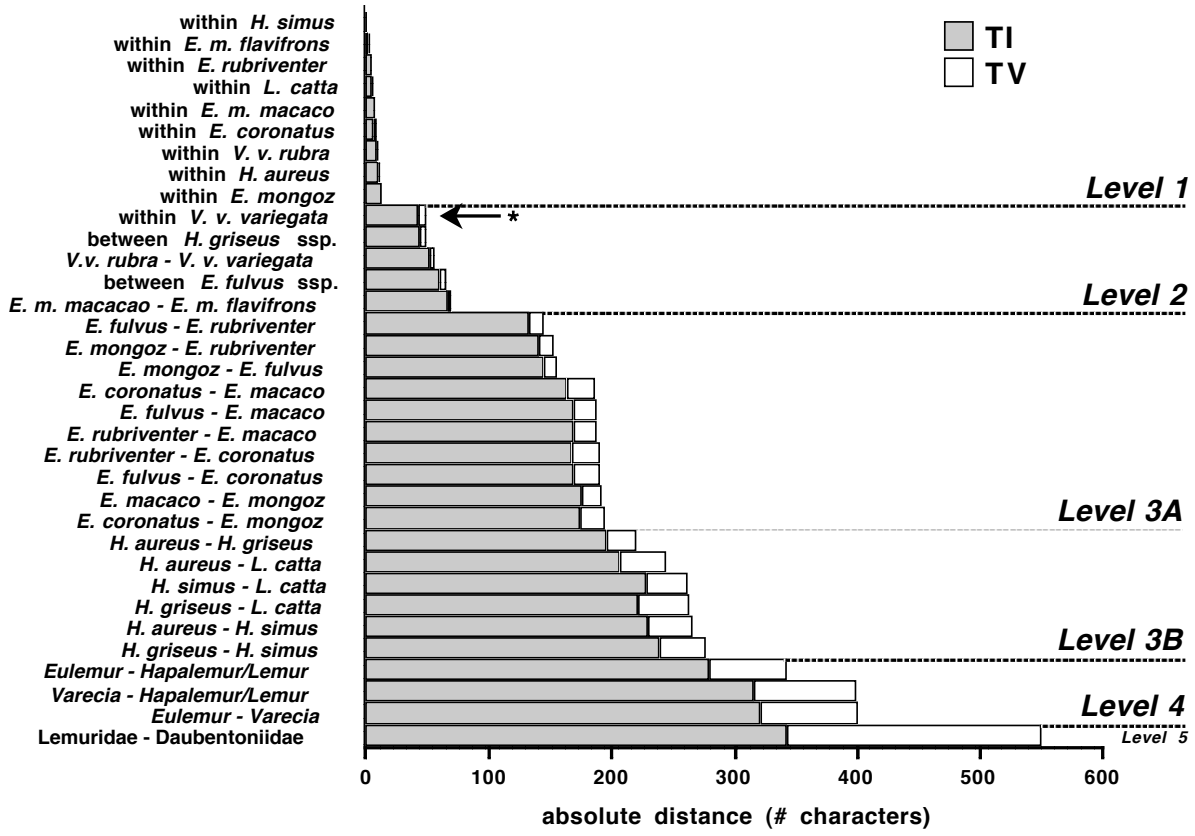

Fig. 5.6 Absolute pairwise distances (transversions and transitions) for five defined taxonomic levels (Level 1 = within species or subspecies, Level 2 = between subspecies, except within *V.v. variegata* (indicated by *), Level 3 = between species, Level 3A = between *Eulemur* species, Level 3B = between *Hapalemur* and *Lemur* species, Level 4 = between genera, Level 5 = between families). Each bar represents the average of all possible comparisons between individuals of the two taxa.

The use of *Daubentonia* as the specified outgroup in all the analyses presented might potentially be problematic due to the large phylogenetic distance between the lemur families Lemuridae and Daubentoniidae. However, the results of the entire analytical suite are not significantly different if *Varecia* is chosen as the outgroup, with *Daubentonia* deleted from the dataset (data not depicted). *Hapalemur* remains paraphyletic with respect to *L. catta*, as seen in maximum parsimony and neighbor-joining analyses which used *Daubentonia* as the outgroup. Rooting with *Varecia* does decrease the BP/JK support in maximum parsimony analyses for grouping *E. fulvus, E. mongoz*, and *E. rubriventer* (55/56%) from values obtained when rooted by *Daubentonia* (90/89%). Maximum parsimony and neighbor-joining analyses using *Varecia* as outgroup place *E. coronatus* and *E. macaco* together. This arrangement is in agreement with maximum likelihood and neighbor-joining analyses using *Daubentonia* as outgroup.

Maximum parsimony and neighbor-joining analyses with only the five *Eulemur* species, using *L. catta* as the outgroup, still fail to resolve relationships among *E. fulvus*, *E. mongoz* and *E. rubriventer* or between *E. coronatus*, *E. macaco* and the subclade containing *E. fulvus*, *E. mongoz* and *E. rubriventer* (data not depicted). Again, the subclade of *E. fulvus*, *E. mongoz* and *E. rubriventer* has good BP/JK support (84–95%).

Table 5.3 Kimura 2-parameter distance (above the diagonal) and absolute distance (under the diagonal) matrices derived from the mtDNA sequence data set.

	Em 1	*Em 2*	*Em 3*	*Ec 1*	*Ec 2*	*Ec 3*	*Er 1*	*Er 2*	*Er 3*	*Emm 1*	*Emm2*	*Emm3*	*Emf 1*	*Emf 2*	*Emf 3*	*Eff*	*Efr 1*	*Efr 2*	*Efa*	*Efc*	*Efa*
E. mongoz 1	-	0.004	0.007	0.087	0.088	0.087	0.068	0.067	0.069	0.089	0.089	0.089	0.086	0.086	0.086	0.069	0.069	0.068	0.068	0.070	0.065
E. mongoz 2	9	-	0.007	0.089	0.090	0.088	0.069	0.068	0.069	0.089	0.088	0.088	0.087	0.087	0.087	0.070	0.070	0.070	0.070	0.072	0.068
E. mongoz 3	16	17	-	0.090	0.090	0.089	0.069	0.069	0.070	0.089	0.089	0.089	0.086	0.086	0.086	0.072	0.070	0.069	0.072	0.074	0.066
E. coronatus 1	192	197	198	-	0.006	0.005	0.086	0.087	0.087	0.084	0.085	0.085	0.084	0.084	0.084	0.085	0.085	0.088	0.089	0.089	0.085
E. coronatus 2	195	198	199	14	-	0.001	0.086	0.087	0.086	0.086	0.086	0.086	0.084	0.085	0.085	0.084	0.085	0.088	0.090	0.089	0.084
E. coronatus 3	192	195	196	11	3	-	0.085	0.086	0.086	0.085	0.085	0.085	0.083	0.084	0.084	0.084	0.084	0.088	0.090	0.088	0.084
E. rubriventer 1	153	154	155	191	190	189	-	0.003	0.000	0.083	0.083	0.083	0.088	0.088	0.088	0.067	0.064	0.064	0.065	0.068	0.063
E. rubriventer 2	151	152	155	193	192	191	6	-	0.003	0.083	0.083	0.083	0.087	0.087	0.087	0.065	0.062	0.062	0.063	0.066	0.063
E. rubriventer 3	154	155	156	192	191	190	1	7	-	0.084	0.083	0.083	0.088	0.089	0.089	0.066	0.064	0.065	0.065	0.068	0.063
E. m. macaco 1	196	195	196	187	191	188	184	184	185	-	0.005	0.005	0.030	0.031	0.031	0.089	0.087	0.087	0.082	0.083	0.084
E. m. macaco 2	195	194	195	189	191	188	183	183	184	12	-	0	0.029	0.030	0.030	0.088	0.087	0.087	0.083	0.084	0.084
E. m. macaco 3	195	194	195	189	191	188	183	183	184	12	0	-	0.029	0.030	0.030	0.088	0.087	0.087	0.083	0.084	0.084
E. m. flavifrons 1	190	191	190	186	187	184	194	192	195	70	68	68	-	0.002	0.002	0.089	0.085	0.085	0.082	0.084	0.086
E. m. flavifrons 2	190	191	190	187	189	186	195	193	196	72	70	70	4	-	0	0.089	0.086	0.085	0.083	0.085	0.086
E. m. flavifrons 3	190	191	190	187	189	186	195	193	196	72	70	70	4	0	-	0.089	0.086	0.085	0.083	0.085	0.086
E. f. fulvus	154	157	160	188	187	186	150	146	149	195	194	194	195	196	196	-	0.024	0.021	0.037	0.039	0.013
E. f. rufus 1	154	157	156	188	187	186	144	140	145	192	191	191	188	189	189	57	-	0.026	0.032	0.035	0.023
E. f. rufus 2	152	157	154	194	195	194	145	141	146	192	193	193	188	189	189	50	60	-	0.037	0.037	0.018
E. f. albocollaris	152	157	160	196	199	198	146	142	147	181	184	184	182	183	183	86	75	85	-	0.017	0.035
E. f. collaris	157	162	165	196	196	195	152	148	153	184	187	187	187	188	188	89	80	86	39	-	0.035
E. f. albifrons	147	152	149	188	187	186	143	141	142	186	185	185	190	191	191	31	54	43	81	82	-
L. catta 1	330	333	336	340	342	341	341	338	340	339	342	342	333	336	336	327	325	327	318	326	321
L. catta 2	332	335	338	340	342	341	339	336	338	339	342	342	333	336	336	329	327	329	318	326	323
L. catta 3	329	332	335	337	339	338	338	335	337	338	341	341	332	335	335	328	326	328	319	327	322
H. g. griseus	348	349	351	366	370	367	353	348	352	353	357	357	359	360	360	336	342	347	335	341	336
H. g. occidentalis 1	360	363	359	375	378	375	362	357	361	356	360	360	356	359	359	352	357	360	344	358	351
H. g. occidentalis 2	354	359	354	369	372	369	354	349	353	350	352	352	344	347	347	339	343	345	330	340	336
H. aureus 1	337	341	344	360	363	360	349	344	348	344	345	345	325	328	328	335	339	337	327	329	329
H. aureus 2	340	346	347	359	362	359	354	349	353	349	350	350	330	333	333	336	342	340	330	334	332
H. simus 1	344	349	350	350	356	353	343	340	342	348	351	351	334	335	335	337	347	337	324	337	336
H. simus 2	345	350	351	351	357	354	343	340	342	349	352	352	335	336	336	338	348	338	325	338	337
V. v. variegata 1	401	403	404	418	414	415	419	415	418	400	402	402	393	394	394	410	402	413	385	387	408
V. v. variegata 2	403	403	408	426	426	425	427	423	426	406	408	408	403	404	404	410	404	413	391	389	410
V. v. variegata 3	405	405	408	418	416	417	413	409	412	401	403	403	400	401	401	401	396	409	383	388	402
V. v. rubra 1	392	394	395	414	410	411	414	410	413	403	403	403	392	393	393	395	393	402	376	383	395
V. v. rubra 2	397	399	400	411	409	410	409	407	408	402	402	402	395	396	396	398	394	403	377	384	398
V. v. rubra 3	394	394	397	412	410	411	414	410	413	401	401	401	394	395	395	395	393	404	378	385	397
V. v. rubra 4	397	399	400	411	409	410	409	407	408	402	402	402	395	396	396	398	394	403	377	384	398
V. v. rubra 5	397	399	400	411	409	410	409	407	408	402	402	402	395	396	396	398	394	403	377	384	398
Daubentonia 1	557	553	559	552	554	553	552	550	551	544	549	549	554	553	553	550	551	551	550	550	555
Daubentonia 2 [a]	553	549	555	548	550	549	548	546	547	539	544	544	549	548	548	546	547	547	546	546	551
Daubentonia 3 [a]	553	549	555	548	550	549	548	546	547	539	544	544	549	548	548	546	547	547	546	546	551

	Lc 1	*Lc 2*	*Lc 3*	*Hgg*	*Hgo 1*	*Hgo 2*	*Ha 1*	*Ha 2*	*Hs 1*	*Hs 2*	*Vvv 1*	*Vvv 2*	*Vvv 3*	*Vvr 1*	*Vvr 2*	*Vvr 3*	*Vvr 4*	*Vvr 5*	*D 1*	*D 2*	*D 3*
E. mongoz 1	0.158	0.159	0.157	0.168	0.175	0.171	0.162	0.163	0.165	0.166	0.198	0.199	0.200	0.192	0.195	0.193	0.195	0.195	0.288	0.289	0.289
E. mongoz 2	0.159	0.161	0.159	0.168	0.176	0.174	0.164	0.167	0.168	0.169	0.199	0.199	0.200	0.193	0.196	0.193	0.196	0.196	0.286	0.286	0.286
E. mongoz 3	0.161	0.162	0.161	0.170	0.174	0.171	0.166	0.167	0.169	0.169	0.200	0.202	0.202	0.194	0.197	0.195	0.197	0.197	0.290	0.291	0.291
E. coronatus 1	0.163	0.163	0.161	0.178	0.183	0.180	0.174	0.174	0.169	0.169	0.208	0.212	0.207	0.205	0.203	0.204	0.203	0.203	0.285	0.285	0.285
E. coronatus 2	0.164	0.164	0.163	0.180	0.185	0.181	0.176	0.176	0.172	0.173	0.205	0.212	0.206	0.203	0.202	0.203	0.202	0.202	0.286	0.287	0.287
E. coronatus 3	0.164	0.164	0.162	0.179	0.183	0.180	0.175	0.174	0.170	0.171	0.206	0.212	0.207	0.203	0.203	0.203	0.203	0.203	0.285	0.286	0.286
E. rubriventer 1	0.164	0.163	0.163	0.171	0.176	0.171	0.169	0.171	0.165	0.165	0.209	0.213	0.205	0.205	0.203	0.205	0.203	0.203	0.285	0.286	0.286
E. rubriventer 2	0.163	0.161	0.161	0.168	0.173	0.169	0.166	0.169	0.164	0.163	0.206	0.211	0.202	0.203	0.201	0.203	0.201	0.201	0.284	0.285	0.285
E. rubriventer 3	0.164	0.163	0.162	0.170	0.175	0.171	0.168	0.171	0.165	0.165	0.208	0.213	0.204	0.205	0.202	0.205	0.202	0.202	0.285	0.285	0.285
E. m. macaco 1	0.163	0.163	0.162	0.171	0.172	0.169	0.166	0.168	0.168	0.168	0.197	0.200	0.197	0.199	0.198	0.197	0.198	0.198	0.279	0.279	0.279
E. m. macaco 2	0.164	0.164	0.164	0.173	0.175	0.170	0.166	0.169	0.169	0.170	0.198	0.202	0.198	0.199	0.198	0.197	0.198	0.198	0.283	0.283	0.283
E. m. macaco 3	0.164	0.164	0.164	0.173	0.175	0.170	0.166	0.169	0.169	0.170	0.198	0.202	0.198	0.199	0.198	0.197	0.198	0.198	0.283	0.283	0.283
E. m. flavifrons 1	0.159	0.159	0.159	0.174	0.172	0.165	0.155	0.158	0.160	0.160	0.193	0.199	0.197	0.192	0.194	0.193	0.194	0.194	0.286	0.286	0.286
E. m. flavifrons 2	0.161	0.161	0.160	0.175	0.174	0.167	0.156	0.159	0.160	0.161	0.193	0.199	0.197	0.193	0.194	0.194	0.194	0.194	0.285	0.285	0.285
E. m. flavifrons 3	0.161	0.161	0.160	0.175	0.174	0.167	0.156	0.159	0.160	0.161	0.193	0.199	0.197	0.193	0.194	0.194	0.194	0.194	0.285	0.285	0.285
E. f. fulvus	0.156	0.157	0.157	0.161	0.170	0.163	0.161	0.161	0.162	0.162	0.203	0.203	0.198	0.194	0.196	0.194	0.196	0.196	0.284	0.284	0.284
E. f. rufus 1	0.155	0.156	0.156	0.165	0.173	0.165	0.163	0.165	0.167	0.168	0.199	0.200	0.195	0.193	0.194	0.193	0.194	0.194	0.284	0.285	0.285
E. f. rufus 2	0.156	0.157	0.157	0.167	0.174	0.166	0.162	0.163	0.161	0.162	0.205	0.205	0.202	0.198	0.199	0.199	0.199	0.199	0.284	0.285	0.285
E. f. albocollaris	0.151	0.151	0.152	0.161	0.165	0.158	0.156	0.158	0.154	0.155	0.189	0.192	0.187	0.183	0.184	0.184	0.184	0.184	0.284	0.285	0.285
E. f. collaris	0.156	0.156	0.156	0.164	0.173	0.163	0.157	0.160	0.162	0.162	0.190	0.191	0.190	0.187	0.188	0.188	0.188	0.188	0.284	0.284	0.284
E. f. albifrons	0.153	0.154	0.153	0.161	0.169	0.161	0.157	0.159	0.161	0.162	0.202	0.203	0.198	0.194	0.196	0.195	0.196	0.196	0.287	0.288	0.288
L. catta 1	-	0.003	0.003	0.122	0.128	0.121	0.112	0.114	0.123	0.123	0.184	0.179	0.181	0.181	0.181	0.179	0.181	0.181	0.281	0.285	0.285
L. catta 2	6	-	0.001	0.122	0.126	0.119	0.113	0.115	0.122	0.123	0.183	0.179	0.180	0.179	0.180	0.178	0.180	0.180	0.281	0.284	0.284
L. catta 3	7	3	-	0.121	0.128	0.121	0.113	0.115	0.122	0.122	0.185	0.181	0.182	0.181	0.182	0.180	0.182	0.182	0.279	0.282	0.282
H. g. griseus	261	261	260	-	0.022	0.021	0.100	0.100	0.127	0.127	0.210	0.205	0.206	0.207	0.207	0.205	0.207	0.207	0.285	0.284	0.284
H. g. occidentalis 1	274	270	273	52	-	0.021	0.105	0.104	0.137	0.138	0.213	0.208	0.209	0.210	0.211	0.209	0.211	0.211	0.291	0.291	0.291
H. g. occidentalis 2	259	257	260	50	48	-	0.101	0.101	0.127	0.127	0.210	0.207	0.207	0.207	0.207	0.207	0.207	0.207	0.290	0.289	0.289
H. aureus 1	241	243	244	218	228	220	-	0.005	0.126	0.126	0.208	0.204	0.205	0.202	0.204	0.201	0.204	0.204	0.291	0.292	0.292
H. aureus 2	245	247	248	218	226	220	12	-	0.125	0.125	0.206	0.204	0.204	0.201	0.203	0.200	0.203	0.203	0.289	0.290	0.290
H. simus 1	263	262	261	270	290	271	268	266	-	0.000	0.203	0.199	0.198	0.199	0.199	0.199	0.199	0.199	0.286	0.287	0.287
H. simus 2	264	263	262	271	291	272	269	267	1	-	0.203	0.200	0.199	0.199	0.200	0.199	0.200	0.200	0.287	0.288	0.288
V. v. variegata 1	377	376	379	422	426	421	417	414	409	410	-	0.015	0.024	0.024	0.026	0.025	0.026	0.026	0.288	0.291	0.291
V. v. variegata 2	370	369	372	414	419	416	412	411	404	405	35	-	0.024	0.027	0.028	0.026	0.028	0.028	0.290	0.293	0.293
V. v. variegata 3	373	371	374	416	420	417	413	412	403	404	56	57	-	0.019	0.020	0.018	0.020	0.020	0.292	0.295	0.295
V. v. rubra 1	372	370	373	416	422	417	408	407	403	404	57	62	44	-	0.006	0.002	0.006	0.006	0.290	0.293	0.293
V. v. rubra 2	373	371	374	417	423	416	411	410	404	405	60	65	47	15	-	0.007	0	0	0.291	0.294	0.294
V. v. rubra 3	370	368	371	414	420	417	406	405	403	404	58	61	42	4	17	-	0.007	0.007	0.289	0.292	0.292
V. v. rubra 4	373	371	374	417	423	416	411	410	404	405	60	65	47	15	0	17	-	0	0.291	0.294	0.294
V. v. rubra 5	373	371	374	417	423	416	411	410	404	405	60	65	47	15	0	17	0	-	0.291	0.294	0.294
Daubentonia 1	546	545	542	552	562	560	560	557	554	555	556	560	562	560	561	558	561	561	-	0.003	0.003
Daubentonia 2 [a]	546	545	542	545	555	553	556	553	550	551	556	559	562	559	560	557	560	560	7	-	0
Daubentonia 3 [a]	546	545	542	545	555	553	556	553	550	551	556	559	562	559	560	557	560	560	7	0	-

[a] 23 bp of COIII are missing

5.4 Discussion

Phylogenetic Relationships Among Genera

The results indicate that *Varecia* is deeply separated from all other lemurid taxa (Fig. 5.5). Pairwise distances between *Varecia* and other genera of the Lemuridae (368–427 bp) are the highest among all within-family comparisons (Fig. 5.6, Table 5.3). The molecular data thus strongly support generic status for *Varecia variegata*. The basal divergence between the genus *Varecia* and other lemurids in this study is in agreement with previously published data on karyotypes (Rumpler et al. 1989) and DNA sequences (Yoder & Irwin 1999) (Fig. 5.1). Thus, conflicting with analyses of morphological characters, that group *Varecia* either as sister to *Eulemur* (Groves & Eaglen 1988; Tattersall 1991; Yoder et al. 1996) or as sister to *Lemur* (Yoder 1994).

The mtDNA sequences resolve a monophyletic genus *Eulemur*, which forms the sister group to a clade containing *L. catta* and *Hapalemur* (Figs. 5.3 - 5.5). The maximum likelihood phylogram confirms the deep divergence of *Eulemur* from *Varecia* or *L. catta*/*Hapalemur* (Fig. 5.5). Pairwise distances (Fig. 5.6, Table 5.3) between *Eulemur* and *L. catta* (318–342 bp) are higher than among the five *Eulemur* species (140–199 bp) and lie in the range of between-genera comparisons (241–427 bp). As in *Varecia*, the sequencing data thus strongly support the classification of *Eulemur* as a genus distinct from *L. catta*. This is in disagreement with Tattersall (1991), who would prefer including *Eulemur* and *Varecia* once again within the genus *Lemur*.

Of special interest was the phylogenetic position of *L. catta*. This study suggests a close relationship between *L. catta* and *Hapalemur*, which has been supported before by analyses of morphological (Groves & Eaglen 1988), communicatory (Macedonia & Stanger 1994), chromosomal (Rumpler et al. 1989; Vezuli et al. 1997), and genetical (Jung et al. 1992; Crovella et al. 1993; Adkins & Honeycutt 1994; Crovella et al. 1995; Del Pero et al. 1995; Stanger-Hall & Cunningham 1998; Yoder & Irwin 1999) characters (Fig. 5.1). On the other hand, some data based on immunology (Sarich & Cronin 1976) or morphology (Tattersall & Schwartz 1974; Tattersall 1991; Yoder 1994; Groves & Trueman 1995; Yoder et al. 1996; Stanger-Hall 1997) fail to support a sister group relationship between *L. catta* and *Hapalemur*.

Paraphyletic* Lemur/Hapalemur *Clade

Interestingly, in maximum parsimony analyses *Hapalemur* is only monophyletic with respect to *L. catta* if transversions are weighted over transitions. If all characters are used, *L. catta* groups within the genus *Hapalemur* (Figs. 5.3 and 5.4). However, both arrangements are only weakly supported by bootstrap or jackknife analyses (62–73%). The results are outgroup-insensitive with regard to relationships within the *Lemur*/*Hapalemur* clade. In the maximum likelihood phylogram a very short branch separates *L. catta* from the clade formed by all three *Hapalemur* species (Fig. 5.5). Pairwise distances (Fig. 5.6, Table 5.3) between *L. catta* and each of the three *Hapalemur* species (241–274 bp) clearly lie in the range of comparisons between *Hapalemur* species (218–290 bp). All genetic distances among *Lemur* and *Hapalemur* species are slightly higher than between *Eulemur* species (140–199 bp), but they do not attain the range observed between other genera of the family Lemuridae (318–427 bp).

In most previous studies, *H. simus* has been placed as sister to other *Hapalemur* species (Sarich & Cronin 1976; Groves & Eaglen 1988; Rumpler et al. 1991; Yoder 1994; Crovella et al. 1995). While communication characters (Macedonia & Stanger 1994) support the phylogenetic arrangement of *L. catta* within *Hapalemur*, chromosome painting (Vezuli et al. 1997) was unable to differentiate among *H. simus*, *L. catta* and *H. aureus*/*H. griseus*. Many studies note features in which *L. catta* appears more similar to *Hapalemur* than to other species originally included in the genus *Lemur* (see Groves & Eaglen 1988 or Simons & Rumpler 1988 for review). While *Eulemur*, *Lemur* and *Varecia* are long-snouted, *Hapalemur* is short-snouted (Groves 1989). If the relatively long-faced *L. catta* really groups within the short-faced *Hapalemur*, then either *H. simus* and the common ancestor of the *H. aureus*/*H. griseus* clade evolved foreshortened faces independently, or the long face of *L. catta* is a reversal to the primitive state (Macedonia & Stanger 1994). In strepsirrhines, variation between taxa in facial length is pronounced. The skull differences, in for example the orbit/muzzle relationships, suggest that facial elongation may have occurred independently in *L. catta* and *Eulemur* (Groves 1989).

If *L. catta* is, indeed, nested within the *Hapalemur* clade, a cladistic approach to classification would require either uniting *L. catta* and *Hapalemur* into one genus, or separating *H. simus* at the genus level. Based on tree topology and pairwise distances, the molecular data presented in this study would favour the former solution. On the other hand, Groves (1989) has

already stated that even if several synapomorphic features are shared by *L. catta* and *Hapalemur*, profound differences between them remain, and it is not productive to combine them taxonomically. In the past, Pocock (1917) and Tattersall and Schwartz (1974) regarded *H. simus* as sufficiently distinct to warrant generic separation under the name *Prolemur* (Gray 1871). From all this it follows that more comparative studies including *L. catta* and all three *Hapalemur* species are needed before a decision can be made.

Phylogenetic Relationships Among* Eulemur *Species

The branching order among *Eulemur* species was of particular interest. All analyses unambiguously linked *E. fulvus*, *E. mongoz*, and *E. rubriventer* into one clade with strong bootstrap or jackknife support (89–99%) (Figs. 5.3 and 5.4). Neighbor-joining and maximum parsimony analyses differ in the arrangement of *E. fulvus*, *E. mongoz* and *E. rubriventer* relative to one another and in the branching order of *E. coronatus*, *E. macaco* and the *E. fulvus*/*E. mongoz*/ *E. rubriventer* clade, which is not well supported by bootstrap or jackknife analyses (<67%). The short branch lengths of the maximum likelihood phylogram separating the *Eulemur* species confirm the results (Fig. 5.5). More proximal outgroup rooting, using *Varecia* or *L. catta* as the outgroup, does not enhance resolution among the five *Eulemur* species. When weighting the transversions by 6 and the transitions by 1, *E. fulvus*, *E. mongoz*, and *E. rubriventer* again form a clade.

Previously published phylogenetic studies have not recovered a clade with *E. fulvus*, *E. rubriventer* and *E. mongoz* apart from *E. macaco* and *E. coronatus*. Only two previous character sets, based on morphology, yield the same tree topology among all five *Eulemur* species (Tattersall 1991; Groves & Trueman 1995) (Fig. 5.1). Those two studies support the sister-group relationship between *E. rubriventer* and *E. mongoz* found in this study. Three phylogenetic studies support a sister-group relationship between *E. coronatus* and *E. rubriventer* (Crovella et al. 1993; Macedonia & Stanger 1994; Stanger-Hall 1997), and two studies group *E. fulvus* and *E. macaco* (Eaglen 1983; Macedonia & Stanger 1994). Both arrangements are incompatible with the present findings. Those results do not exclude the possibility that *E. macaco* (Crovella et al. 1993; 1995) or *E. coronatus* (Tattersall 1991; Groves & Trueman 1995) diverged first among *Eulemur* species, as found in some other studies.

Subspecies Level Considerations

For *E. macaco*, two subspecies have been described, both of which have been included in the data set. Tree topology clearly resolves those two subspecies (Figs. 5.3 - 5.5). In all analyses, the clades of *E.m. macaco* and *E.m. flavifrons* have complete bootstrap and jackknife support (100%). Pairwise distances (Fig. 5.6, Table 5.3) between *E.m. macaco* and *E.m. flavifrons* (68–72 bp) are in the same range as between *E. fulvus* subspecies (29–90 bp, see Chapter 3). This study thus supports the subspecies status of *E.m. macaco* and *E.m. flavifrons*.

All analyses resolve the five *V.v. rubra* individuals into a monophyletic clade. However, the three *V.v. variegata* individuals fail to form a single subclade. The genetic distance between *V.v. variegata* and *V.v. rubra* (42–65 bp) is, as expected, of the same order as between *Eulemur* subspecies (29–90 bp). However, the fairly high genetic distances among *V.v. variegata individuals* (35–57 bp), together with tree topology, indicates more phylogenetic structure in this taxon than current taxonomy would depict.

Considerable variation in pelage pattern and colour occurs within *V. variegata*. Hill (1953) recognised four subspecies in the single species, all of which were confirmed by Petter, Albignac, and Rumpler (1977). Today, however, only two subspecies — a northern one with red-and-black coat coloration (*V.v. rubra*) and a southern one with black-and-white pelage (*V.v. variegata*) — are generally recognised (Tattersall 1982; Groves 1989; Harcourt & Thornback 1990). The latter, however, shows at least four distinct coat patterns, and better knowledge of the distributions of these varieties might ultimately suggest their recognition as distinct subspecies (Tattersall 1982; Groves 1989). The possibility of diagnosing the structure in wild *V.v. variegata* populations is limited, as the samples are from captive animals without adequate locality data; however, this level of phylogenetic structure is equivalent to subspecific differentiation in related lemurid genera.

Concluding Remarks

Molecular data presented here strongly support the generic status of *Varecia* and *Eulemur*, a sister-group relationship between *Eulemur* and *Lemur*/*Hapalemur*, and a basal divergence of *Varecia* among Lemuridae. Subspecific status for *E.m. macaco* and *E.m. flavifrons* and for *V.v. rubra* and *V.v. variegata* is supported by these results. However, considerable genetic differentiation among the individual *V.v. variegata* was found, which indicates that more detailed research on *V.v. variegata* might reveal more subspecies.

H. griseus and *H. aureus* form a clade with strong support, but the sequence data do not yield clear resolution of the trichotomy involving *H. simus*, *H. aureus*/*H. griseus* and *L. catta*. There is obviously a potential taxonomic problem regarding paraphyly of these two genera which needs further investigation. These data do not clarify the specific relationships among the *Hapalemur* species and *L. catta*; however, it is clear that *Lemur* and *Hapalemur* are closely related and represent a monophyletic unit.

Like data in previous investigations, the data presented here failed to yield clear resolution of phylogenetic relationships among the five *Eulemur* species. It can generally be excluded that this is due to the genes chosen for sequencing. Other phylogenetic studies on reptiles (Forstner et al. 1998) and primates (Wang et al. 1997; Pastorini et al. 1998), including lemurs (see other Chapters), using the same genes clearly resolved the species within a genus. It can therefore be concluded that evolution within the genus *Eulemur* was initiated in two very rapid steps: First *E. coronatus* (North) and *E. macaco* (North) separated from a trunk, then the latter split into *E. mongoz* (Northwest), *E. rubriventer* (East) and *E. fulvus*. *E. fulvus* finally radiated step-by-step throughout Madagascar (see Chapter 3). Based on the molecular phylogeny and geographic distribution, it would seem that radiation from *E. fulvus* started in the Southeast. This is the only region where the distribution of *E. fulvus* (*E.f. collaris* and *E.f. albocollaris*) does not overlap with the distributions of the other four *Eulemur* species. It is therefore possible that *Eulemur* first radiated from North to South, and later *E. fulvus* back from South to North.

6. Family Cheirogaleidae

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J. Pastorini, R.D. Martin, P. Ehresmann, E. Zimmermann, M.R.J. Forstner (resubmitted)
Molecular phylogeny of the lemur family Cheirogaleidae (Primates) based on mitochondrial DNA sequences.
Molecular Phylogenetics and Evolution.

6.1 Introduction

The Cheirogaleidae, one of five endemic families of Madagascar lemurs, is currently classified into five genera (*Allocebus*, *Cheirogaleus*, *Microcebus*, *Mirza*, *Phaner*) and at least nine species. The fork-marked lemur, *Phaner furcifer* (Blainville 1839), was assigned to its own genus by Gray in 1870 (Tattersall 1982) and some authors even classify it as the only member of a distinct subfamily Phanerinae (Rumpler & Albignac 1973; Petter et al. 1977). Today, *Phaner* is widely but discontinuously distributed through Madagascar, including at least four different subspecies (Groves & Tattersall 1991). The genus *Cheirogaleus* (Geoffroy 1812) currently contains two species: the greater dwarf lemur *C. major* with at least two subspecies, and the fat-tailed dwarf lemur *C. medius* (Tattersall 1982). The hairy-eared dwarf lemur, *Allocebus trichotis*, was originally described as *Cheirogaleus trichotis* by Günther in 1875, but was assigned to its own genus by Petter-Rousseaux and Petter in 1967 (Tattersall 1982). Despite its early discovery, for more than a century knowledge of *A. trichotis* was confined to five museum specimens. Recently, two populations have been found in northeastern and eastern Madagascar (Meier & Albignac 1991; Rakotoarison et al. 1996). Coquerel's dwarf lemur, *Mirza coquereli*, was first described as a member of *Cheirogaleus* by Grandidier (1867), but Schlegel and Pollen (1868) allocated it to *Microcebus* while Gray (1870) assigned it to its own genus *Mirza* (Tattersall 1982). Some authors continue to include it within the genus *Microcebus* (Petter et al. 1977; Napier & Napier 1985; Rowe 1996).

The genus *Microcebus* (Geoffroy 1828) at present includes four species. Until recently, only two forms were recognised: a grey long-eared form from western Madagascar (*M. murinus*; Miller 1777), and a brown short-eared form from the east (*M. rufus*; Lesson 1840) (Fig. 6.2). These two forms were long considered to be subspecies of the single species *M. murinus*, but clear evidence for their separation emerged (see Martin 1995 for review). In 1994, Schmid and Kappeler (1994) found a much smaller and more gracile species in the Forêt

de Kirindy in central western Madagascar and identified it as *M. myoxinus* (Peters 1852), the pygmy mouse lemur. Recently, a golden-brown mouse lemur was discovered in northwestern Madagascar and was described as *M. ravelobensis* (Zimmermann et al. 1998). Both *M. myoxinus* and *M. ravelobensis* occur sympatrically with *M. murinus*.

Little is known about evolutionary relationships within the family Cheirogaleidae. A comparative karyological study of the Cheirogaleidae revealed only two different karyotypes within the family, that of *Phaner* (2N=46) and that of the four other genera (2N=66) (Rumpler & Albignac 1973; Rumpler et al. 1995; Rumpler et al. 1998). Morphological studies indicate that *Phaner* possesses several derived character states distinguishing it from the common ancestor of *Microcebus* and *Cheirogaleus* (Tattersall & Schwartz 1974). By contrast, immunological data from albumin and transferrin group *Phaner* and *Cheirogaleus* together, while indicating that *Microcebus* is the earliest diverging genus of Cheirogaleidae (Sarich & Cronin 1976). Analyses of 125 morphological and behavioural characters opposed *Microcebus* to a sister clade including *Cheirogaleus* and *Mirza* (Yoder et al. 1996). A cladistic analysis of 25 morphological and behavioural characters identified *Microcebus* as sister group to a clade containing *Mirza* and *Phaner*, while *Cheirogaleus* was the most basal offshoot (Stanger-Hall 1997). However, *Allocebus* was not included in any of these studies. A comparative analysis of vocalisations suggested closer affinities of *Allocebus* to a clade containing *Cheirogaleus* and *Microcebus* than to *Phaner* (Rakotoarison et al. 1996). A cladistic analysis of 13 morphological and behavioural characters placed *Cheirogaleus* as a sister taxon to a clade including *Allocebus*, *Microcebus* and *Mirza*, with the later two genera forming a subclade (Stanger 1993). *Phaner* again seemed to represent the earliest offshoot among cheirogaleids. Analyses of highly repeated DNA yielded exactly the same branching order among cheirogaleid genera as that reported by Stanger (1993), but Crovella et al. (1995) concluded that the taxonomic position of *Allocebus* within the Cheirogaleidae required further confirmation. Prior to the present study, DNA sequences were available only for the three genera *Mirza*, *Microcebus* and *Cheirogaleus*: Analyses of sequence data for 1140 bp of the cytochrome b gene (Yoder et al. 1996), for the IRBP gene (Yoder 1997) and for 461 bp of the large ribosomal subunit (Stanger-Hall & Cunningham 1998) all indicated that *Mirza* and *Microcebus* constitute a sister group to *Cheirogaleus*.

Not much is known concerning phylogenetic relationships among the four *Microcebus* species currently recognised. RAPD analyses cluster *M. rufus* as sister group to a clade containing *M. murinus* and *M. myoxinus* (Tomiuk et al. 1998), while a cluster analysis of morphological characters places *M. rufus* as a sister taxon to a clade including *M. murinus* and *M. ravelobensis* (Zimmermann et al. 1998).

In the present study, a large fragment of mitochondrial DNA was sequenced and the data examined in an attempt to clarify nodal relationships among four genera within the Cheirogaleidae. Close attention was paid to the phylogenetic position of *Allocebus*, from which no DNA sequence data were previously available. An additional aim was to assess the generic status of *Mirza coquereli*. A further goal was to evaluate the taxonomic status of the recently discovered *M. ravelobensis* and to determine its phylogenetic position within the genus *Microcebus*. A final objective was to attempt species identification of two captive *Microcebus* of unknown origin. Previous successful resolution of other problematic taxa using this region of mtDNA (Forstner et al. 1995; Wang et al. 1997; Forstner et al. 1998; Pastorini et al. 1998) indicated that this fragment could resolve the phylogenetic relationships among cheirogaleid genera and among different species of *Microcebus*.

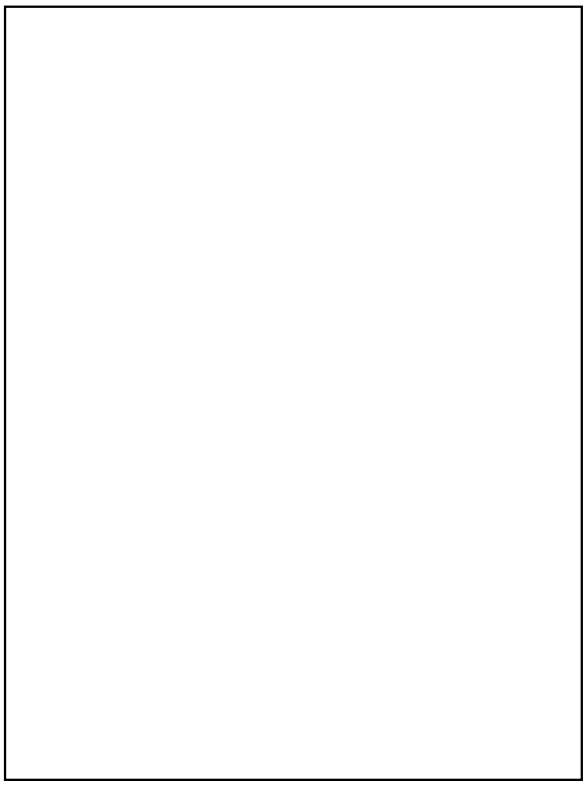


Fig. 6.1 *Microcebus rufus* from Parc Zoologique et Botanique de Tsimbazaza (July 1997).

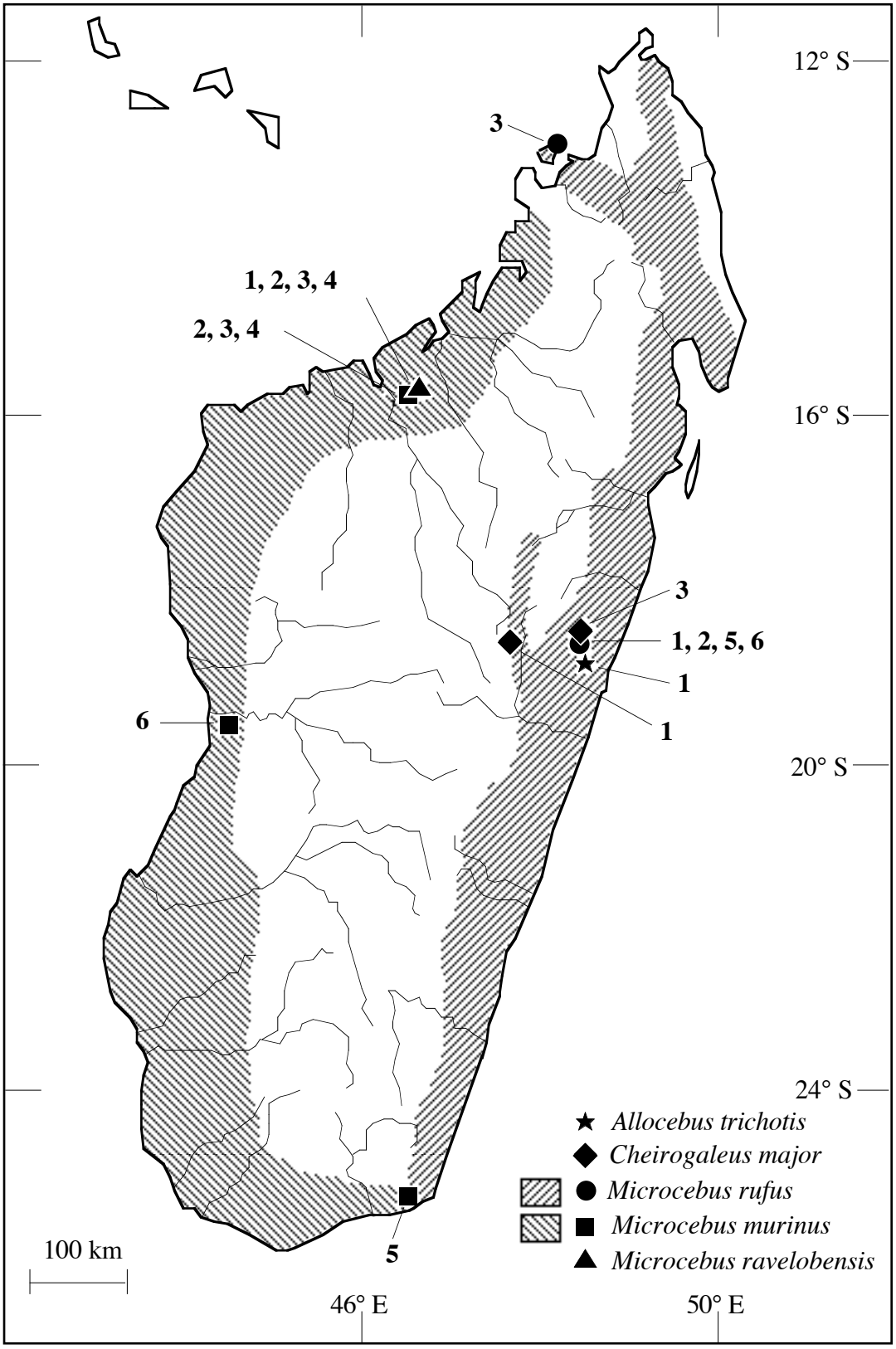


Fig. 6.2 Map of Madagascar showing approximate distribution of the two main *Microcebus* species (Tattersall 1982; Martin 1995). Symbols indicate individuals with exact locality data that were included in the present study.

6.2 Material

Samples were collected from 1 *A. trichotis*, 3 *C. major*, 3 *C. medius*, 6 *M. murinus*, 4 *M. ravelobensis*, 6 *M. rufus*, and 3 *Mirza coquereli*. Unfortunately, no samples from the fifth genus *Phaner* or from the fourth species *Microcebus myoxinus* were available. Three samples from *Daubentonia madagascariensis* were sequenced to serve as an outgroup. All samples utilised are listed in Table 6.1.

The records from 'Parc Zoologique et Botanique de Tsimbazaza' indicated that one *C. major* was captured at Mantasoa, while the second *C. major* and 2 *M. rufus* were from Andasibe (Perinet). All but one individual of *M. rufus* at the 'Tierärztliche Hochschule Hannover' analysed in this study also originated from Andasibe. One *M. rufus* analysed was captured on the island of Nosy Be. All 4 *M. ravelobensis* and 3 *M. murinus* sampled for this study were from Ampijoroa. One *M. murinus* sample originated from Kirindy and one from Mandena. One *M. murinus* and one *M. rufus* held at the 'Tierärztliche Hochschule Hannover' were of unknown origin. The single *A. trichotis* individual came from Vohidrazana. No locality data were available for the samples from 3 *C. medius*, 1 *C. major*, and 3 *Mirza coquereli*. All known sample localities are shown in Figure 6.2.

Table 6.1 Taxa, origin, identification numbers, and GenBank accession numbers for all individuals sequenced.

Taxon	Origin	ID #	GenBank #
Allocebus trichotis	Vohidrazana (East) [a]	JP349	AF224620
Cheirogaleus medius 1	unknown [b]	JP6, AIMUZ 8128	AF224614
Cheirogaleus medius 2	unknown [b]	JP70, AIMUZ 10095	AF224615
Cheirogaleus medius 3	unknown [c]	JP282	AF224616
Cheirogaleus major 1	Mantasoa (East) [d]	JP137	AF224617
Cheirogaleus major 2	unknown [d]	JP138	AF224618
Cheirogaleus major 3	Andasibe (East) [d]	JP118	AF224619
Microcebus murinus 1	unknown [e]	JP285	AF224624
Microcebus murinus 2	Ampijoroa (Northwest) [a]	JP288	AF224625
Microcebus murinus 3	Ampijoroa (Northwest) [a]	JP289	AF224626
Microcebus murinus 4	Ampijoroa (Northwest) [a]	JP292	AF224627
Microcebus murinus 5	Mandena (South) [a]	JP308	AF224628
Microcebus murinus 6	Kirindy (West) [a]	JP313	AF224629
Microcebus ravelobensis 1	Ampijoroa (Northwest) [a]	JP299	AF224630
Microcebus ravelobensis 2	Ampijoroa (Northwest) [a]	JP301	AF224631
Microcebus ravelobensis 3	Ampijoroa (Northwest) [a]	JP303	AF224632
Microcebus ravelobensis 4	Ampijoroa (Northwest) [a]	JP321	AF224633
Microcebus rufus 1	Andasibe (East) [d]	JP141	AF224634
Microcebus rufus 2	Andasibe (East) [d]	JP142	AF224635
Microcebus rufus 3	Nosy Be (North) [a]	JP309	AF224636
Microcebus rufus 4	unknown [e]	JP315	AF224637
Microcebus rufus 5	Andasibe (East) [e]	JP316	AF224638
Microcebus rufus 6	Andasibe (East) [e]	JP317	AF224639
Mirza coquereli 1	unknown [f]	JP268	AF224621
Mirza coquereli 2	unknown [f]	JP269	AF224622
Mirza coquereli 3	unknown [f]	JP270	AF224623
Daubentonia madagascariensis 1	Andratamarina (Northeast) [b]	JP7, AIMUZ 11902	AF224640
Daubentonia madagascariensis 2	Anjiamangirana (Northwest) [d]	JP119	AF224641
Daubentonia madagascariensis 3	Anjiamangirana (Northwest) [d]	JP120	AF224642

[a] wild-caught animals with verified origin, immediately released after capture and sampling (Tierärztliche Hochschule Hannover)

[b] specimen held at Anthropological Institute and Museum of the University of Zürich (AIMUZ), Switzerland

[c] held at Köln Zoo, Germany

[d] held at Parc Zoologique et Botanique de Tsimbazaza, Madagascar

[e] held at Tierärztliche Hochschule Hannover, Germany

[f] held at Duke University Primate Center, U.S.A.

6.3 Results

The new mtDNA sequences generated for the taxa examined have been deposited in GenBank (Table 6.1). Aligned sequences are available from the first author upon request. The nucleotide sequences span a total of 2379 base positions (bp). The analysed dataset consists of 3' end of the COIII gene (29 bp), the complete NADH-dehydrogenase subunits ND3 (348 bp), ND4L (297 bp) and ND4 (1378 bp) along with the glycine (73 bp), arginine (69 bp), histidine (70 bp), serine (65 bp), and part of leucine (47 bp) tRNA genes. The partition-homogeneity test showed no significant incongruence among those nine genes (P=0.99). The sequences obtained provided 962 parsimony-informative characters with a transition:transversion ratio of 3.9:1. A summary of the frequencies of invariant, parsimony uninformative, and informative characters along the segment sequenced is given in Table 6.2.

In the members of family Cheirogaleidae examined, the ND3 gene is terminated by 'TAA', whereas in *Daubentonia* 'TA' and in *Homo* only 'T' serve as the stop codons via polyadenylation. Cheirogaleids have an insertion of 2 or 3 bp between the ND3 gene and the $tRNA^{Arg}$. The mtDNA genomes of *Homo* or *Daubentonia* do not contain any untranslated bp between these genes. *Allocebus* has 4 additional bp, while other cheirogaleids and *Daubentonia* have one additional base position not present in *Homo* between $tRNA^{Arg}$ and ND4L. *Allocebus* has an insertion of 3 bp between the tRNAs histidine and serine that is not present in other lemurs or *Homo*. *Daubentonia* has a deletion of 6 bp, coding for two amino acids in the ND4 gene (positions 49 and 50, Leu and Phe in the human genome). All other insertions or deletions are limited to loops of tRNA genes. Table 6.2 indicates how many gaps are found in each gene for the lemurs analysed in this study (exclusive of *Homo* outgroup sequences). Finally, *C. medius* has a repetition 1 (8 bp) to 8 (64 bp) times of the A-stem of the $tRNA^{Leu}$ between the tRNAs for serine and leucine. The $tRNA^{Leu}$ beyond this repetitive sequence element retains

Table 6.2 Summary of sequence variation across the 29 lemurs examined.

Genes	All	COIII	ND3	ND4L [a]	ND4 [a]	tRNAs	Not translated
characters (nucleotides)	2379	29	348	297	1378	324	10
constant	1345	23	192	159	759	218	0
parsimony-uninformative	72	0	10	10	38	8	6
parsimony-informative	962	6	146	128	581	98	4
informative proportion	0.40	0.21	0.42	0.43	0.42	0.30	0.40
insertions/deletions	32	0	1	0	6	16	9

[a] ND4L and ND4 overlap for 7 bp

correct structure and anticodon. No other anomalies were seen and this insertion has been excluded from all analyses, tables or graphs presented in this paper.

The maximum parsimony heuristic search with all characters weighted equally results in four trees, each 2265 steps in length with a consistency index of 0.59 and a retention index of 0.85 (Fig. 6.3). The distance matrices constructed using Kimura 2-parameter corrections (Table 6.3) were analysed by neighbor-joining and reconstruct the same topology with respect to the

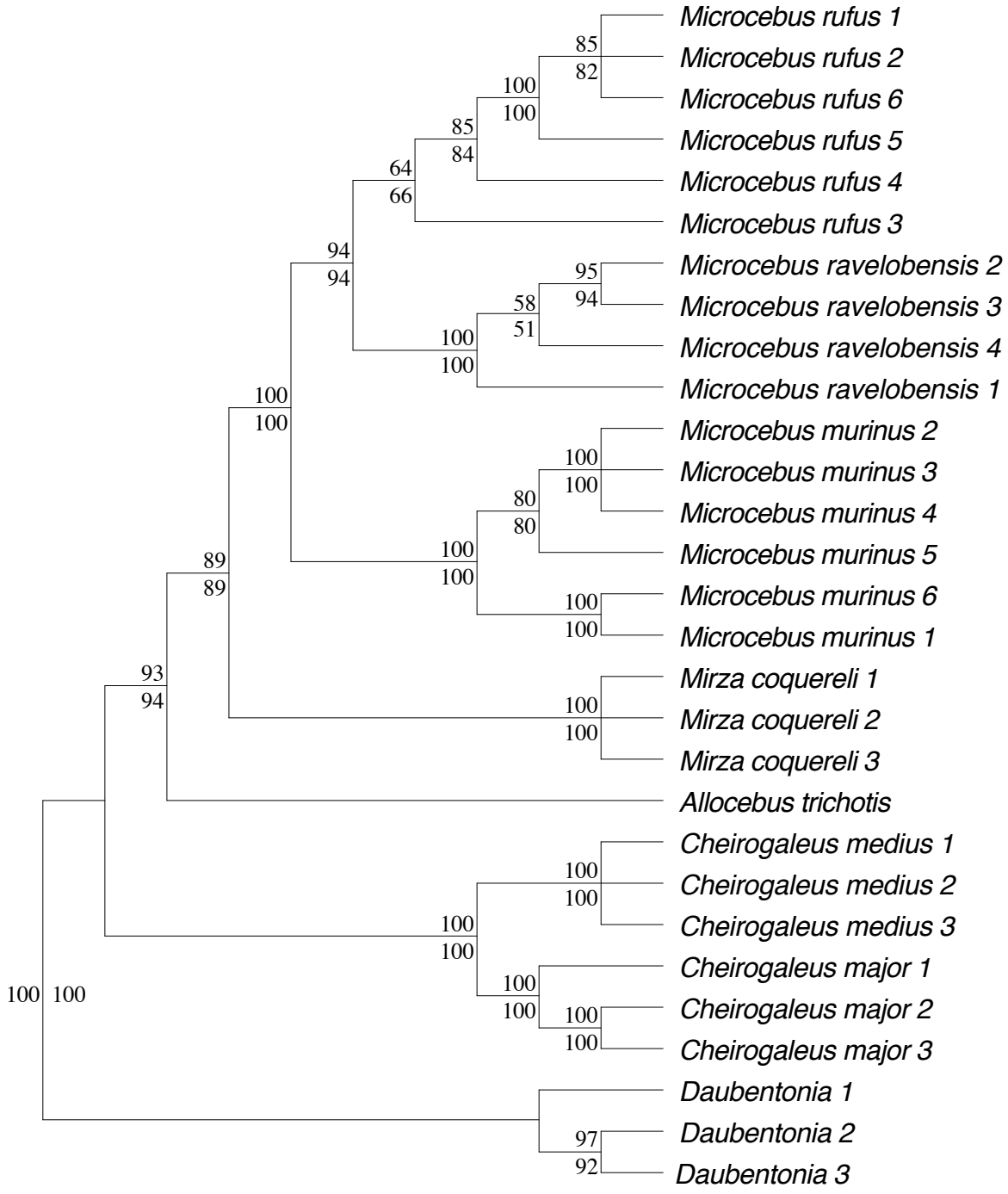


Fig. 6.3 Maximum parsimony tree with bootstrap values (as percentages, above nodes) obtained in 2500 replicates and with jackknife values (below nodes) from 2500 iterations with 50% deletion.

arrangement of species and genera (Fig. 6.4). The support values from bootstrap and jackknife analyses of 2500 replicates are in the same range as for maximum parsimony analyses.

The results of the maximum likelihood analysis are presented in Figure 6.5. The phylogram presented maintains branch lengths proportional to the number of changes. The phylogenetic relationships among clades are virtually identical to those from the other analyses presented in Figures 6.3 and 6.4. The final maximum likelihood tree (–ln likelihood = 12521.45) was obtained from a transition/transversion ratio of 7.08 (kappa = 15.55) and gamma

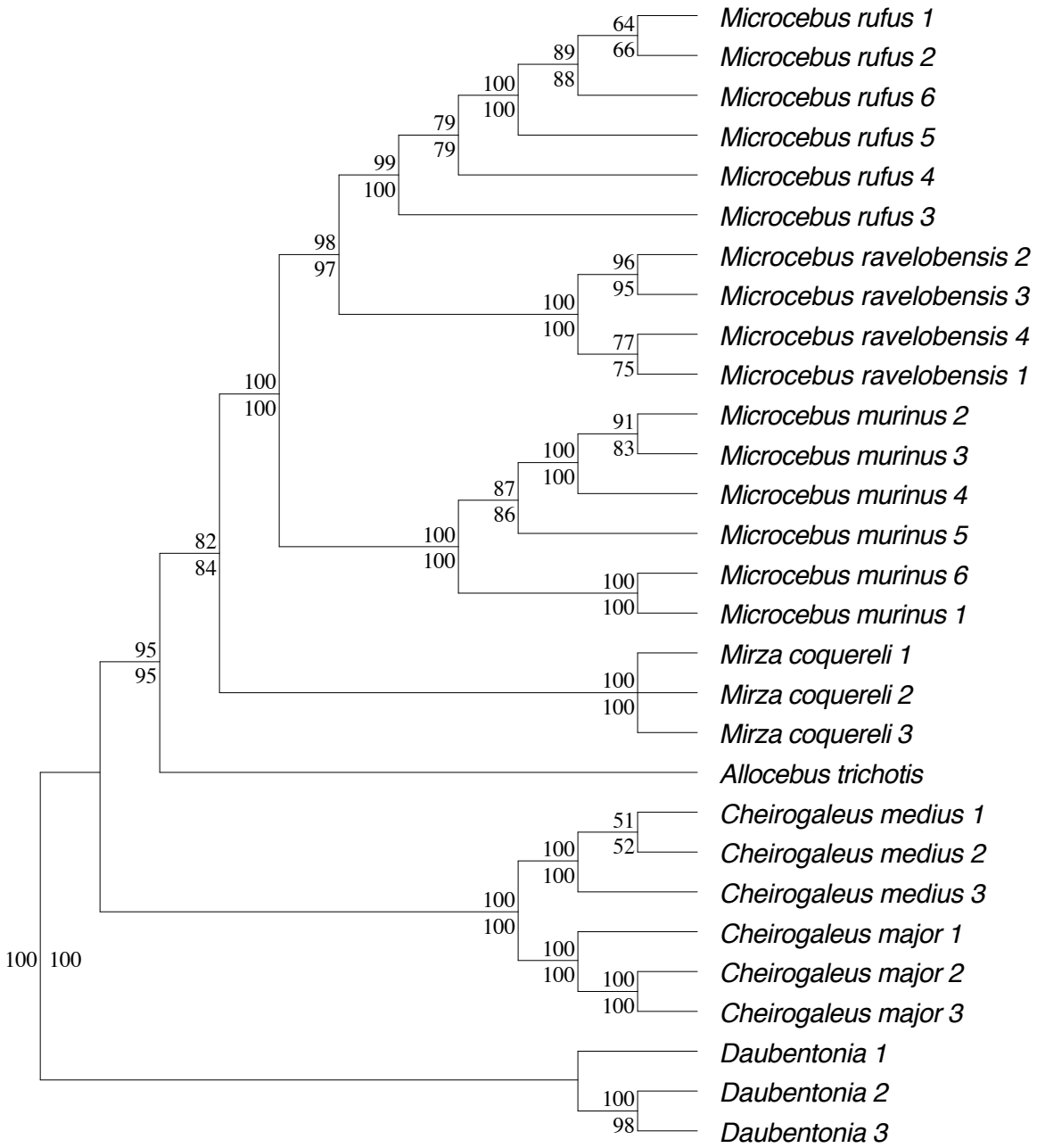


Fig. 6.4 Neighbour-joining tree with bootstrap values (as percentages, above nodes) obtained in 2500 replicates and with jackknife values (below nodes) from 2500 iterations with 50% deletion using Kimura 2-parameter distance correction.

shape parameter of 0.21.

Relationships among species or genera hence remain consistent in all analyses. Generally, there are very high bootstrap (BP) and jackknife (JK) supports for both maximum parsimony and neighbor-joining analyses with respect to the branching order of genera and species (Figs. 6.3 and 6.4). Both analyses unambiguously linked *Mirza* and *Microcebus* with 82–89% BP or JK support. The sister-group relationship between *Allocebus* and *Mirza*/*Microcebus* is supported in both maximum parsimony and neighbor-joining analyses with BP or JK values of 93–95%, such that the genus *Cheirogaleus* branches away first among the four genera examined. The clade containing the two *Cheirogaleus* species is supported in all analyses by 100% BP or JK support, as is the clade comprising the three *Microcebus* species.

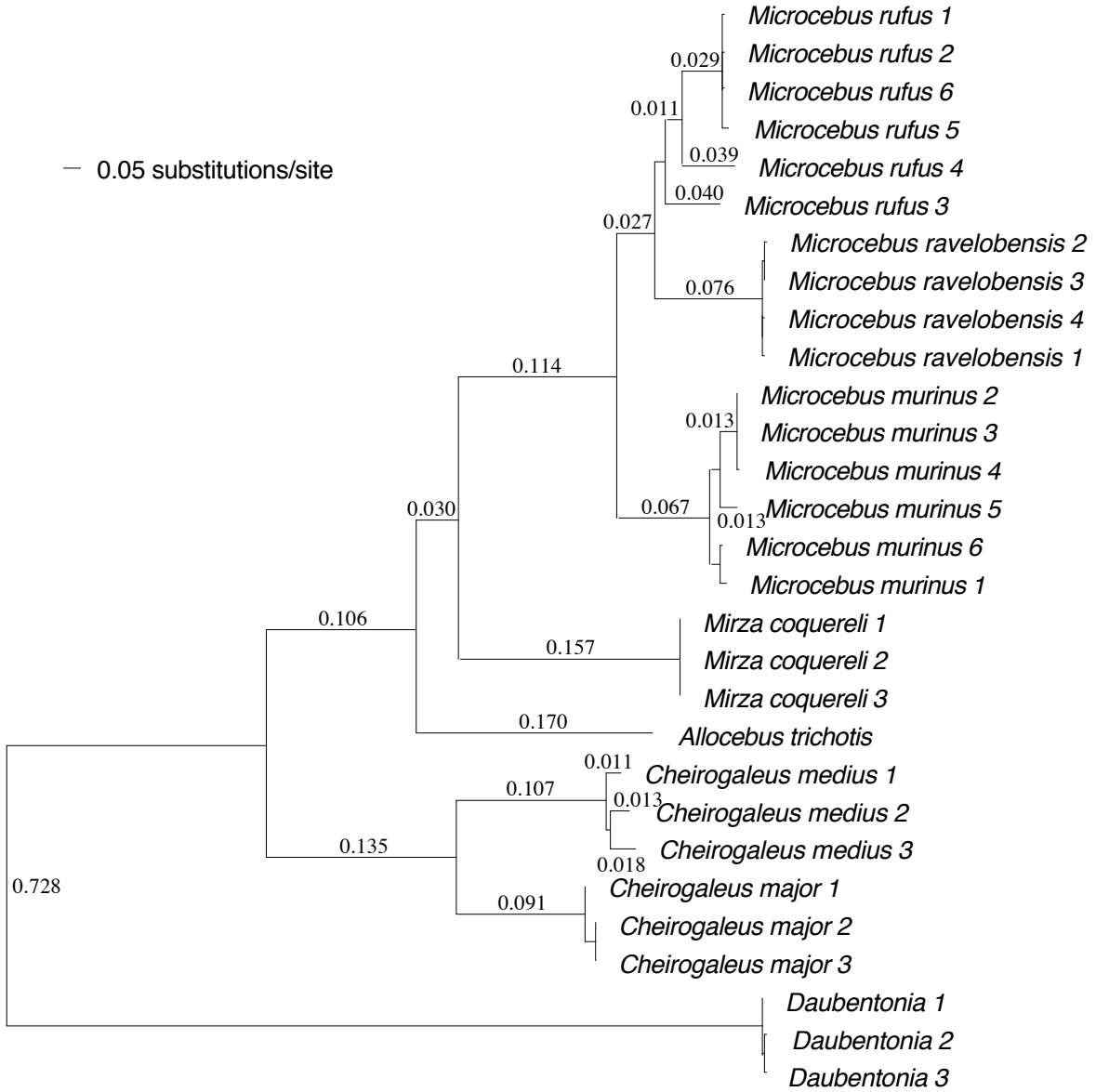


Fig. 6.5 Maximum likelihood phylogram with proportional branch lengths (values provided on each branch).

M. rufus and *M. ravelobensis* form a subclade within *Microcebus* with high BP or JK support (94–98%). The clades containing individuals of the species *C. medius*, *C. major*, *M. murinus*, *M. ravelobensis* and *Mirza coquereli* always have complete BP or JK support (100%). While the *M. rufus* clade as a whole is not supported with 100% BP or JK values, four individuals within *M. rufus* are unambiguously linked with 100% BP or JK support. Within *M. murinus*, two subclades containing three individuals and two individuals, respectively, have 100% BP or JK support in maximum parsimony and neighbor-joining analyses. Branching arrangements within *M. ravelobensis*, *M. rufus* or *C. medius* differ slightly between the different analyses.

Absolute pairwise distances are presented in Table 6.3 and range from a maximum of 586 bp between *Daubentonia* and members of the ingroup to 0–473 bp within the family Cheirogaleidae. Examination of absolute pairwise distances within the family Cheirogaleidae reveals seven levels of differentiation (Fig. 6.6): Divergences between *Cheirogaleus* and other genera of the family are higher (436–470 bp) than divergences between *Allocebus*, *Mirza* and *Microcebus* (376–409 bp). Pairwise distances between *C. medius* and *C. major* range from 315 to 332 bp, whereas distances between the three *Microcebus* species are of the order of 215 to

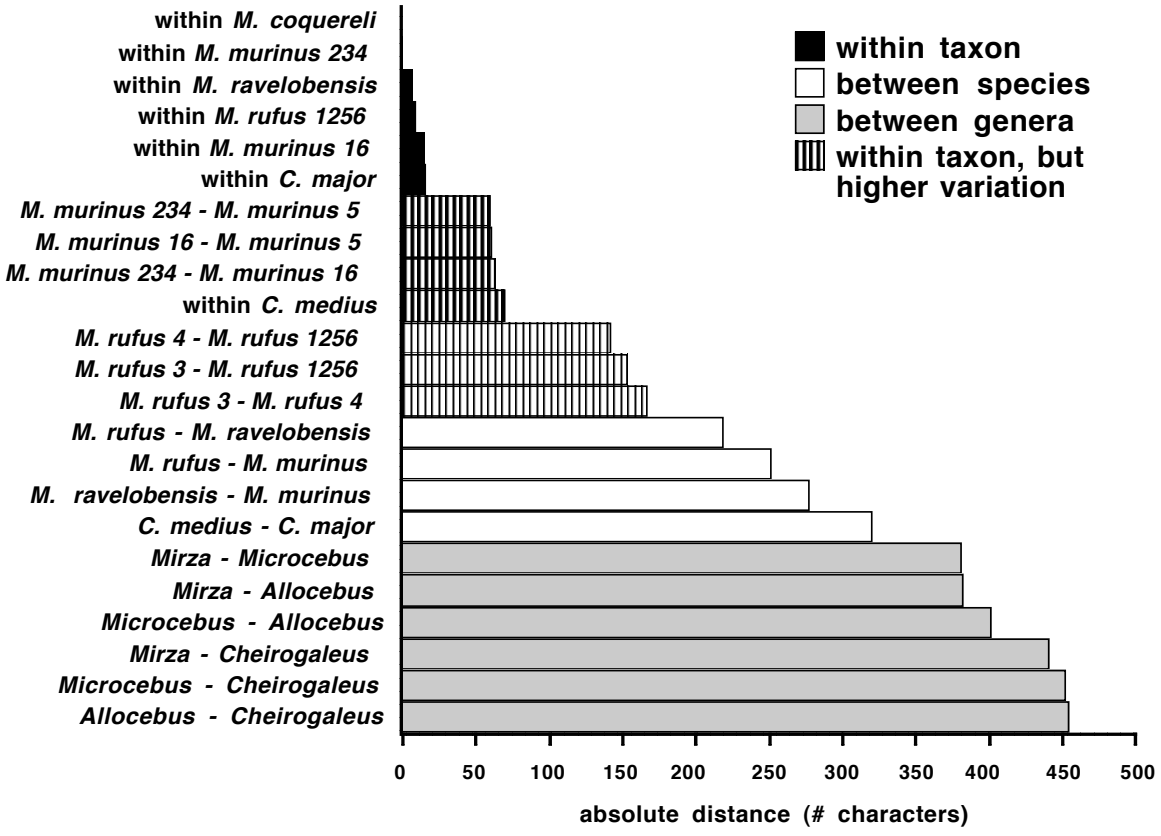


Fig. 6.6 Absolute pairwise distances over three defined taxonomic levels. Each bar represents the average of all possible comparisons between individuals of the two taxa. Single values are presented in Table 6.3.

282 bp. Within *M. rufus*, three subclades can be separated that differ from each other on the order of 142 to 168 bp. Similarly, within *M. murinus* three different subclades can be recognised with smaller pairwise distances ranging from 58 to 70 bp. Pairwise comparisons of the three individuals within *C. medius* give values in the same range (66–75 bp) as the different subclades within *M. murinus*. Within *C. major*, *Mirza coquereli*, *M. ravelobensis*, each subclade of *M. murinus* or *M. rufus,* and *Daubentonia* pairwise distances range from 0 to 24 bp.

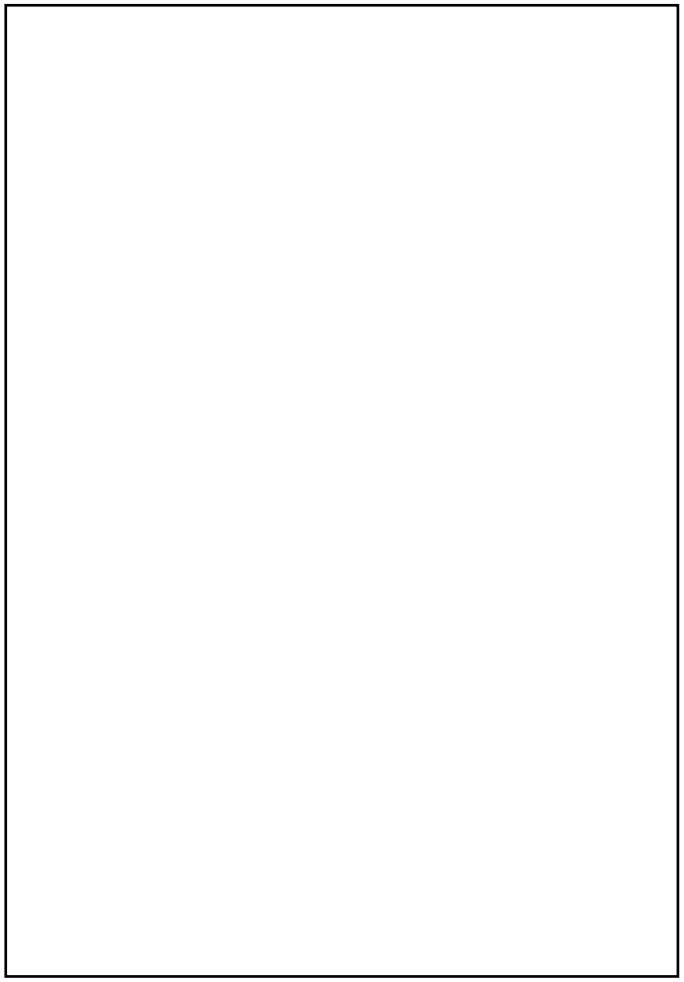


Fig. 6.7 *Cheirogaleus major* from Parc Zoologique et Botanique de Tsimbazaza (July 1997).

Table 6.3 Kimura 2-parameter distance (above the diagonal) and absolute distance (under the diagonal) matrices derived from the 2389 bp mitochondrial DNA sequence data set, with gaps treated as missing data.

	C.me. 1	*C.me. 2*	*C.me. 3*	*C.ma. 1*	*C.ma. 2*	*C.ma. 3*	*A.tr.*	*M.co. 1*	*M.co. 2*	*M.co. 3*	*M.mu. 1*	*M.mu. 2*	*M.mu. 3*	*M.mu. 4*	*M.mu. 5*
Cheirogaleus medius 1	-	0.029	0.033	0.152	0.154	0.153	0.231	0.224	0.224	0.224	0.238	0.238	0.238	0.237	0.239
Cheirogaleus medius 2	66	-	0.032	0.152	0.153	0.152	0.231	0.222	0.222	0.222	0.236	0.239	0.239	0.238	0.236
Cheirogaleus medius 3	75	73	-	0.158	0.161	0.160	0.228	0.227	0.227	0.227	0.238	0.240	0.240	0.239	0.240
Cheirogaleus major 1	315	315	327	-	0.010	0.010	0.228	0.216	0.216	0.216	0.218	0.220	0.220	0.220	0.220
Cheirogaleus major 2	320	318	332	24	-	0.001	0.228	0.218	0.218	0.218	0.224	0.225	0.225	0.225	0.225
Cheirogaleus major 3	318	316	330	23	3	-	0.228	0.216	0.216	0.216	0.223	0.225	0.225	0.225	0.224
Allocebus trichotis	458	458	454	454	454	454	-	0.188	0.188	0.188	0.197	0.203	0.203	0.203	0.196
Mirza coquereli 1	448	445	453	435	438	436	384	-	0	0	0.187	0.192	0.192	0.192	0.189
Mirza coquereli 2	448	445	453	435	438	436	384	0	-	0	0.187	0.192	0.192	0.192	0.189
Mirza coquereli 3	448	445	453	435	438	436	384	0	0	-	0.187	0.192	0.192	0.192	0.189
Microcebus murinus 1	469	467	470	438	448	447	400	381	381	381	-	0.030	0.030	0.030	0.029
Microcebus murinus 2	470	472	473	442	450	449	409	390	390	390	68	-	0	0.001	0.026
Microcebus murinus 3	470	472	473	442	450	449	409	390	390	390	68	0	-	0.001	0.026
Microcebus murinus 4	468	470	471	442	450	449	409	390	390	390	70	2	2	-	0.027
Microcebus murinus 5	472	467	473	442	450	449	398	385	385	385	66	60	60	62	-
Microcebus murinus 6	468	466	469	433	443	442	403	375	375	375	16	60	60	62	58
Microcebus ravelobensis 1	459	451	455	443	442	443	405	383	383	383	280	279	279	279	282
Microcebus ravelobensis 2	458	452	456	444	443	444	408	386	386	386	280	279	279	279	282
Microcebus ravelobensis 3	458	452	456	443	442	443	407	387	387	387	279	278	278	278	281
Microcebus ravelobensis 4	458	452	456	442	441	442	403	384	384	384	279	276	276	276	279
Microcebus rufus 1	447	447	448	455	461	460	401	378	378	378	243	256	256	254	258
Microcebus rufus 2	446	446	447	456	462	461	402	377	377	377	242	255	255	253	257
Microcebus rufus 3	458	459	460	449	455	454	387	376	376	376	245	258	258	258	255
Microcebus rufus 4	456	445	442	444	450	448	396	379	379	379	251	254	254	252	265
Microcebus rufus 5	444	442	445	460	464	463	404	384	384	384	250	261	261	259	265
Microcebus rufus 6	449	447	448	455	461	460	403	380	380	380	243	256	256	254	258
Daubentonia 1	560	565	570	536	544	542	538	576	576	576	562	561	561	559	561
Daubentonia 2	558	563	568	536	544	542	542	580	580	580	562	562	562	560	563
Daubentonia 3	558	563	568	536	544	542	542	580	580	580	562	562	562	560	563

	M.mu. 6	*M.ra. 1*	*M.ra. 2*	*M.ra. 3*	*M.ra. 4*	*M.ru. 1*	*M.ru. 2*	*M.ru. 3*	*M.ru. 4*	*M.ru. 5*	*M.ru. 6*	*D. 1*	*D. 2*	*D. 3*
Cheirogaleus medius 1	0.237	0.231	0.230	0.230	0.231	0.224	0.223	0.230	0.229	0.222	0.225	0.293	0.292	0.292
Cheirogaleus medius 2	0.236	0.226	0.226	0.226	0.227	0.224	0.223	0.231	0.222	0.221	0.224	0.297	0.295	0.295
Cheirogaleus medius 3	0.238	0.228	0.229	0.229	0.229	0.225	0.224	0.231	0.221	0.223	0.225	0.300	0.299	0.299
Cheirogaleus major 1	0.215	0.221	0.222	0.221	0.220	0.229	0.230	0.225	0.222	0.232	0.229	0.277	0.277	0.277
Cheirogaleus major 2	0.221	0.220	0.221	0.220	0.220	0.233	0.233	0.228	0.225	0.235	0.233	0.282	0.282	0.282
Cheirogaleus major 3	0.220	0.221	0.221	0.221	0.220	0.232	0.233	0.228	0.224	0.234	0.232	0.281	0.281	0.281
Allocebus trichotis	0.199	0.200	0.202	0.201	0.199	0.198	0.199	0.189	0.195	0.200	0.199	0.278	0.281	0.281
Mirza coquereli 1	0.184	0.188	0.190	0.190	0.189	0.185	0.185	0.183	0.186	0.189	0.186	0.302	0.305	0.305
Mirza coquereli 2	0.184	0.188	0.190	0.190	0.189	0.185	0.185	0.183	0.186	0.189	0.186	0.302	0.305	0.305
Mirza coquereli 3	0.184	0.188	0.190	0.190	0.189	0.185	0.185	0.183	0.186	0.189	0.186	0.302	0.305	0.305
Microcebus murinus 1	0.007	0.133	0.133	0.132	0.132	0.113	0.113	0.114	0.117	0.117	0.113	0.294	0.294	0.294
Microcebus murinus 2	0.026	0.132	0.132	0.132	0.131	0.120	0.120	0.121	0.119	0.123	0.120	0.293	0.294	0.294
Microcebus murinus 3	0.026	0.132	0.132	0.132	0.131	0.120	0.120	0.121	0.119	0.123	0.120	0.293	0.294	0.294
Microcebus murinus 4	0.027	0.132	0.132	0.132	0.131	0.119	0.119	0.121	0.118	0.122	0.119	0.292	0.292	0.292
Microcebus murinus 5	0.025	0.134	0.134	0.133	0.132	0.121	0.120	0.119	0.124	0.125	0.121	0.293	0.294	0.294
Microcebus murinus 6	-	0.133	0.133	0.132	0.131	0.112	0.112	0.112	0.116	0.116	0.112	0.289	0.291	0.291
Microcebus ravelobensis 1	280	-	0.004	0.004	0.002	0.102	0.102	0.100	0.099	0.103	0.100	0.295	0.296	0.296
Microcebus ravelobensis 2	280	9	-	0.001	0.004	0.104	0.104	0.103	0.100	0.105	0.103	0.296	0.296	0.296
Microcebus ravelobensis 3	279	10	3	-	0.004	0.103	0.104	0.102	0.100	0.104	0.102	0.296	0.296	0.296
Microcebus ravelobensis 4	277	4	9	10	-	0.103	0.103	0.101	0.100	0.104	0.102	0.293	0.294	0.294
Microcebus rufus 1	241	219	223	222	221	-	0.001	0.069	0.064	0.007	0.002	0.307	0.306	0.306
Microcebus rufus 2	240	220	224	223	222	3	-	0.069	0.064	0.007	0.002	0.307	0.307	0.307
Microcebus rufus 3	241	217	222	221	219	154	153	-	0.076	0.071	0.069	0.310	0.310	0.310
Microcebus rufus 4	248	215	217	216	217	142	143	168	-	0.066	0.063	0.296	0.296	0.296
Microcebus rufus 5	248	221	225	224	223	16	17	159	148	-	0.008	0.307	0.307	0.307
Microcebus rufus 6	241	217	221	220	219	4	5	154	142	18	-	0.307	0.307	0.307
Daubentonia 1	555	564	566	566	561	580	581	586	566	581	581	-	0.003	0.003
Daubentonia 2	557	566	566	566	563	580	581	586	566	581	581	7	-	0
Daubentonia 3	557	566	566	566	563	580	581	586	566	581	581	7	0	-

6.4 Discussion

Phylogenetic Relationships Among Genera

One goal of the present study was to clarify nodal relationships among four genera of Cheirogaleidae. According to these results, *Mirza* and *Microcebus* consistently form a sister group relationship to *Allocebus*. The two *Cheirogaleus* species constitute the earliest diverging clade. These arrangements are strongly supported by bootstrap and jackknife analyses in maximum parsimony and neighbor-joining methods (82–100%), as well as by the maximum likelihood phylogram (Figs. 6.3 - 6.5).

Unfortunately, no sample from the genus *Phaner* could be obtained. Previous studies generally agree, however, that *Phaner* is the earliest genus to diverge within the Cheirogaleidae. This is supported by morphology (Tattersall & Schwartz 1974; Stanger 1993), behaviour (Stanger 1993), vocalisations (Rakotoarison et al. 1996), chromosomes (Rumpler & Dutrillaux 1979), and highly repeated DNA (Crovella et al. 1995). The only exceptions to this arrangement were derived from immunological data, which grouped *Phaner* and *Cheirogaleus* and indicated that *Microcebus* is the earliest genus to diverge among Cheirogaleidae (Sarich & Cronin 1976), and from morphological and behavioural characters, which resolved *Microcebus* as the sister group to a clade containing *Mirza* and *Phaner*, while *Cheirogaleus* was the most basal offshoot (Stanger-Hall 1997).

The deep divergence of *Cheirogaleus* from the other genera was an unexpected result and one which further emphasises the need for DNA sequence evaluation of the genus *Phaner*. Basal divergence of the genus *Cheirogaleus* and the sister relationship between *Microcebus* and *Mirza* identified in this study are in agreement with previously published results from studies of morphological features (Tattersall & Schwartz 1974; Stanger 1993), behaviour (Stanger 1993), highly repeated DNA (Crovella et al. 1995), and DNA sequences (Yoder et al. 1996; Yoder 1997; Stanger-Hall & Cunningham 1998). Analyses of 125 morphological and behavioural characters, however, indicated that *Microcebus* is sister group to a clade containing *Cheirogaleus* and *Mirza* (Yoder et al. 1996). In contrast, a cladistic analysis of 25 morphological and behavioural characters resolved *Cheirogaleus* sister to a clade containing *Microcebus*, *Mirza* and *Phaner* (Stanger-Hall 1997).

Of special interest was the phylogenetic position of *Allocebus*. A sister group relationship with *Microcebus*/*Mirza* is in agreement with a previous cladistic analysis of

13 morphological and behavioural characters (Stanger 1993). Other studies either did not include *Allocebus* (Tattersall & Schwartz 1974; Sarich & Cronin 1976; Yoder et al. 1996; Stanger-Hall & Cunningham 1998) or could not resolve its phylogenetic position (Yoder 1994; Crovella et al. 1995; Rumpler et al. 1995). This is the first genetic study clearly resolving the phylogenetic position of *Allocebus* within the Cheirogaleidae.

The generic status of *Mirza coquereli* is still debated. Coquerel's dwarf lemur has been alternatively classified as a member of *Cheirogaleus* (Grandidier 1867; Tattersall & Schwartz 1974), as a member of *Microcebus* (Schlegel & Pollen 1868; Petter et al. 1977; Napier & Napier 1985; Jenkins 1987; Rowe 1996), or as the sole species within the genus *Mirza* (Gray 1870; Tattersall 1982; Groves 1989; Harcourt & Thornback 1990; Mittermeier et al. 1994). According to these results, *Mirza* is the sister group to *Microcebus* and these two genera together form the sister group of *Allocebus*, which conflicts with a taxonomic position within the genus *Cheirogaleus*. Using the proportional branch lengths as visual aids (Fig. 6.5), it is obvious that *Mirza* is more deeply divergent from *Microcebus* than the different species of *Microcebus* are from one another. Pairwise distances (Fig. 6.6, Table 6.3) between *Mirza* and *Microcebus* (375–390 bp) are higher than among the three *Microcebus* species (315–332 bp) and reach the range of between-genera comparisons (384–473 bp). From this it follows that the genetic data analysed here support the generic status of *Mirza coquereli*.

Phylogenetic Relationships Among Mouse Lemur Species

Within the genus *Microcebus*, a subclade is formed by *M. ravelobensis* and *M. rufus*, with *M. murinus* being the sister taxon to these two. This arrangement has strong bootstrap and jackknife support (89–100%) using maximum parsimony or neighbor-joining searches (Figs. 6.3 and 6.4). In contrast, comparative morphological analysis places *M. rufus* as sister group to a clade containing *M. murinus* and *M. ravelobensis* (Zimmermann et al. 1998). Unfortunately, it was not possible to investigate *M. myoxinus*. However, RAPD analyses cluster *M. rufus* as sister group to a clade containing *M. murinus* and *M. myoxinus* (Tomiuk et al. 1998). Apparently, no other phylogenetic studies have been published for *Microcebus*.

One aim of this study was to examine the taxonomic status of the recently discovered *M. ravelobensis*. Discrimination of this new species from its sibling species, the sympatric western grey mouse lemur (*M. murinus*), from the western pygmy mouse lemur (*M. myoxinus*) and from the eastern rufous mouse lemur (*M. rufus*) has been previously supported by

morphological characters (Zimmermann et al. 1998). Furthermore, the sympatric species *M. murinus* and *M. ravelobensis* in northwestern Madagascar differ in features of microhabitat usage (Randrianambinina 1997; Ehresmann & Zimmermann 1998; Rendigs & Zimmermann 2000), communication (Zietemann et al. 2000) and reproduction (Schmelting et al. 2000). Furthermore, *M. rufus* and *M. murinus* exhibit distinct differences in communication and reproduction (Zimmermann et al. in press). The maximum likelihood phylogram from the analysis of mtDNA in fact shows three well-separated *Microcebus* clades (Fig. 6.5). Pairwise distances between *M. ravelobensis* and *M. murinus* (276–282 bp) or between *M. ravelobensis* and *M. rufus* (215–224 bp) clearly lie in the same range as those between *M. murinus* and *M. rufus* (240–261 bp) (Fig. 6.6). Moreover, three of the six *M. murinus* samples used in this study are from the same locality in northwestern Madagascar as the four *M. ravelobensis* samples studied. Genetic divergences between the two sympatric forms of *Microcebus* are at the level separating other cheirogaleid species. Consequently, the molecular data provide additional support for the species-level distinction of these three *Microcebus* species, two of which exist as well-delineated sympatric taxa at Ampijoroa.

Genetic Differentiation Among Brown Mouse Lemurs

Within the sample of *M. rufus*, three subclades differ from each other on the order of 142–168 bp (Fig. 6.6). This is not as high as divergences between *M. murinus, M. rufus* and *M. ravelobensis* (215–282 bp). Within *C. major*, *M. ravelobensis, Mirza coquereli*, and *Daubentonia*, on the other hand, pairwise distances range from 0–24 bp. *M. rufus* is the only clade containing individuals attributed to only one species that is not completely (100%) supported by bootstrap or jackknife values (Figs. 6.3 and 6.4). The three subclades within *M. rufus* are also separated by relatively long branches in maximum likelihood analyses (Fig. 6.5).

Four *M. rufus* in the sample come from Andasibe in eastern Madagascar (Fig. 6.2), one (#3) from Nosy Be in the north, and one (#4) is a captive individual of unknown origin held at the 'Tierärztliche Hochschule Hannover'. Interestingly, this latter individual, a female, was socially incompatible with various *M. rufus* males from Andasibe, but compatible with a *M. murinus* male. However, although that female showed regular oestrous cycles, she failed to reproduce with males from either species.

The data presented here imply that separation between those three subclades within *M. rufus* occurred later than those among *M. rufus*, *M. murinus* and *M. ravelobensis*. However, genetic, reproductive, and distributional data indicate that speciation may already be complete. Pairwise distances among the three subclades of brown mouse lemurs (142–168 bp) reach the level of differentiation among the five well-accepted *Eulemur* species (138–201 bp, Chapter 5). This suggests that brown mouse lemurs from eastern Madagascar (Andasibe) may be a different species from those of northern Madagascar (Nosy Be). Furthermore, the results indicate the existence of at least one more previously unrecognised species of brown mouse lemur, which is currently represented only by one captive animal of unknown origin.

Genetic Differentiation Among Grey Mouse Lemurs

Within *M. murinus*, three different subclades can be recognised on the basis of tree topology, branch lengths, and pairwise distances. Two subclades contain 3 and 2 individuals, respectively, and consistently show 100% bootstrap or jackknife support in maximum parsimony and neighbor-joining analyses (Figs. 6.3 and 6.4). Pairwise distances between the three *M. murinus* subclades are on the order of 58 to 70 bp, which is higher than the 0–24 bp found within *C. major*, *Mirza coquereli*, *M. ravelobensis,* or *Daubentonia* (Fig. 6.6).

The three subclades are composed of: (1) three individuals from Ampijoroa (northwest), (2) one individual from Mandena (south), and (3) one individual from Kirindy (west) together with one captive animal of unknown origin. The mtDNA sequence data suggest that the ancestors of the latter captive *M. murinus* most likely come from western Madagascar.

Genetic differentiation among the three subclades within *M. murinus* does not reach the species level, but indicates potentially significant divergences within *M. murinus*. Pairwise distances between the subclades (58–70 bp) are in the same range as between *Eulemur fulvus* subspecies (29–90 bp, see Chapter 3). Subspecific differentiation of these populations of *M. murinus* is also indicated by differences in acoustic structure of the trill advertisement call that is uttered during mating (Hafen et al. 1998).

The samples for *C. medius* also demonstrate stronger than expected molecular divergences (Figs. 6.3 - 6.6), suggesting that phylogeographic differentiation may exist in this taxon as well. Unfortunately, nothing is known about the origin of the three captive animals used in this study.

Concluding Remarks

Analyses of mtDNA sequences in this study facilitated clear resolution of phylogenetic relationships among genera, species and subclades within species of the family Cheirogaleidae. Molecular data strongly support the generic status of *Mirza coquereli,* a sister-group relationship between *Allocebus* and *Mirza/Microcebus*, and a basal divergence of *Cheirogaleus* among the four cheirogaleid genera studied. Furthermore, specific status of the recently described species *M. ravelobensis* is supported by these results.

Within both *M. rufus* and *M. murinus*, genetic differentiation among different localities was found to exceed the range of intraspecific variation seen to occur among lemur samples thus far examined. The molecular data suggest that differentiation among *M. rufus* localities may have already reached the species level, while among *M. murinus* localities subspecific differentiation is suggested. Evolutionary diversity within the genus *Microcebus* seem to be comparable to diversity within the genus *Eulemur* (Lemuridae). Taxonomy of the lemurs of Madagascar at the species level and below has in general attracted less attention for nocturnal forms (e.g. Cheirogaleidae) than for diurnal ones (e.g. Lemuridae) (Groves & Tattersall 1991). As a result, fewer species per genus and fewer subspecies per species have been recognised on average among the nocturnal Malagasy lemurs than among their diurnal relatives. The molecular data strengthen the suspicion of Groves and Tattersall (1991) that systematists have probably underestimated the taxonomic diversity of at least some of the nocturnal lemurs.

In the case of *Lepilemur*, there is only limited morphological evidence supporting any distinction between species (Martin 1995). The main impetus towards modern recognition of several species came from chromosomal studies which yielded a strong indication of separation among distinct populations. In *Microcebus*, on the other hand, chromosomal evidence supporting the proposed distinction between species is completely lacking, and it is morphological evidence that strongly indicates a separation between species. The lesson to be learned from this is that the study of species differences should be a multidisciplinary undertaking (Martin 1995). DNA sequences can be a valuable tool in this respect.

Further molecular studies should include the genus *Phaner* to test its purported basal position among the Cheirogaleidae. While the genus *Microcebus* clearly requires far more detailed studies in multiple disciplines to allow determination of specific and subspecific components, the same may also be true for the genus *Cheirogaleus.*

7. Family Indridae

This chapter is to be published in a modified form:
J. Pastorini, M.R.J. Forstner, R.D. Martin (in press)
Phylogenetic history of sifakas (*Propithecus*: Lemuriformes) derived from mtDNA sequences.
American Journal of Primatology.

7.1 Introduction

The primate fauna of Madagascar presents us with numerous problems in the delineation of species and subspecies populations. Some of the longest-standing uncertainties concern the taxonomic status of lemur populations classified in the indrid genus *Propithecus* (Tattersall 1986).

Three species of *Propithecus* are currently recognised. The largest sifaka species *P. diadema* (Bennett 1832) is found in the humid forests of eastern Madagascar (Fig. 7.1). The second, *P. verreauxi* (Grandidier 1867) inhabits the drier seasonal forests of the island's west and south. *P. verreauxi* has a slightly smaller body but a longer tail than *P. diadema*. Both of these species are polytypic and reliable information on variation and distribution of the recognisable phenotypes is lacking. Five subspecies of *P. diadema* and four or five subspecies of *P. verreauxi* have been recognised (e.g. Petter et al. 1977; Tattersall 1982).

P. tattersalli (Simons 1988) is a recently described species which is extremely rare and occurs in a very small and fragmented range (Fig. 7.1). It was diagnosed on the basis of its unique appearance, geographic isolation from other *Propithecus* populations and possession of characters intermediate between those of the other two species. *P.tattersalli* is similar in size and vocalisations to *P. verreauxi,* but its karyotype is closer to that of *P. diadema* (Simons 1988).

In the last century, four forms of western sifakas (*P. verreauxi*) were recognised. The face of *P.v. verreauxi* (Grandidier 1867) is hairless and black. The crown of the head is typically dark brown and the remaining body fur is white. It occurs throughout the forested regions of south and southwest Madagascar from just west of Fort-Dauphin to the Tsiribihina river (Fig. 7.1). *P.v. coquereli* (Milne-Edwards 1867) has a black face, but generally with a patch of very short white hairs on the muzzle. The fur on most of the body is white, but there are large chocolate-brown areas on the chest and on the front of the arms and thighs. The range of *P.v. coquereli* is north and east of the Betsiboka river in northwest Madagascar. The two other

subspecies, *P.v. deckeni* and *P.v. coronatus*, occupy the intervening part of western Madagascar between the Betsiboka and Tsiribihina rivers. *P.v. deckeni* (Peters 1870) has a black face, naked, largely hidden ears and a relatively blunt, rounded muzzle. Many individuals are completely white, but others show varying degrees of dark coloration on shoulders, back and limbs. *P.v. coronatus* (Milne-Edwards 1871) has a somewhat blunt, rounded muzzle and its face is naked, black or with some short whitish hairs on perianal skin. The body fur is white apart from tinting of the shoulders and back (for review, see Tattersall 1982).

Milne-Edwards and Grandidier (1875) separated *P.v. coronatus* at the species level on the basis of chromatic differences and cranial distinctions, chief among them an inflation of the anterior part of the maxilla. Subsequent authors such as Schlegel (1876) and Forbes (1894) additionally raised *P.v. verreauxi*, *P.v. deckeni* and *P.v. coquereli* to full species, and it was left to Elliot (1907; 1913) to reduce them all to subspecies of *P. verreauxi* (for review, see Tattersall 1986). A fifth form, *P.v. majori* (Rothschild 1894), was subsumed into *P.v. verreauxi* in 1982 (Tattersall 1982). Today, the taxonomic status of the remaining four variants, particularly the relationship between *P.v. deckeni* and *P.v. coronatus*, is still unclear.

Little is known about evolutionary relationships within the family Indridae generally or among sifakas in particular. A comparative study of highly repeated DNA confirmed the specific status of *P. tattersalli*, placing it as the sister group to the *P. verreauxi*/*P. diadema* clade (Razafindraibe et al. 1997). A chromosomal banding study showed an early separation of *Avahi* and the relatively late divergence of the other indrids (Rumpler et al. 1988). *P. verreauxi* and *P. diadema* are distinguished by Robertsonian translocations, whereas *Indri* is differentiated from *Propithecus* by more complex rearrangements of much more selective importance. All four *P. verreauxi* subspecies have the same karyotype (Rumpler 1975), so no delineation is possible on this basis. To date, no genetic data have been available for sifaka subspecies.

In the present study, a large fragment of mitochondrial DNA was sequenced and the data examined in an attempt to clarify phylogenetic relationships among species and subspecies of sifakas. A further aim was to assess the validity of the status of the four subspecies currently recognised in *P. verreauxi*. Previous successful resolution of problematic taxa using this region of mtDNA (Forstner et al. 1995; Wang et al. 1997; Forstner et al. 1998; Pastorini et al. 1998) indicated that this fragment could potentially resolve phylogenetic relationships among the species and subspecies of the genus *Propithecus*.

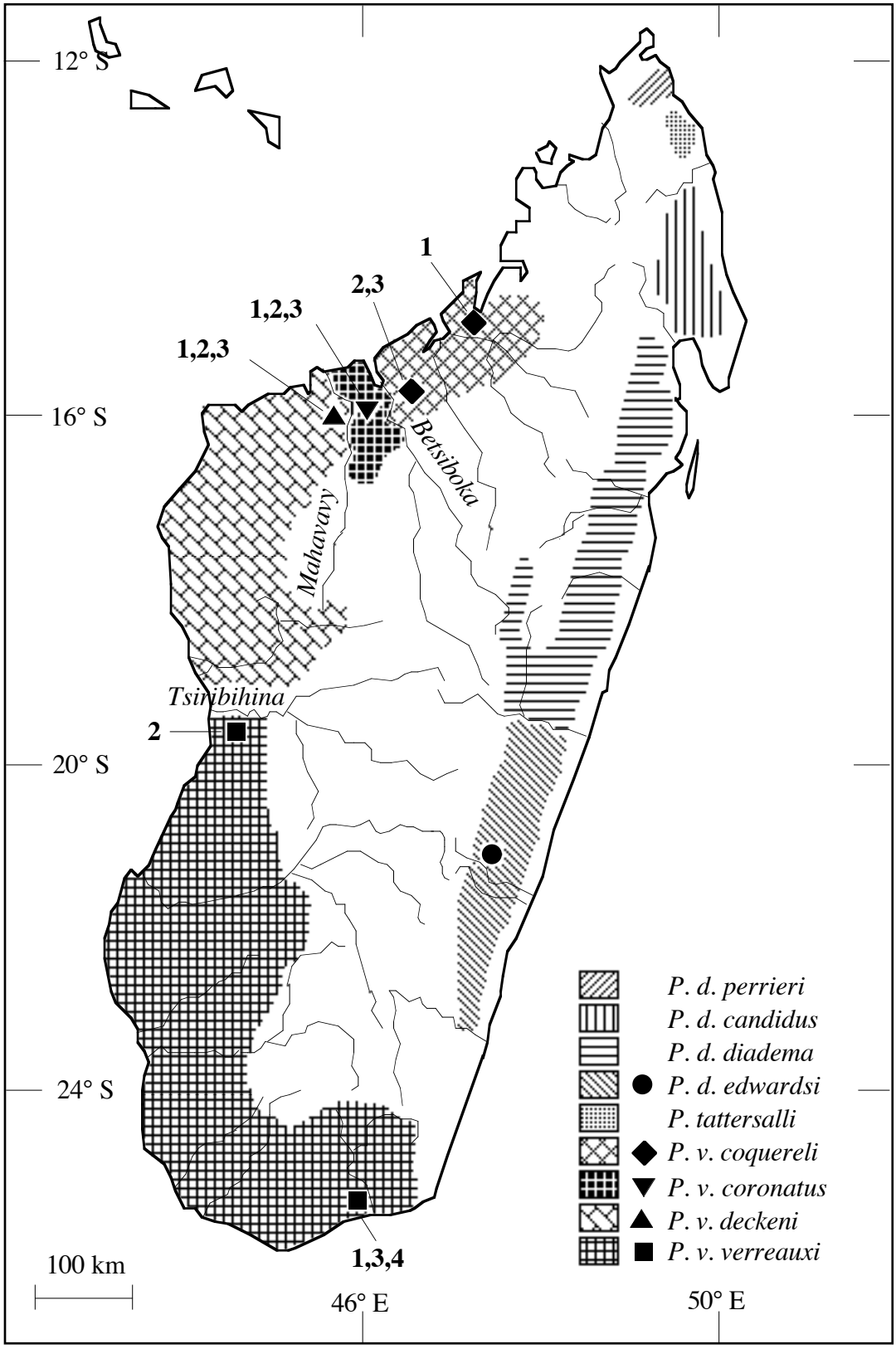


Fig. 7.1 Map of Madagascar showing approximate distribution of the *Propithecus* species and subspecies (Tattersall 1982; Mittermeier et al. 1994). Symbols indicate individuals with exact locality data that were included in the present study.

7.2 Material

Samples from two genera of the family Indridae (*Avahi* and *Propithecus*) were obtained. Specifically, samples were collected from 1 *A. laniger*, 1 *P. diadema*, 1 *P. tattersalli*, and from all 4 subspecies of *P. verreauxi* (4 *P.v. verreauxi*, 3 *P.v. deckeni*, 3 *P.v. coronatus*, 3 *P.v. coquereli*). Unfortunately, no sample from the third indrid genus *Indri* was available. Single samples from *Eulemur macaco* and *E. fulvus* were sequenced for subsequent use as the outgroup taxa. All samples utilised are listed in Table 7.1.

The 3 *P.v. deckeni* and 3 *P.v. coronatus* individuals were captured west (Anadabomandry) and east (Anjamena), respectively, of the Mahavavy river in northwestern Madagascar. Two *P.v. coquereli* sampled for this study were collected in Ampijoroa. The records from 'Parc Zoologique et Botanique de Tsimbazaza' indicated that the third *P.v. coquereli* was captured at Andrevorevo-Antsohihy, which lies further to the north. Two *P.v. verreauxi* samples were collected in the Berenty Private Reserve. Another *P.v. verreauxi* originated from Kirindy, while the fourth *P.v. verreauxi* was from a semifree-ranging group

Table 7.1 Taxa, origin, identification numbers, and GenBank accession numbers for the 18 individuals sequenced.

Taxon	Origin	ID #	GenBank #
Avahi laniger	Ranomafana (Southeast) [a]	JP345	AF224598
Propithecus tattersalli	unknown [b]	JP344	AF224600
P. diadema edwardsi	Ranomafana (Southeast) [a]	JP343	AF224599
P. verreauxi verreauxi 1	Amboasary Reserve (South) [c]	JP237	AF224601
P. verreauxi verreauxi 2	Kirindy forest (West) [a]	JP271	AF224602
P. verreauxi verreauxi 3	Berenty Private Reserve (South) [a]	JP350	AF224603
P. verreauxi verreauxi 4	Berenty Private Reserve (South) [a]	JP351	AF224604
P. verreauxi deckeni 1	Anadabomandry, west of Mahavavy River (Northwest) [a]	JP172	AF224605
P. verreauxi deckeni 2	Anadabomandry, west of Mahavavy River (Northwest) [a]	JP208	AF224606
P. verreauxi deckeni 3	Anadabomandry, west of Mahavavy River (Northwest) [a]	JP209	AF224607
P. verreauxi coronatus 1	Anjamena, east of Mahavavy River (Northwest) [a]	JP147	AF224608
P. verreauxi coronatus 2	Anjamena, east of Mahavavy River (Northwest) [a]	JP154	AF224609
P. verreauxi coronatus 3	Anjamena, east of Mahavavy River (Northwest) [a]	JP166	AF224610
P. verreauxi coquereli 1	Andrevorevo-Antsohihy (Northwest) [d]	JP136	AF224611
P. verreauxi coquereli 2	Ampijoroa (Northwest) [a]	JP212	AF224612
P. verreauxi coquereli 3	Ampijoroa (Northwest) [a]	JP217	AF224613
Eulemur macaco macaco	Ambato (North) [e]	JP83	AF224530
Eulemur fulvus fulvus	Ampijoroa (Northwest) [a]	JP218	AF224536

[a] wild-caught animals with verified origin

[b] held at Duke University Primate Center, U.S.A.

[c] held in a semi-wild environment in the Amboasary Reserve in southern Madagascar

[d] held at Parc Zoologique et Botanique de Tsimbazaza, Madagascar

[e] held at Université Louis Pasteur, Strasbourg, France

kept at Amboasary Reserve. Samples from one *P. diadema* and one *A laniger* were collected in Ranomafana. No locality data were available for the *P. tattersalli* sample from 'Duke University Primate Center', but the known geographical range of this species is very limited. All known sample localities are shown in Figure 7.1.

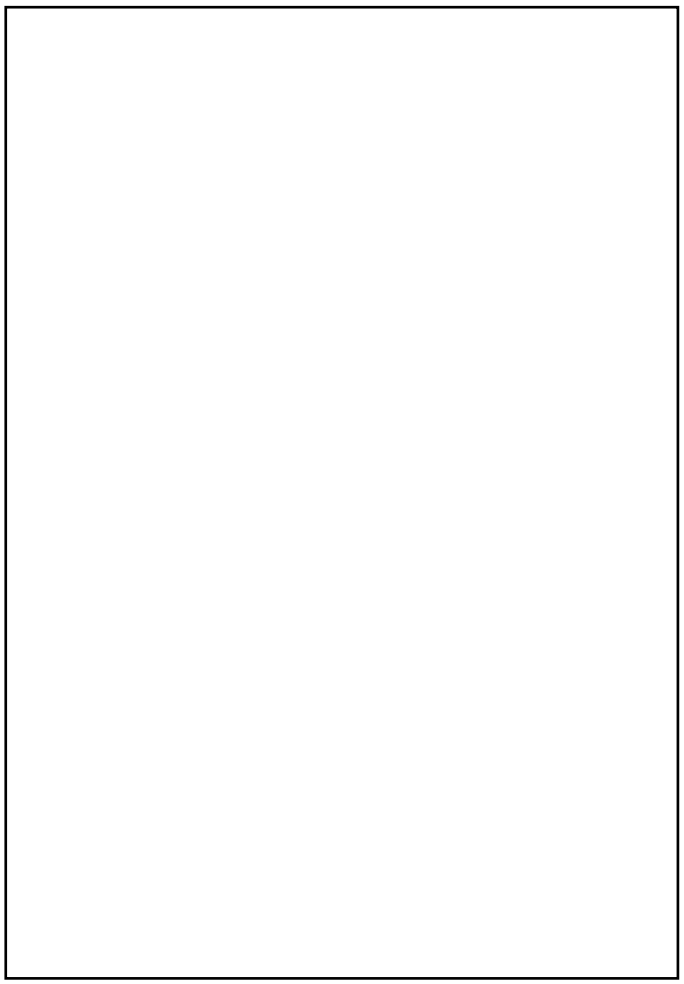


Fig. 7.2 *Propithecus verreauxi coronatus* (top left), *P.v. deckeni* (top right), *P.v. verreauxi* (bottom left) and *P.v. coquereli* (bottom right).

7.3 Results

The new mtDNA sequences generated for the taxa examined have been deposited in GenBank (Table 7.1). Aligned sequences are available from the first author upon request. The nucleotide sequences span a total of 2389 base positions (bp). The analysed dataset consists of 3' end of the COIII gene (53 bp), the complete NADH-dehydrogenase subunits ND3 (348 bp), ND4L (297 bp) and ND4 (1378 bp) along with the glycine (71 bp), arginine (68 bp), histidine (69 bp), serine (62 bp), and part of leucine (47 bp) tRNA genes. The partition-homogeneity test showed no significant incongruence among those nine genes (P=0.15).

The sequences obtained provided 500 parsimony-informative characters with a transition:transversion ratio of 5.3:1. A summary of the frequencies of invariant, parsimony uninformative, and informative characters along the segment sequenced is given in Table 7.2.

The maximum parsimony branch-and-bound search with all characters weighted equally results in two trees, each 1119 steps in length with a consistency index of 0.80 and a retention index of 0.82 (Fig. 7.3). The distance matrices constructed using Kimura 2-parameter

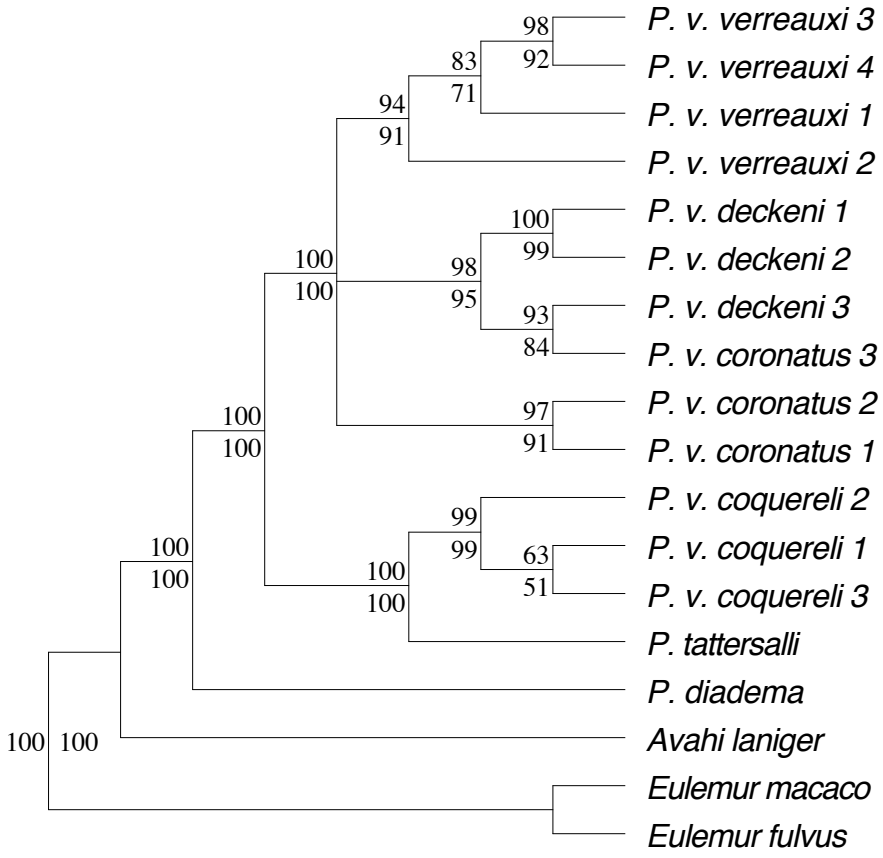


Fig. 7.3 Maximum parsimony strict consensus tree with bootstrap values (as percentages, above nodes) obtained in 2500 replicates and with jackknife values (below nodes) from 2500 iterations with 50% deletion.

Table 7.2 Summary of variation for the sequences across the 18 lemurs examined.

Genes	All	COIII	ND3	ND4L [a]	ND4 [a]	tRNAs	Not translated
characters (nucleotides)	2389	53	348	297	1378	317	3
constant	1614	41	228	194	914	243	0
parsimony-uninformative	275	3	39	40	162	31	1
parsimony-informative	500	9	81	63	302	43	2
informative proportion	0.21	0.17	0.23	0.21	0.22	0.14	0.67
insertions/deletions	5	0	0	0	0	5	0

[a] ND4L and ND4 overlap for 7 bp

corrections (Table 7.3) were analysed by neighbor-joining and reconstruct the same topology with respect to the arrangement of species and genera (Fig. 7.4). The support values from bootstrap and jackknife analyses of 2500 replicates are in the same range as for maximum parsimony analyses.

The results of the maximum likelihood analysis are presented in Figure 7.5. The phylogram presented maintains branch lengths proportional to the number of changes. The phylogenetic relationships among clades are virtually identical to those from maximum parsimony and neighbor-joining analyses. The final maximum likelihood tree (–ln likelihood =

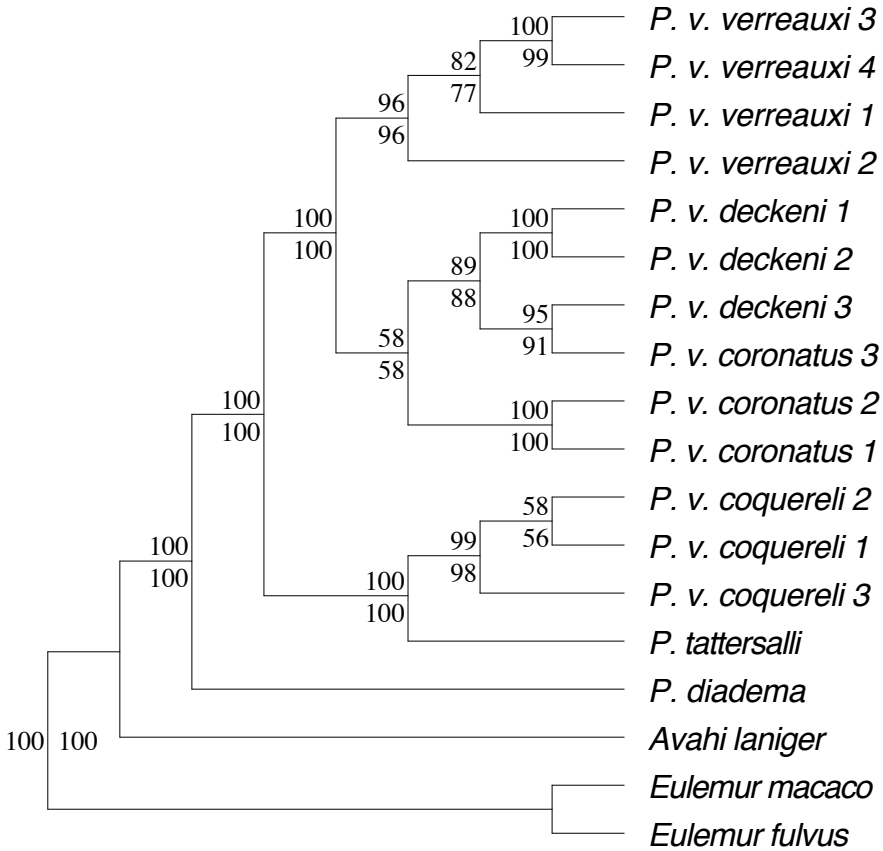


Fig. 7.4 Neighbor-joining tree with bootstrap values (as percentages, above nodes) obtained in 2500 replicates and with jackknife values (below nodes) from 2500 iterations with 50% deletion using Kimura 2-parameter distance correction.

8006.46) was obtained with a previously estimated transition/transversion ratio of 10.68 (kappa = 22.73) and gamma shape parameter of 0.19.

Relationships among species or genera remain consistent in all analyses. Generally, there is very high bootstrap (BP) and jackknife (JK) support in both maximum parsimony and neighbor-joining analyses with respect to the branching order of genera and species (Figs. 7.3 and 7.4). All analyses group *Avahi* as sister to the clade containing all sifakas, which is supported in both maximum parsimony and neighbor-joining analyses with BP or JK values of 100%. *P. diadema* diverges first among *Propithecus* with 100% BP or JK support. Among the remaining sifakas, one subclade is formed by *P.v. coquereli* and *P. tattersalli*, while *P.v. verreauxi*, *P.v. deckeni* and *P.v. coronatus* form the second subclade. Both clades have complete BP and JK support (100%) using maximum parsimony or neighbor-joining searches. All analyses fail to resolve *P.v. coronatus* and *P.v. deckeni* into two clades. The four *P.v. verreauxi* individuals always form a subclade with high BP or JK support (91–96%). The clade containing 1 *P.v. coronatus* and 3 *P.v. deckeni* individuals is supported with BP or JK values of 88–98%. The subclade formed by the remaining 2 *P.v. coronatus* is supported with 91–100% BP or JK values in maximum parsimony and neighbor-joining analyses.

Absolute pairwise distances are presented in Table 7.3 and range from a maximum of 435–507 bp between *Daubentonia* and members of the ingroup to 0–396 bp within the family

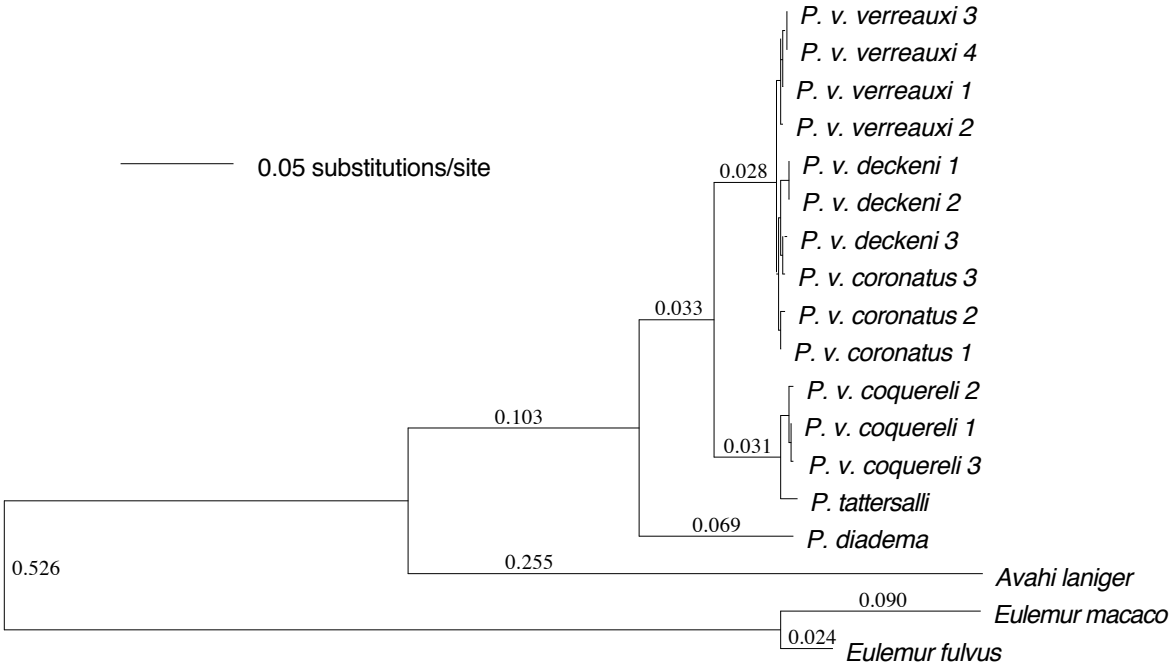


Fig. 7.5 Maximum likelihood phylogram with proportional branch lengths (values provided on each branch).

Indridae. Examination of pairwise distances within the family Indridae reveals four levels of differentiation (Fig. 7.6). Divergences between *Avahi* and *Propithecus* are much higher (367–396 bp) than divergences between *P. diadema* and *P. verreauxi* or *P. tattersalli* (216–222 bp). Pairwise distances between *P. tattersalli* and *P.v. verreauxi*, *P.v. coronatus* or *P.v. deckeni* are in the same range (131–137 bp) as distances between *P.v. coquereli* and *P.v. verreauxi*, *P.v. coronatus* or *P.v. deckeni* (126–133 bp). Absolute pairwise distances within each *P. verreauxi* subspecies or among the three subspecies *P.v. verreauxi*, *P.v. coronatus* and *P.v. deckeni* never exceed 24 bp. *P. tattersalli* and *P.v. coquereli* differ from each other by 27–28 bp.

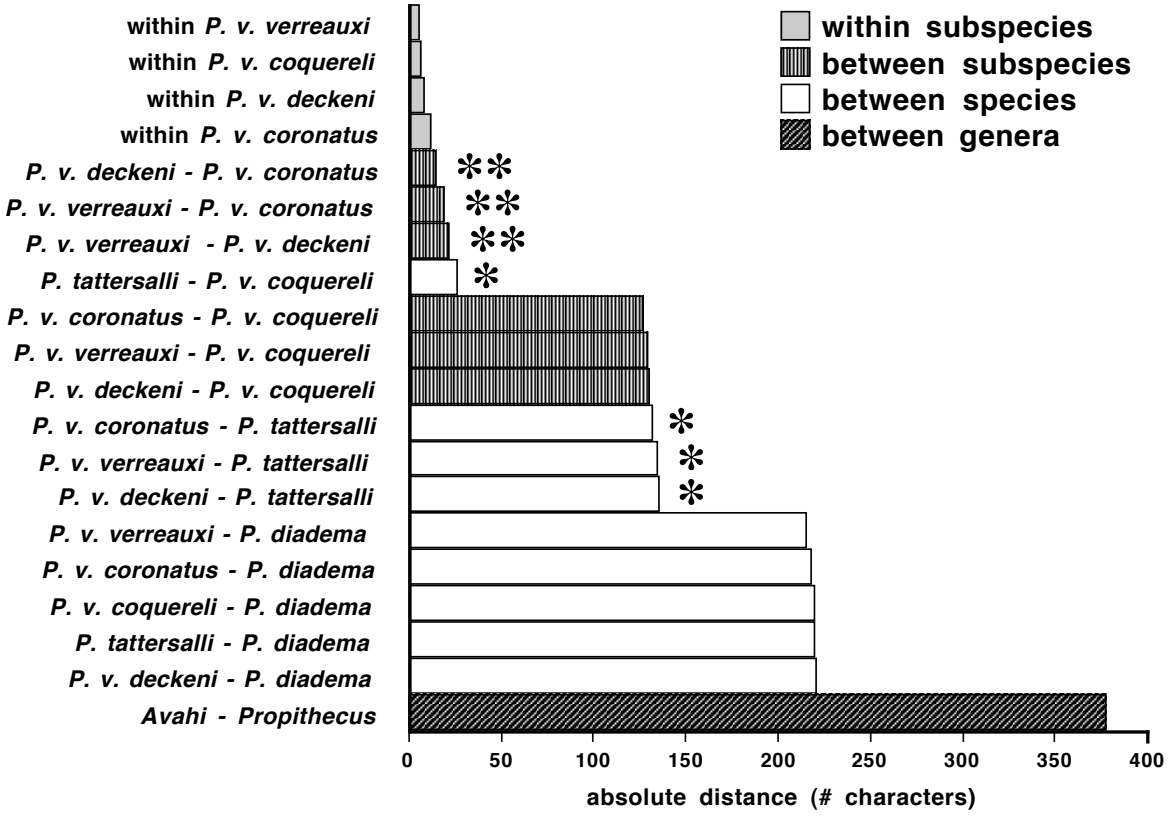


Fig. 7.6 Absolute pairwise distances over four defined taxonomic levels. Each bar represents the average of all possible comparisons between individuals of the two taxa. Single values are presented in Table 7.3. The unexpected small genetic distances between *P. tattersalli* and *P. verreauxi* are indicated by *, those between *P.v. verreauxi*, *P.v. deckeni* and *P.v. coronatus* by **.

Table 7.3 Kimura 2-parameter distance (above the diagonal) and absolute distance (under the diagonal) matrices derived from the 2389 bp mitochondrial DNA sequence data set, with gaps treated as missing data.

	A.l.	*P.d.*	*P.t.*	*P.v.v. 1*	*P.v.v. 2*	*P.v.v. 3*	*P.v.v. 4*	*P.v.d. 1*	*P.v.d. 2*	*P.v.d. 3*	*P.v.cr. 1*	*P.v.cr. 2*	*P.v.cr. 3*	*P.v.cq. 1*	*P.v.cq. 2*	*P.v.cq. 3*	*E.m.*	*E.f.*
Avahi laniger	-	0.194	0.178	0.186	0.187	0.185	0.185	0.188	0.188	0.185	0.182	0.183	0.184	0.178	0.177	0.178	0.258	0.249
P. diadema	396	-	0.101	0.099	0.099	0.099	0.099	0.102	0.102	0.102	0.100	0.101	0.100	0.101	0.101	0.102	0.226	0.217
P. tattersalli	368	221	-	0.061	0.060	0.060	0.060	0.061	0.061	0.060	0.058	0.060	0.058	0.011	0.012	0.011	0.234	0.224
P. v. verreauxi 1	381	217	137	-	0.003	0.003	0.003	0.009	0.009	0.009	0.006	0.008	0.009	0.057	0.059	0.058	0.226	0.216
P. v. verreauxi 2	384	217	136	8	-	0.004	0.004	0.008	0.008	0.009	0.007	0.008	0.009	0.058	0.058	0.057	0.225	0.215
P. v. verreauxi 3	380	216	135	6	10	-	0	0.010	0.010	0.010	0.008	0.009	0.010	0.056	0.058	0.057	0.224	0.214
P. v. verreauxi 4	380	216	135	6	10	0	-	0.010	0.010	0.010	0.008	0.009	0.010	0.056	0.058	0.057	0.224	0.214
P. v. deckeni 1	385	222	137	22	20	24	24	-	0	0.005	0.007	0.008	0.005	0.058	0.059	0.058	0.225	0.218
P. v. deckeni 2	385	222	137	22	20	24	24	0	-	0.005	0.007	0.008	0.005	0.058	0.059	0.058	0.225	0.218
P. v. deckeni 3	380	221	135	21	21	23	23	13	13	-	0.006	0.008	0.003	0.057	0.058	0.057	0.225	0.216
P. v. coronatus 1	375	218	131	14	16	18	18	16	16	15	-	0.002	0.006	0.056	0.056	0.055	0.226	0.220
P. v. coronatus 2	376	220	135	18	20	22	22	20	20	19	4	-	0.008	0.057	0.058	0.057	0.226	0.220
P. v. coronatus 3	378	217	132	21	21	23	23	13	13	6	15	19	-	0.056	0.056	0.056	0.226	0.217
P. v. coquereli 1	368	220	27	130	131	128	128	132	132	130	126	130	127	-	0.003	0.003	0.235	0.230
P. v. coquereli 2	367	220	28	133	132	131	131	133	133	131	127	131	128	7	-	0.004	0.235	0.230
P. v. coquereli 3	369	222	27	131	130	129	129	131	131	129	125	129	126	6	9	-	0.234	0.227
Eulemur macaco	507	456	468	455	454	452	452	453	453	453	455	456	455	469	469	468	-	0.088
Eulemur fulvus	492	440	452	438	436	435	435	442	442	438	444	445	440	461	461	456	194	-

7.4 Discussion

Phylogenetic Relationships

One goal of the present study was to clarify nodal relationships among indrid genera, species and subspecies. As expected, *Avahi* constitutes the earliest diverging taxon compared to *Propithecus* species (Figs. 7.3 - 7.5). This arrangement is strongly supported by bootstrap and jackknife analyses with both maximum parsimony and neighbor-joining methods (100%). In the maximum likelihood phylogram *Avahi* and *Propithecus* are separated by relatively long branches, which supports an early divergence (Fig. 7.5). Unfortunately, no sample from the genus *Indri* could be obtained for this analysis. Previous studies place either *Indri* (Tattersall & Schwartz 1974; Yoder 1994), *Avahi* (Sarich & Cronin 1976; Rumpler et al. 1988; Razafindraibe et al., 1997), or *Propithecus* (Tattersall 1982; Jungers et al. 1991; Stanger-Hall 1997) as the earliest genus to diverge within the Indridae.

According to the results obtained, *P. tattersalli* and *P. verreauxi* consistently form a sister group relationship to *P. diadema* (Figs. 7.3 - 7.5). The basal divergence of the species *P. diadema* among sifakas identified in this study is not in agreement with previously published results from a study of highly repeated DNA band patterns, which resolved *P. tattersalli* sister to a clade containing *P. verreauxi* and *P. diadema* (Razafindraibe et al. 1997). So far, no other phylogenetic study has included all three sifaka species.

Paraphyletic* P.v. verreauxi/P.v. deckeni/P.v. coronatus *Clade

The two taxa *P.v. deckeni* and *P.v. coronatus* consistently fail to resolve into separate monophyletic lineages (Figs. 7.3 - 7.5). Additionally, average genetic distances between *P.v. deckeni* and *P.v. coronatus* (6–20 bp) clearly lie in the range of within-taxon comparisons (0–19 bp, Table 7.3). The same is true for genetic divergence of *P.v. verreauxi* and *P.v. deckeni* (20–24 bp) or *P.v. coronatus* (14–23 bp). No individual in this *coronatus*/*deckeni*/*verreauxi* clade is distinctly delineated in the maximum likelihood phylogram (Fig. 7.5).

Based on tree topology and pairwise distances, the molecular data presented in this study thus do not support monophyletic lineages for *P.v. verreauxi*, *P.v. deckeni* or *P.v. coronatus*. Other phylogenetic studies of lemurs using the same genes clearly resolve the subspecies (see other Chapters), thus excluding the possibility that the lack of resolution among sifaka subspecies is due simply to the genes chosen for sequencing. The genetic data therefore provide

strong support for combination of *P.v. verreauxi*, *P.v. deckeni* and *P.v. coronatus* into a single subspecies.

***Taxonomic Status of* P.v. coronatus**

As mentioned above, both the distribution and the taxonomic relationships of *P.v. deckeni* and *P.v. coronatus* are uncertain. *P.v. deckeni* and *P.v. coronatus* occupy the region of northwestern Madagascar between the Betsiboka and Tsiribihina rivers (Fig. 7.1). Originally, the range of *P.v. coronatus* was placed between the Betsiboka and Mahavavy rivers and that of *P.v. deckeni* to the west and south of the Mahavavy river all the way south to the Tsiribihina river. However, this neat geographic separation of *P.v. coronatus* and *P.v. deckeni* has been questioned (Petter et al. 1977; Tattersall 1982). Variation in pelage coloration of *P.v. deckeni* has been reported especially from the Bongolava Massif (Petter et al. 1977). Recently, *P.v. coronatus* and *P.v. deckeni* have been sighted on the southern banks of the river Manambolo, which is south of the distribution range of *P.v. deckeni* (Thalmann & Rakotoarison 1994). Clearly, *P.v. deckeni* is not confined to the area west of the Mahavavy, as sightings at Katsepy attest (Tattersall 1986).

The Mahavavy river therefore does not appear to genetically isolate *P.v. deckeni* from *P.v. coronatus*. In *Eulemur fulvus*, the Mahavavy river also fails to act as a genetic barrier (see Chapter 3). So far, no lemur taxa seem to be isolated by this river. The Mahavavy river, which originates in the Bongolava Massif, constitutes a potential distributional barrier for lemurs but its different tributaries do not serve as efficient barriers in the Bongolava Massif (Thalmann & Rakotoarison 1994). As a result, the Bongolava Massif may serve as a contact zone between the different subregions (Thalmann & Rakotoarison 1994).

Because of the puzzling variation in pelage coloration in *P.v. deckeni* and *P.v. coronatus*, and their observed sympatry, Tattersall (1988b) suggested synonymy of *P.v. deckeni* with *P.v. coronatus*. The genetic data strongly support this conclusion.

***Taxonomic Status of* P.v. deckeni**

No previously published studies have suggested unifying *P.v. verreauxi* with *P.v. deckeni* or *P.v. coronatus* within a single subspecies. Along with the distinct pelage colour, *P.v. deckeni* and *P.v. coronatus* possess an inflated muzzle. This morphological feature distinguishes *P.v. deckeni* and *P.v. coronatus* from all other subspecies of *P. verreauxi*, which

retain the primitive uninflated muzzle (Tattersall 1986). Based on the present study, the Tsiribihina river does not seem to be a barrier to gene flow for *P.v. verreauxi* and *P.v. deckeni*. This is in contrast to *Lepilemur*, for which the Tsiribihina river separates the distribution areas of *L. edwardsi* and *L. ruficaudatus* (Petter et al. 1977; Tattersall 1982). Likewise, in *Eulemur fulvus* there are indications that the Tsiribihina river acts as a barrier to gene flow (see Chapter 3).

The recognition of *P.v. deckeni* as a separate subspecies has been supported on the basis of pelage characteristics, but pelage variation is only weakly diagnostic at best for sifakas. Because of the considerable variation in pelage pattern and colour within sifakas, the history of subspecies designations for *P. diadema* and *P. verreauxi* is rather complex. In 1982, Tattersall placed *P.v. majori* in synonymy with *P.v. verreauxi*. "*P.v. majori*" has been observed on several occasions in mixed groups with *P.v. verreauxi*, which strongly indicated that *P.v. majori* is merely a melanistic variant of *P.v. verreauxi*. *P.d. edwardsi* and *P.d. holomelas* were also presumed to be only colour variants, because analysis of all available locality information showed that the two supposedly distinct subspecies were sympatric in at least one forest (Tattersall 1986).

***Taxonomic Status of* P. tattersalli**

Another aim of this study was to examine the taxonomic status of the recently discovered *P. tattersalli*. Interestingly, in all analyses *P. tattersalli* and *P.v. coquereli* form a subclade which groups as sister to the remaining *P. verreauxi* subspecies (Figs. 7.3 - 7.5). This arrangement has very strong bootstrap or jackknife support (100%). In the maximum likelihood phylogram, long branches separate those two clades. However, only a very short branch separates *P. tattersalli* from the clade formed by the three *P.v. coquereli* individuals (Fig. 7.5). Pairwise distances (Fig. 7.6) between *P. tattersalli* and *P. verreauxi* (27–137 bp) never reach the range of comparison between *P. diadema* and *P. tattersalli* or *P. verreauxi* (216–222 bp). Furthermore, genetic distances between *P. tattersalli* and *P.v. coquereli* do not exceed 28 bp, which suggests a very recent divergence of those two taxa.

Discrimination of the new species *P. tattersalli* from *P. verreauxi* and from *P. diadema* was previously supported by morphological characters (Simons 1988) and repeated DNA band patterns (Razafindraibe et al. 1997). However, the only available skull length of *P. tattersalli* (83 mm) is comparable to an intermediate-sized subspecies of *P. verreauxi* (80.2–83.9 mm) and

is smaller than *P. diadema* (85.7–91.2 mm) (Simons 1988; Albrecht et al. 1990). Also ear length, body weight, tail length and head & body length of *P. tattersalli* all lie in the range of *P. verreauxi* (see measurements table in Simons 1988). The ground predator alarm call of *P. tattersalli* closely resembles that of *P. verreauxi* (Simons 1988). Indisputably, however, *P. tattersalli* has completely furred ears with long hair tufts. Such conspicuously furred ears do not occur in any other kind of sifaka. Additionally, *P. tattersalli* and *P. diadema* are cytogenetically more closely related than either is to *P. verreauxi* (Simons 1988).

If *P. tattersalli* is, indeed, nested within the *P. verreauxi* clade, a cladistic approach to classification would require either uniting *P. tattersalli* and *P. verreauxi* into one species or separating *P.v. coquereli* at the species level. Based on tree topology and pairwise distances, the molecular data presented in this study would seem to indicate that *P.v. coquereli* and *P. tattersalli* should be united into the same subspecies. More comparative studies including *P. tattersalli* are required before a decision can be made, but on the basis of the genetic data so far available it is suggested that *P. tattersalli* does not deserve species status.

***Taxonomic Status of* P.v. coquereli**

The deep divergence between the *P.v. coquereli*/*P. tattersalli* clade and the clade containing the other three *P. verreauxi* subspecies is an unexpected result. Sifakas of the two clades differ from each other on the order of 126–137 bp (Fig. 7.6, Table 7.3). This is not as high as divergences among *P. diadema* and *P. verreauxi* or *P. tattersalli* (216–222 bp). Nonetheless, pairwise distances between the *coquereli*/*tattersalli* clade and the *verreauxi*/*deckeni*/*coronatus* clade nearly reach the level of differentiation found among the five well-accepted *Eulemur* species (138–201 bp, see Chapter 5). Within each clade, on the other hand, pairwise distances do not exceed 28 bp.

The data imply that separation between the *coquereli*/*tattersalli* clade and the *verreauxi*/*deckeni*/*coronatus* clade occurred later than among *P. diadema* and other sifaka species. However, genetic data indicate that speciation among sifakas from northwestern/northern and southwestern/southern Madagascar may already be complete. A large river drainage system, the Betsiboka, lies between those two localities (Fig. 7.1). This river is a known isolation barrier to gene flow for *Eulemur fulvus* (see Chapter 3), *Hapalemur griseus* (Chapter 4), and *Lepilemur* (Chapter 8).

Evolution of Sifakas

The molecular data strongly support the early divergence of *P. diadema* among sifakas. *P.v. coquereli* and *P. tattersalli* together form the sister group to a clade containing *P.v. verreauxi*, *P.v. deckeni* and *P.v. coronatus*. Considerable genetic differentiation exists among those two clades, which indicates that they may represent two species of sifakas along the western coast. The sequence data, however, do not yield clear resolution of *P.v. coronatus* from *P.v. deckeni* or *P.v. verreauxi*, thus indicating that *P.v. deckeni* and *P.v. coronatus* do not deserve subspecific rank. Furthermore, *P. tattersalli* is clearly resolved within *P. verreauxi*. There is obviously a taxonomic problem regarding paraphyly of these two species. Genetic differentiation of *P. tattersalli* from *P.v. coquereli* is very low, suggesting that those two taxa diverged very recently if at all.

The data set suggests that evolution within the genus *Propithecus* took place in two steps. Originally *P. diadema* (east) separated from *P. verreauxi* (west), then the latter split into the *verreauxi-deckeni-coronatus* clade (southwest) and the *coquereli-tattersalli* clade (north-west). These two clades might already have been separated long enough to have achieved specific rank. However, within each of these two clades no further significant genetic differentiation has occurred in the populations sampled. The molecular data do not support any subspecific divergence among western sifakas.

Relatively few previous examinations have investigated the evolutionary relationships among sifakas. No other DNA sequence data sets have been published which examine the phylogenetic relationships among the different populations within this genus. To achieve a better understanding of the evolution of *Propithecus*, the present data set should be supplemented by additional samples that expand on the current work. Of particular interest would be samples from the different pelage forms of *P. diadema* that occur along the eastern coast. It might also be valuable to add additional individuals of *P. tattersalli* to the data set, should samples become available.

8. Infraorder Lemuriformes

This chapter is to be published in a modified form.

8.1 Introduction

The evolution of the lemurs represents a spectacular example of adaptive radiation among primates, providing an excellent model for studies of evolutionary biology (Martin 1972, 1995). Madagascar provided the natural experimental conditions required to produce this outstanding radiation. Madagascar is the world's fourth largest island and has a diverse geology, climate and vegetation. Most of the flora and fauna of Madagascar is unique to this island. Madagascar can be divided into seven major zones of species distribution, each of which has distinctive climatic and vegetational characteristics (Martin 1972). Those climatic and vegetational factors are important in understanding the zoogeographic, evolutionary, and general biology of the Malagasy prosimians.

The infraorder Lemuriformes is part of the primate suborder Strepsirrhini. Today, five distinct extant lemur families are recognised (Cheirogaleidae, Indridae, Lemuridae, Lepilemuridae, Daubentoniidae), all of which are endemic to the island of Madagascar. Evolutionary relationships and taxonomical classification of the lemurs continues to be a highly controversial topic. With five endemic primate families, including 14 genera, 33 species and 50 distinct taxa, Madagascar's diversity ranks third highest on the world list of primate species (Mittermeier et al. 1994). In addition to the extant lemurs at least 17 species of extinct lemurs have been found on Madagascar (Godfrey et al. 1999).

In this study, representatives of all five lemur families have been analysed. Three of those families have been addressed in previous chapters. In this chapter, only the overall relationship among those major families, relationships within the family Lepilemuridae, and the monophyly of the lemurs are addressed. At present, a tentative consensus accepts four genera (*Eulemur*, *Hapalemur*, *Lemur*, *Varecia*) in the family Lemuridae, which includes 10 species. For additional details on the taxonomy and systematics of the taxa within this family see Chapters 3, 4 and 5. The historical literature and the results of this investigation of phylogenetic relationships within the Lemuridae are also addressed in those chapters. The Cheirogaleidae are currently classified into five genera (*Allocebus*, *Cheirogaleus*, *Microcebus*, *Mirza*, *Phaner*),

which contain at minimum nine species. The taxonomic and evolutionary history of this family and the phylogenetic results obtained in this study are described in Chapter 6. The family Indridae includes six species in three genera (*Avahi*, *Indri*, *Propithecus*). Chapter 7 contains an overview of the different taxa of this family and the results obtained in this study.

Lepilemur (Geoffroy 1851) is the only genus within the family Lepilemuridae. The classification, specifically the nomenclature and taxonomy, of *Lepilemur* has been the subject of much controversy and discussion. The genus is common across Madagascar and is currently divided into a maximum of seven different species (Petter et al. 1977; Harcourt & Thornback 1990; Groves 1989; Mittermeier et al. 1994). However, some authors prefer to unite all forms in the single species *L. mustelinus* with 6 different subspecies (Tattersall 1982). *L. leucopus* (Major 1894) occurs in the dry southern portion of Madagascar. *L. dorsalis* (Gray 1870) can be found on Nosy Be Island and on the small area of rain forest on the northwest coast. *L. mustelinus* (Geoffroy 1851) is confined to the northern half of the east coast. *L. microdon* (Major 1894) inhabits the southern half of the eastern rain forest. *L. septentrionalis* (Rumpler & Albignac 1975) inhabits the far north of Madagascar. *L. edwardsi* (Major 1894) lives in the northern half, and *L. ruficaudatus* (Grandidier 1867) in the southern half of the western dry forest zone (Fig. 8.1).

The family Daubentoniidae contains only one extant lemur species. Since its early description as a rodent, *Daubentonia* (Geoffroy 1795) has been a challenge to primate systematists. Morphologically, *Daubentonia madagascariensis* (Gmelin 1788) is the most peculiar of Madagascar's lemurs. Long, coarse blackish-brown guard hairs overlay a dense layer of relatively short white hair, giving the overall impression of dark brown pelage suffused with white. The tail is bushy, and the ears are large, naked and mobile. The incisors (one in each tooth row) are long, chisel-like and continually growing. The digits of the hand, all claw-bearing, are elongated and the middle finger is attenuated to serve as a probe (Martin 1990). *Daubentonia* is probably still widely, but apparently at very low densities, distributed throughout the eastern rain forests. It also occurs in the north, northwest and west of Madagascar (Tattersall 1982; Harcourt & Thornback 1990; Mittermeier et al. 1994).

Phylogenetic relationships among the five extant lemur families remain an issue of controversy. Data sets based on morphology (Tattersall & Schwartz 1974; Yoder 1994; Yoder et al. 1996; Stanger-Hall 1997), behaviour (Stanger-Hall 1997), immunodiffusion (Dene et al.

1980), proteins (Sarich & Cronin 1976), chromosomal banding patterns (Rumpler et al. 1989) and DNA sequences (Adkins & Honeycutt 1994; Yoder 1994; Del Pero et al. 1995; Porter et al. 1995; Yoder et al. 1996; Stanger-Hall & Cunningham 1998) have been used to analyse evolutionary relationships among lemur families. Despite all those efforts, there is, as yet, no general consensus on lemur evolution.

Monophyly of the Lemuridae, as well as of the Lepilemuridae, has been questioned several times. *Varecia* has been placed apart from the family Lemuridae (Stanger-Hall 1997). *Hapalemur* and *Lepilemur* together have been grouped in the family Lepilemuridae (Tattersall & Schwartz 1974). Another broad problematic issue in primate systematics concerns the monophyly of the Malagasy lemurs. In the past, the Cheirogaleidae have been considered to be a member of the lorisiform clade (Szalay & Katz 1973; Tattersall & Schwartz 1974). Additionally, the Daubentoniidae have been placed at the base of all Strepsirrhini (Adkins & Honeycutt 1994). However, at present the monophyly of lemurs is generally accepted (Martin 1990, 2000; Yoder 1997).

The primary goal of this study was to generate a mitochondrial DNA sequence data set to clarify the phylogenetic relationships among the five lemur families. This also includes an assessment of the phylogenetic status of each of the families, especially the Lepilemuridae. A further aim was to confirm the affiliation of each genus to its family. Of special interest was the phylogenetic position of the genus *Hapalemur*, which has sometimes been considered a member of the family Lepilemuridae. A further aim of this study was to clarify the specific status of the different *Lepilemur* forms. Finally, the analyses seek to verify the phylogenetic position of *Daubentonia* among the Strepsirrhini in order to test the monophyly of the Malagasy lemurs. To achieve this goal, samples from a total of 131 lemurs from 12 genera, 25 species and 18 subspecies have been sequenced. Different weighting schemes and outgroup analyses have been applied in the attempt to resolve the families and to test the monophyly of lemurs.

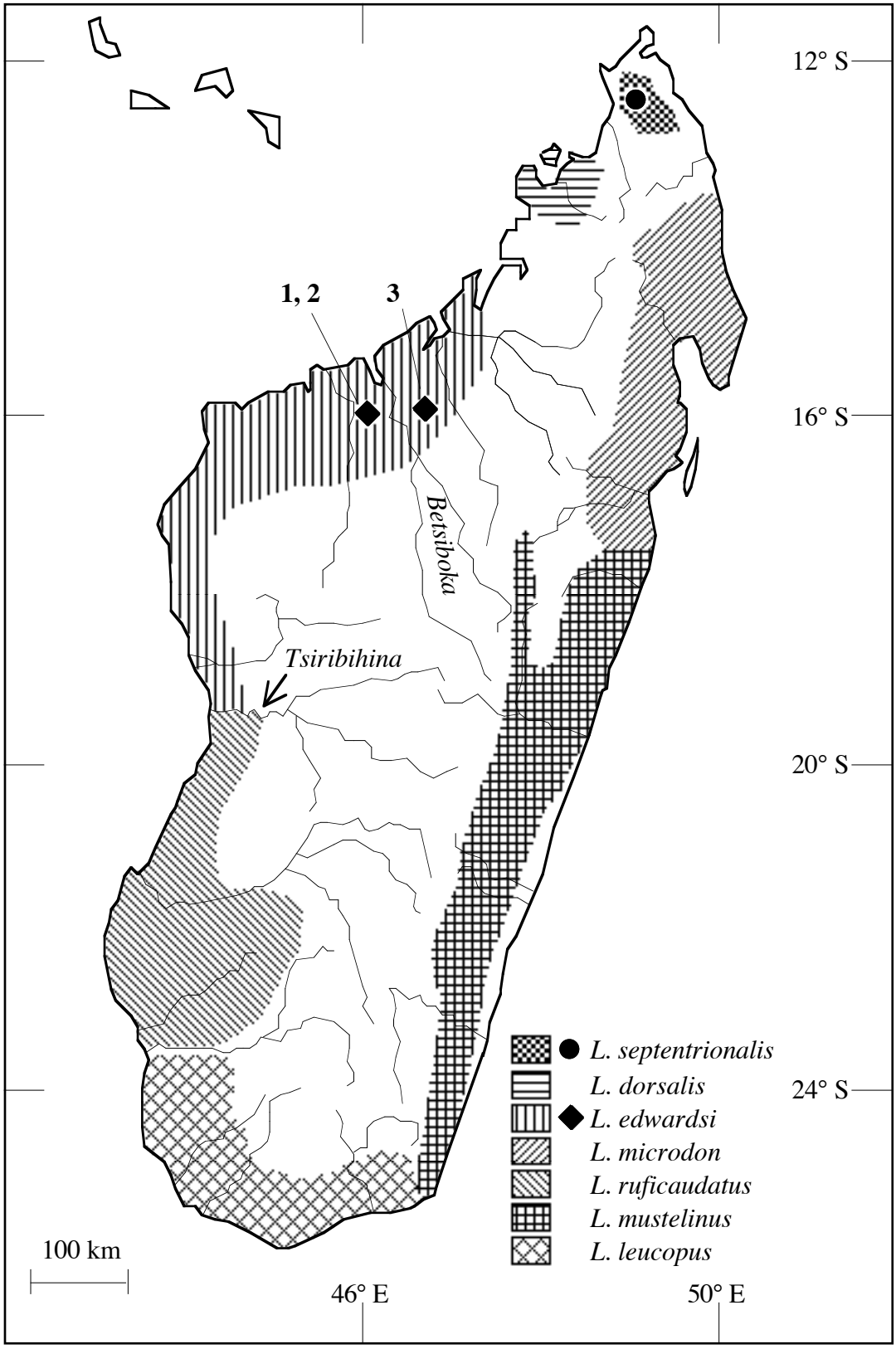


Fig. 8.1 Map of Madagascar showing approximate areas of distribution of the species of *Lepilemur* according to Tattersall (1982) and Mittermeier et al. (1994). Symbols indicate individuals with exact locality data that were included in the present study.

8.2 Material

This study includes 12 (86%) of the 14 extant lemur genera. Unfortunately, no samples from the genera *Indri* (Indridae) and *Phaner* (Cheirogaleidae) could be obtained. The data set includes 25 (76%) of the 33 currently recognised species. *Indri indri*, *Phaner furcifer*, *Microcebus myoxinus*, *Avahi occidentalis* and four *Lepilemur* species are the only species missing. Additionally, a total of 18 subspecies (ssp.) from *Hapalemur griseus* (3 of 4 ssp.), *Eulemur fulvus* (7 ssp.), *E. macaco* (2 ssp.), *Varecia variegata* (2 ssp.) and *Propithecus verreauxi* (4 ssp.) could be included in this study. No different subspecies could be investigated from *Propithecus diadema* (4 ssp.) or *Phaner furcifer* (4 ssp.). Two galagos (*Otolemur crassicaudatus* and *Galago senegalensis*) were sequenced to serve as an outgroup. Table 8.1 lists the new samples not used for analyses presented in the previous chapters (see Tables 3.1, 4.1, 5.1, 6.1, 7.1). The complete set of samples utilised is also listed in the Appendix. All known localities of the samples from the genera *Lepilemur* and *Daubentonia* are shown in Figure 8.1. Other known sample localities can be found in Figures 3.1, 4.1, 6.2 and 7.1 of the previous chapters.

Table 8.1 Taxa, sex, origin, identification numbers, and GenBank accession numbers for 12 lemurs and 2 galagos sequenced.

Taxon	Origin	ID #	GenBank #
Eulemur mongoz 4	unknown [a]	JP1, AIMUZ 9208	AF224512
Eulemur mongoz 5	unknown [b]	JP49	AF224513
Eulemur mongoz 6	unknown [c]	JP240	AF224521
Eulemur mongoz 7	Anjamena (Northwest) [d]	JP178	AF224516
Eulemur mongoz 8	Anjamena (Northwest) [d]	JP196	AF224517
Eulemur mongoz 9	Anadabomandry (Northwest) [d]	JP211	AF224518
Eulemur mongoz 10	Ampijoroa (Northwest) [d]	JP221	AF224520
Lepilemur edwardsi 1	Anjamena (Northwest) [d]	JP163	AF224593
Lepilemur edwardsi 2	Anjamena (Northwest) [d]	JP207	AF224594
Lepilemur edwardsi 3	Ampijoroa (Northwest) [d]	JP259	AF224595
Lepilemur ruficaudatus	unknown	JP233	AF224596
Lepilemur septentrionalis	Ankarana, Mahamasina (North) [d]	JP280	AF224597
Otolemur crassicaudatus	unknown [a]	JP8, AIMUZ 10112	AF224643
Galago senegalensis	unknown [a]	JP53, AIMUZ 9982	AF224644

[a] specimen held at Anthropological Institute and Museum of the University of Zürich (AIMUZ), Switzerland

[b] held at Parc Zoologique et Botanique de Mulhouse, France

[c] specimen held at Museum Koenig in Bonn, Germany (previously held at Zoo Köln)

[d] wild-caught animals with verified origin

8.3 Results

As many taxa (genera, species and subspecies) as possible have been included in the data set to provide the most complete possible data set from which to examine lemur evolution. The new mtDNA sequences generated for the 133 taxa examined have been deposited in GenBank (Appendix). The nucleotide sequences span a total of 2387 base positions (bp). The analysed data set consists of the 3' end of the COIII gene (30 bp), the complete NADH-dehydrogenase subunits ND3 (348 bp), ND4L (297 bp) and ND4 (1381 bp) along with the glycine (73 bp), arginine (71 bp), histidine (71 bp), serine (66 bp), and partial leucine (47 bp) tRNA genes. The sequences obtained provided 1270 parsimony-informative characters. A summary of the frequencies of invariant, parsimony uninformative, and informative characters along the segment sequenced is given in Tables 8.2 and 8.3.

mtDNA Sequence Variation

In most lemurs examined, the ND3 gene is terminated by 'TAA'. However, in *Avahi* and *Propithecus verreauxi coquereli* the stop codon is 'TAG'. In *Daubentonia* and galagos 'TA' and in *Homo* only 'T' serve as the stop codons via polyadenylation.

Lemurs have an insertion of 2 or 3 bp between the ND3 gene and the $tRNA^{Arg}$. The mtDNA genomes of *Homo*, galagos, or *Daubentonia* do not contain any untranslated bp between these genes. Most lemurs have 1 additional bp between $tRNA^{Arg}$ and ND4L. The galagos have 2 additional bp and *Allocebus* is the only genus with 4 additional bp between those genes. No additional bp are present in *Hapalemur griseus*, *H. aureus*, *Lepilemur* and *Homo* between $tRNA^{Arg}$ and ND4L. *Allocebus* has an insertion of 3 bp between the tRNAs histidine and serine that is not present in other lemurs, galagos or *Homo*. Finally, *C. medius* has a repetition 1 (8 bp) to 8 (64 bp) times of the A-stem of the $tRNA^{Leu}$ between the tRNAs for

Table 8.2 Summary of variation for the sequences across the 131 lemurs and 2 galagos examined.

Genes	All	COIII	ND3	ND4L[a]	ND4[a]	tRNAs	Not translated
characters (nucleotides)	2387	30	348	297	1381	328	10
constant	1016	20	147	122	567	166	0
parsimony-uninformative	101	2	11	15	49	19	5
parsimony-informative	1270	8	190	160	765	143	5
informative proportion	0.53	0.27	0.55	0.54	0.55	0.44	0.50
insertions/deletions	49	0	1	0	12	26	10

[a] ND4L and ND4 overlap for 7 bp

Table 8.3 Summary of variation for the sequences of the five different tRNAs across the 131 lemurs and 2 galagos examined.

Genes	tRNAs	Gly	Arg	His	Ser	Leu
characters (nucleotides)	328	73	71	71	66	47
constant	166	37	40	31	24	34
parsimony-uninformative	19	6	5	2	3	3
parsimony-informative	143	30	26	38	39	10
informative proportion	0.44	0.41	0.37	0.54	0.59	0.21
insertions/deletions	26	7	7	3	9	0

serine and leucine. The $tRNA^{Leu}$ beyond this repetitive sequence element retains correct structure and anticodon. No other anomalies were seen and this insertion has been excluded from all analyses, tables or graphs presented in this study.

Daubentonia has a deletion of 6 bp, coding for 2 amino acids in the ND4 gene (positions 49 and 50, Leu and Phe in the human genome). *H.g. griseus* , *H.g. alaotrensis* and *H. aureus* have a deletion of 3 bp, coding for the 48th amino acid (Asn in the human genome), and the galagos have 1 amino acid deleted at position 49. Galagos have an insertion of 3 bp or 1 amino acid in the ND4 gene which is not present in any lemur or *Homo*. Several insertions or deletions have occurred in the loops of tRNA genes. Tables 8.2 and 8.3 indicate how many gaps are found in each gene for the lemurs analysed in this study (exclusive of *Homo*).

Genetic Distances

Absolute pairwise distances range from a maximum of 645–733 bp between *Homo* and the ingroup to between 0 and 586 bp within the Malagasy lemurs. Pairwise distances between galagos and lemurs range from 576–650 bp. The divergences between *Daubentonia* and other lemurs are slightly higher (518–586 bp) than between the 4 other lemur families (421–560 bp).

Examination of absolute genetic distances within the genus *Lepilemur* reveals different levels of differentiation (Table 8.4). Two *L. edwardsi* individuals differ from each other by only 4 bp, while they differ from the third *L. edwardsi* individual by 261 and 265 bp. Pairwise comparisons of *L. ruficaudatus* with the first two *L. edwardsi* give values of 142 and 146 bp.

Table 8.4 Kimura 2-parameter distance (above the diagonal) and absolute distance (under the diagonal) matrices derived from the mitochondrial DNA sequence data set, with gaps treated as missing data.

	L. edwardsi 1	*L. edwardsi 2*	*L. edwardsi 3*	*L. ruficaudatus*	*L. septentrion.*
Lepilemur edwardsi 1	–	0.002	0.124	0.064	0.128
Lepilemur edwardsi 2	4	–	0.126	0.066	0.130
Lepilemur edwardsi 3	261	265	–	0.126	0.101
Lepilemur ruficaudatus	142	146	265	–	0.131
Lepilemur septentrionalis	270	274	218	275	–

The divergences between *L. septentrionalis* and the third *L. edwardsi* are lower (218 bp) than between *L. septentrionalis* and the two *L. edwardsi* or *L. ruficaudatus* (270–275 bp).

Unweighted Analyses

The maximum parsimony heuristic search with all characters weighted equally results in 544 trees (Fig. 8.2). The distance matrices constructed using Kimura 2-parameter corrections were analysed by neighbor-joining (Fig. 8.3). The support values from bootstrap and jackknife analyses are in the same range as for maximum parsimony analyses. The results of the maximum likelihood analysis are presented in Figure 8.4. The phylogram presented maintains branch lengths proportional to the number of changes. The final maximum likelihood tree (–ln likelihood = 31425.92) was obtained from a transition/transversion ratio of 6.61 (kappa = 14.23) and gamma shape parameter of 0.32.

Relationships among subspecies, species or genera of Lemuridae, Cheirogaleidae or Indridae have been discussed in the previous chapters. For each of these chapters the analyses were restricted to only that subset of the data set. The results from the simultaneous analyses of all 12 genera, 25 species and 18 subspecies are presented in this chapter. Analyses from partial data sets generally reconstruct the same topology for the arrangement of taxa within a family as do analyses from the whole data set. Furthermore, bootstrap support values are in the same range, no matter how many taxa are included into the analyses.

Generally, there is good resolution among genera, species and subspecies across all the taxa examined, by maximum parsimony, neighbor-joining or maximum likelihood methods. Additionally, there are very high bootstrap supports for both maximum parsimony and neighbor-joining analyses with respect to the branching order of genera, species or subspecies. However, there have been two problematic zones in the phylogenetic trees analysed in the previous chapters. One is the phylogenetic arrangement of the five *Eulemur* species and the other is the trichotomy of *L. catta*, *H. simus* and the *H. griseus*/*H. aureus* clade (see Chapter 5). No further resolution of those two problematic areas in the phylogenetic tree could be obtained by increasing the data set to 131 individuals from all five lemur families. In the current chapter, neither the phylogenetic relationships among *Eulemur* species nor those among the *Hapalemur* species and *L. catta* will be further discussed. This chapter focuses only on the family Lepilemuridae, the phylogenetic relationships among the five lemur families and the monophyly of lemurs.

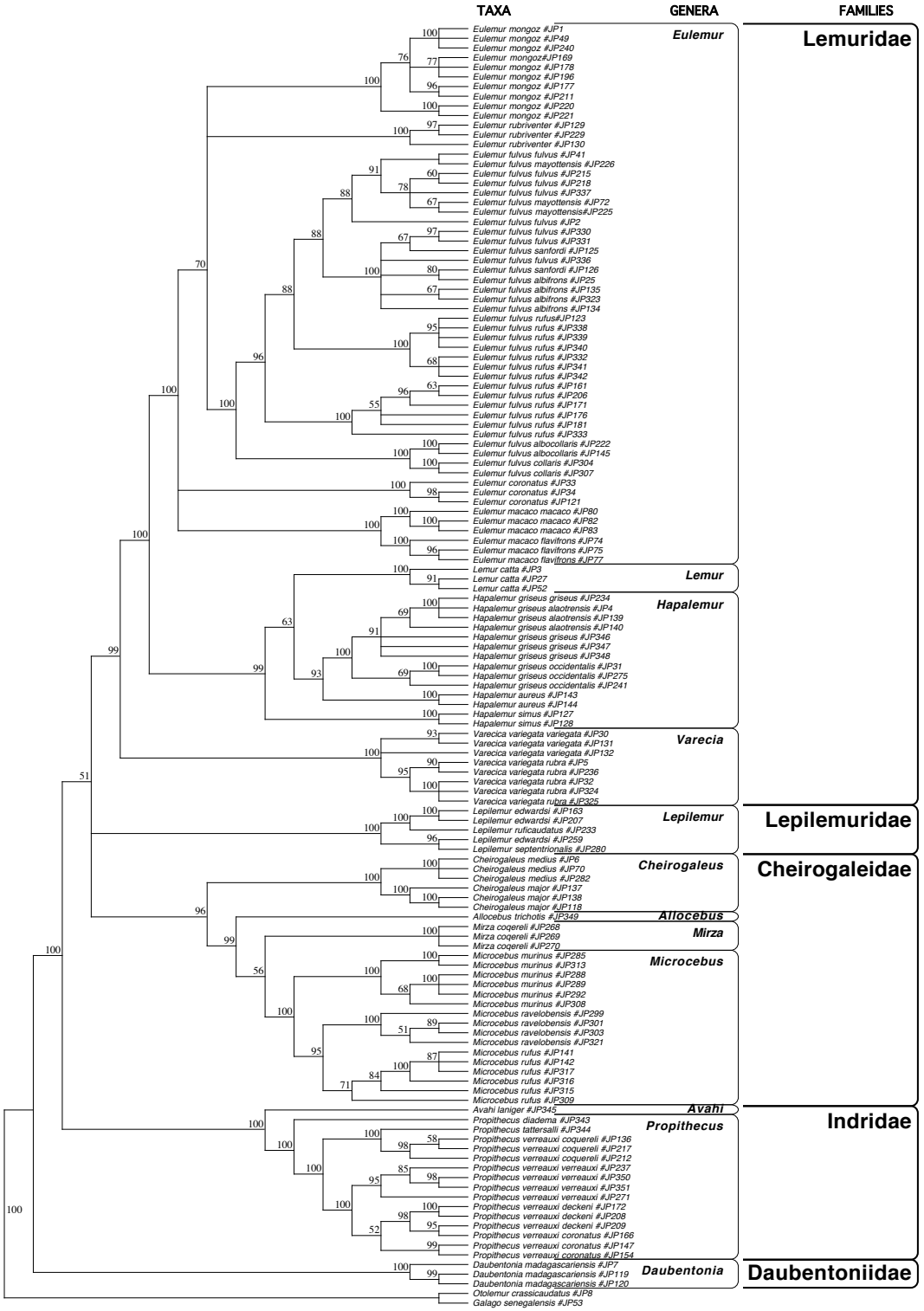


Fig. 8.2 Maximum parsimony tree with bootstrap values (as percentages, above nodes) obtained in 100 replicates.

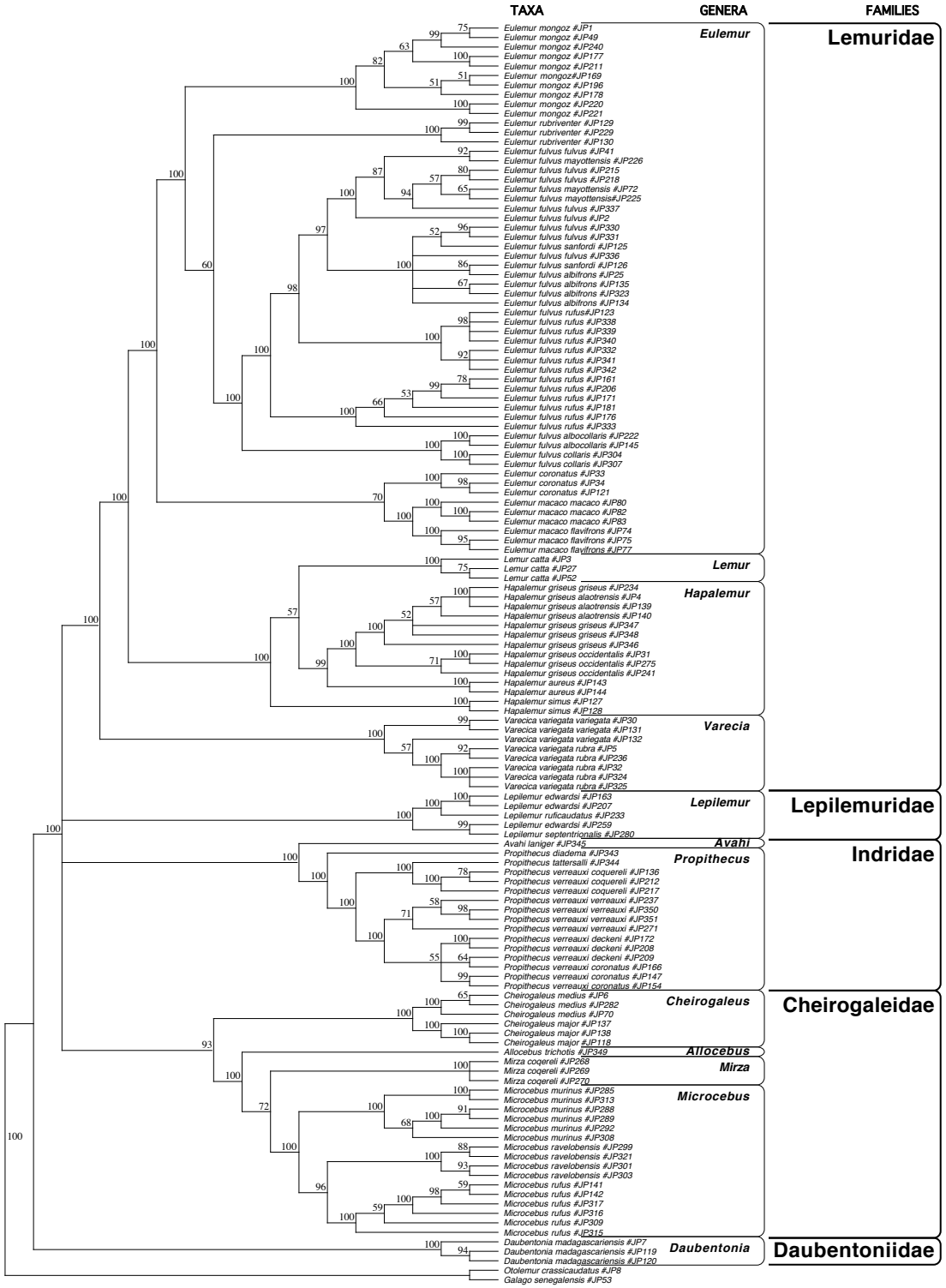


Fig. 8.3 Neighbor-joining tree with bootstrap values (as percentages, above nodes) obtained in 1000 replicates using Kimura 2-parameter distance correction.

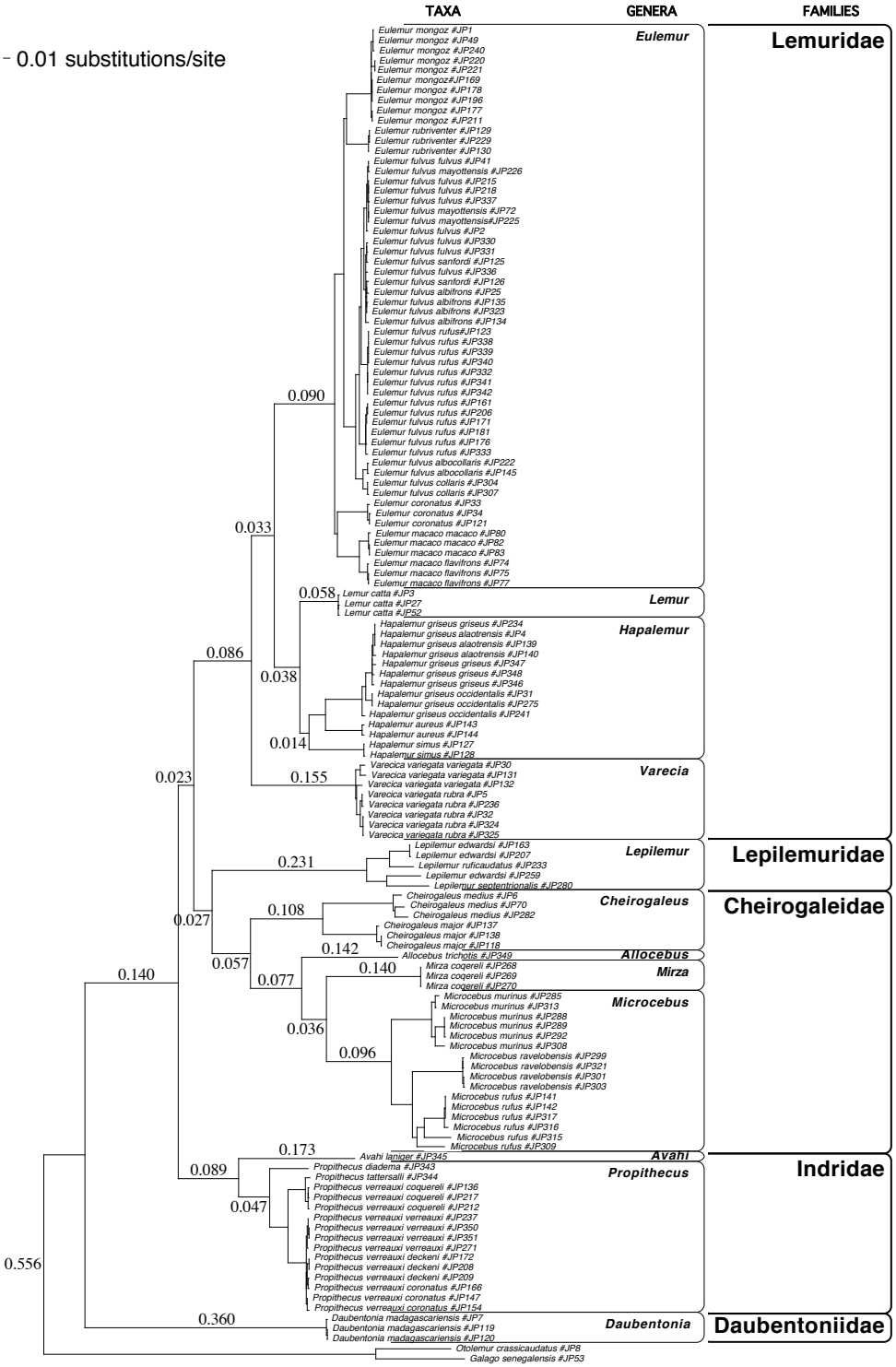


Fig. 8.4 Maximum likelihood phylogram with proportional branch lengths (values provided on each branch).

In maximum parsimony, neighbor-joining and maximum likelihood analyses, two *Lepilemur edwardsi* individuals form a clade which groups as the sister to *L. ruficaudatus*, while the third *L. edwardsi* forms a clade with *L. septentrionalis*. Those arrangements are strongly supported by bootstrap analyses (96–100%) using maximum parsimony and neighbor-joining searches.

In all analyses, *Daubentonia* is unambiguously the first genus to diverge among lemurs. The clade including the Cheirogaleidae, Indridae, Lemuridae and Lepilemuridae is supported in both maximum parsimony and neighbor-joining analyses with bootstrap values of 100%, such that the family Daubentoniidae was separated first from the other lemur families. Branching arrangements among Cheirogaleidae, Indridae, Lemuridae and Lepilemuridae differ between the different analyses. However, there is no bootstrap support in maximum parsimony and neighbor-joining analyses for any of those phylogenetic relationships. The unweighted sequence data do not permit conclusive resolution of the phylogenetic relationships among four of the five lemur families.

Reduction of the Number of Taxa

Because so many taxa have been included into this study, the data set provides the unique possibility to investigate influences of taxon sampling. A smaller data set would significantly reduce computing times. However, before running analyses with only part of the data set it is important to test whether the resolution changes in the modified data set.

In a first step, the data set is reduced in a way that only two individuals per subspecies are included into the analyses (SSP data set). For this selection, *Eulemur fulvus sanfordi*, *E.f. mayottensis*, *Hapalemur griseus alaotrensis*, *Propithecus verreauxi deckeni* and *P.v. coronatus* are not recognised as subspecies. For *E.f. rufus*, *H.g. occidentalis*, *Varecia variegata variegata*, *Cheirogaleus medius*, *Microcebus murinus*, *M. rufus* and *Lepilemur edwardsi* more than two individuals have been included because genetic differentiation strongly indicates that more than one subspecies is actually involved within each of those taxa (see previous chapters for details). This reduced SSP data set includes 71 lemurs representing 54% of the original data set (131 lemurs).

A third data set is produced including only two individuals per species (SP data set). If more than one subspecies is available in the data set, the two individuals are chosen from different subspecies. *Propithecus tattersalli* is only considered as a subspecies of *P. verreauxi*

coquereli. For *P. verreauxi*, *Microcebus rufus* and *Lepilemur edwardsi* more than two individuals have been included, because the genetic data presented here provide strong evidence that those taxa include more than one species (see previous chapters for details). The SP data set contains 37% of the lemurs from the original data set (48 of 131 lemurs).

The fourth data set includes only one representative per genus (GE data set). The GE data set contains 12 individuals from all 12 genera of the original data set.

Analyses of the four different data sets yield very similar phylogenetic trees. Table 8.5 gives an overview of the supported clades for all the different analyses. In all maximum parsimony and neighbor-joining analyses, *Daubentonia* is the first family to diverge among lemurs. This arrangement is consistently supported by 100% BP values. There is no congruence among the different analyses for the phylogenetic relationships among the other four lemur families. However, there is virtually no BP support for any of those clades. Only the arrangement of Indridae sister to a clade containing Lemuridae, Cheirogaleidae and Lepilemuridae receives weak BP support in five of the eight analyses performed (51–67%). There is no conclusive increase or decrease in resolution among the four problematic lemur families by reducing the number of taxa in the data set.

With the GE data set, which includes only one representative per genus, maximum likelihood analysis was carried out. The final maximum likelihood tree (–ln likelihood =

Table 8.5 Comparison of clades found in the phylogenetic trees revealed from different sized data sets.

Taxa [a]	ALL			SSP		SP		GE		
Analysis [d]	MP BP	NJ BP	ML	MP BP	NJ BP	MP BP	NJ BP	MP BP	NJ BP	ML
Ch-In [e]										
Ch-Lm		x			x					
Ch-Lp			x					x		
In-Lm	x			x						x
In-Lp										
Lm-Lp						x	x		59	
Ch-In-Lm	x	x		x	x					
Ch-In-Lp										
Ch-Lm-Lp	51		x			52	67	59	78	
In-Lm-Lp										x
Ch-In-Lm-Lp	100	100	x	100	100	100	100	100	100	x

[a] Taxa included in the data set: ALL = 131 lemurs; SSP = 2 individuals per subspecies (71 lemurs); SP = 2 individuals per species (48 lemurs); GE = 1 individual per genus (12 lemurs). In all analyses, two galagos have been used as the outgroup.

[b] Analysis: MP = maximum parsimony strict consensus tree; NJ = neighbor-joining tree; ML = maximum likelihood tree; BP = bootstrap; x = clade found in the tree; number = bootstrap support value

[c] Lemur families: Ch = Cheirogaleidae; Da = Daubentoniidae; In = Indridae; Lm = Lemuridae; Lp = Lepilemuridae

17351.12) was obtained with previously estimated transition/transversion ratio of 5.40 (kappa = 11.56) and gamma shape parameter of 0.30 (Fig. 8.5). The phylogenetic relationships among the families are completely different from those derived from the maximum likelihood analyses with the complete data set (Fig. 8.4). However, in both maximum likelihood phylograms only very short branches separate the four lemur families from each other.

Examination of the Data Set

The potential necessity for *a posteriori* weighting of the data to obtain better phylogenetic resolution was examined. One commonly applied method is differential weighting of transversions (TV) over transitions (TI) (Miyamoto & Cracraft 1991; Hillis et al. 1996).

Figure 8.6 plots the absolute number of TV and TI over the absolute distance. While the TV show no saturation, the TI climb rapidly and seem to begin to plateau at the higher absolute distances (Fig. 8.6A). Those high absolute distances surely include comparisons among the lemur families. This indicates that the TI will give only limited information for questions among lemur families, and the resulting homoplasy may account for the lack of resolution in the unweighted analyses. Figure 8.6B plots the absolute number of TV over TI, showing how TI climb rapidly and quickly saturate while the number of TV continues to increase. Figure 8.6C plots the relative genetic distances obtained from the proteins and the tRNAs versus those

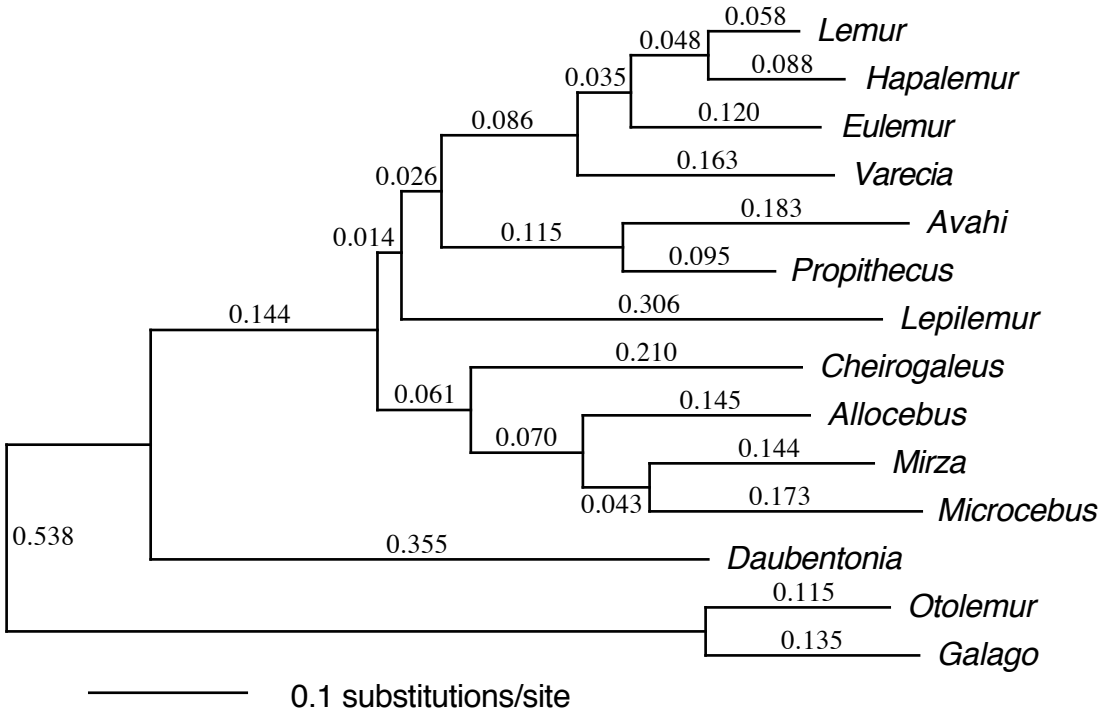


Fig. 8.5 Maximum likelihood phylogram with proportional branch lengths (values provided on each branch) obtained from the GE data set, which includes only one individual per genus.

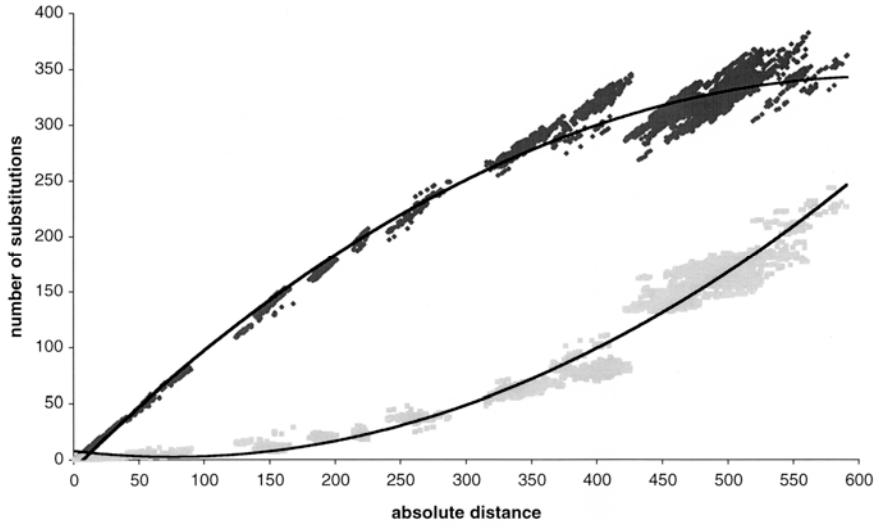
obtained from the whole data set. The protein genes are evolving faster than the tRNAs, but both genes show a linear relationship.

Figure 8.7 shows the results from the protein-coding genes ND3, ND4 and ND4L. First the relative distance of each single protein is plotted against the relative distance of all proteins (Fig. 8.7A). All three protein-coding genes show the same rate of evolution, so no further differentiation among those three ND subunit genes seems to be necessary. Another potential weighting takes into account positional substitution bias among the codons of protein-coding genes. In Figure 8.7B, the numbers of substitutions at first, second and third codon positions are plotted against the absolute distance for the protein-coding genes. As expected, the substitution rate at the third codon positions is much higher than at first or second positions. When plotting the absolute number of TV and TI over the absolute distance, the TI again show saturation, while the TV do not (Fig. 8.7C).

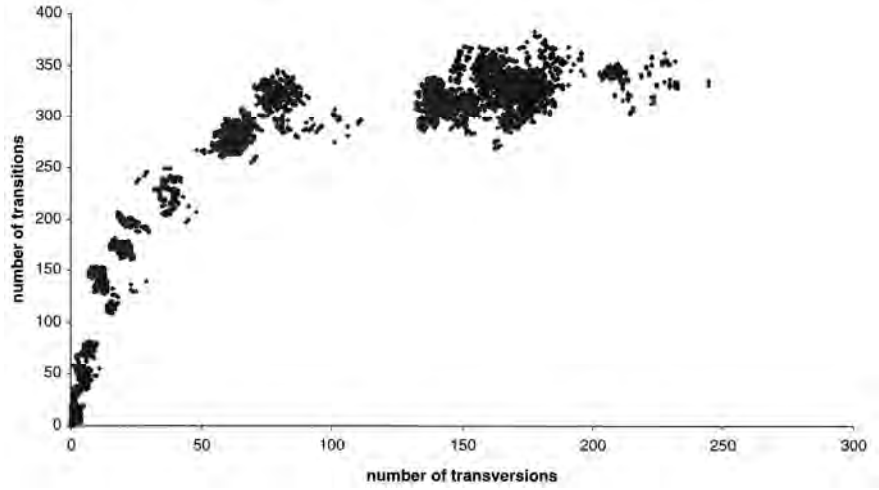
In Figure 8.8, TV and TI are plotted against the absolute distance for first (Fig. 8.8A), second (Fig. 8.8B) and third (Fig. 8.8C) codon positions separately. The TV and TI at second positions remain linear (non-saturated) over the entire evaluation. First-position TV show a linear substitution rate while TI begin to saturate at the higher absolute distances. At third positions both, substitutions resulting in a TV or in a TI, are saturated at high absolute distances. Thus, all third position changes and TI at first positions may be uninformative for the questions specifically of concern here, namely phylogenetic relationships among the distantly related lemur families.

Figure 8.9 shows the substitution rates from the four tRNAs (Gly, Arg, His, Ser). First TV and TI are plotted against the absolute distance (Fig. 8.9A). No saturation seems to occur among the tRNAs. Based on the restrictions on their tertiary structure, stem region nucleotides are more conserved than the nucleotides in the loops (Kumazawa & Nishida 1993). In Figure 8.9B, substitutions restricted to stems and to loops are plotted separately against the absolute distance. There is no observed difference in the substitution rate of stems versus loops across the lemur families.

A



B



C

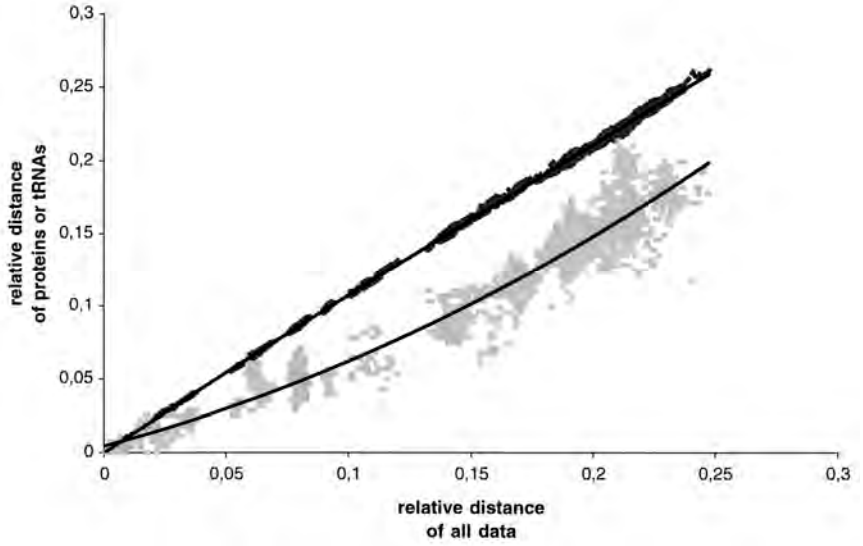
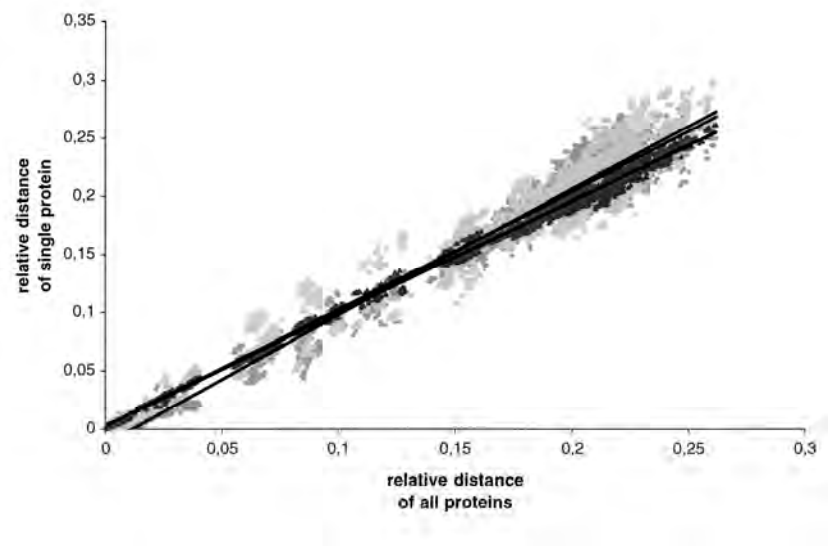
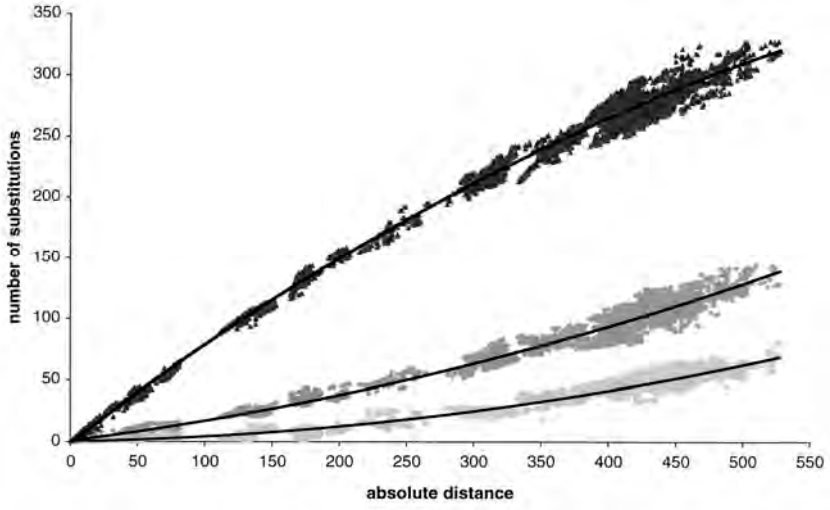


Fig. 8.6 **A** Absolute number of TI (dark diamonds) and TV (pale squares) plotted against absolute distance. **B** Absolute number of TI against absolute number of TV. **C** Relative genetic distances from protein-coding genes (dark diamonds) and tRNA genes (pale squares) against relative genetic distances from the whole data set.

A



B



C

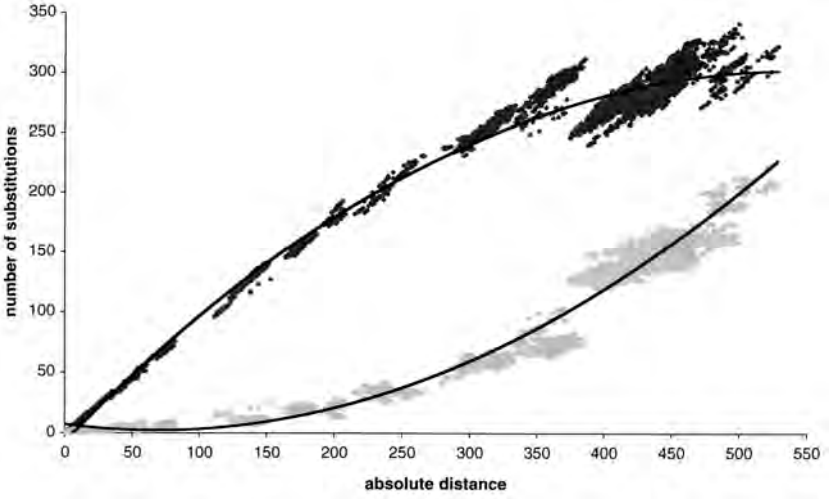
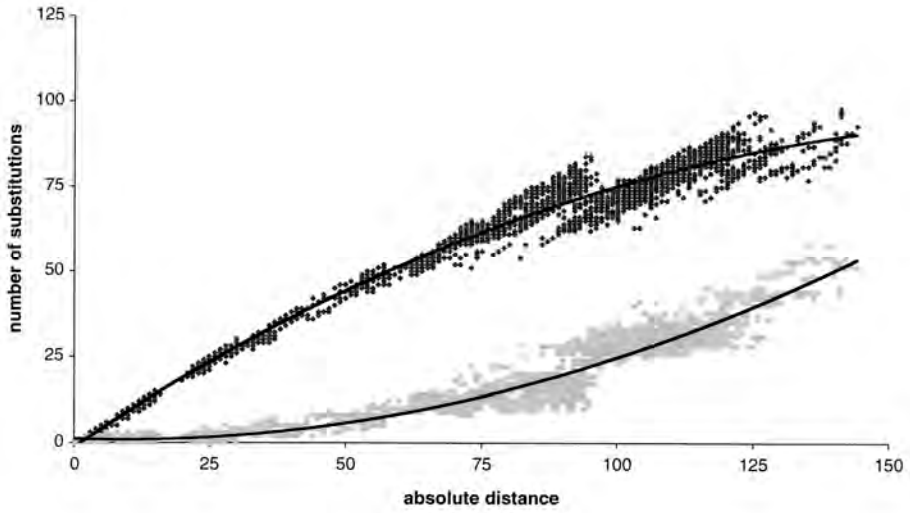
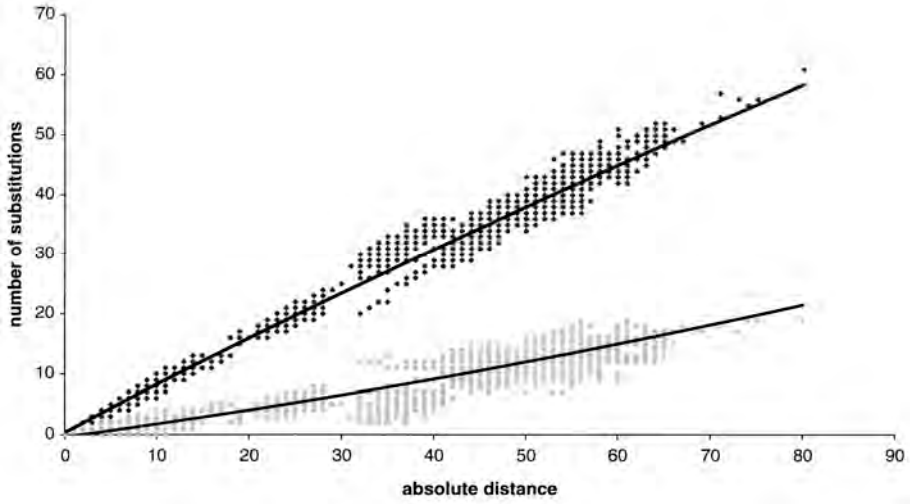


Fig. 8.7 **A** Relative distance of ND3 (diamonds), ND4L (squares) and ND4 (triangles) against the relative distance of all three protein-coding genes. **B** Number of substitutions at first (diamonds), second (squares) and third (triangles) codon positions against the absolute distance of all three protein-coding genes. **C** Number of TV (dark diamonds) and TI (pale squares) against the absolute distance in protein-coding genes.

A



B



C

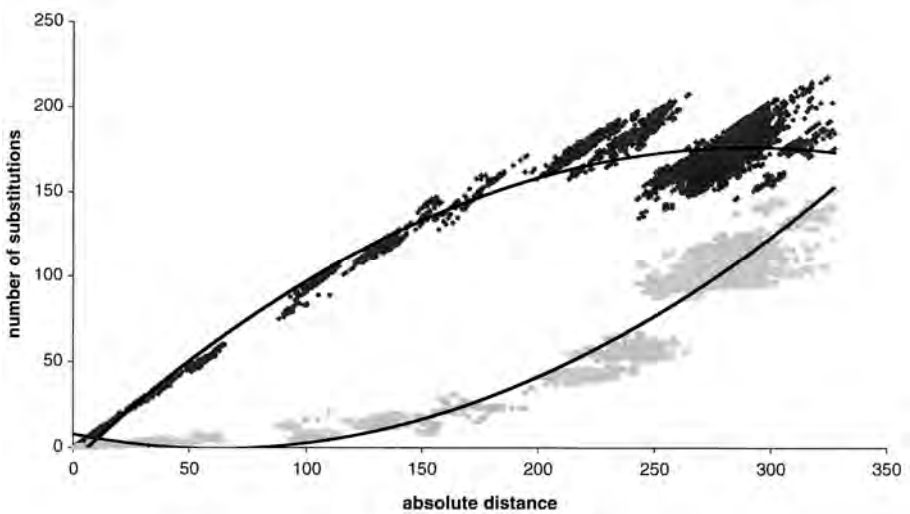


Fig. 8.8 Number of TI (dark diamonds) and TV (pale squares) at **A** first codon positions, **B** second codon positions and **C** third codon positions plotted against the absolute distances of all three protein-coding genes.

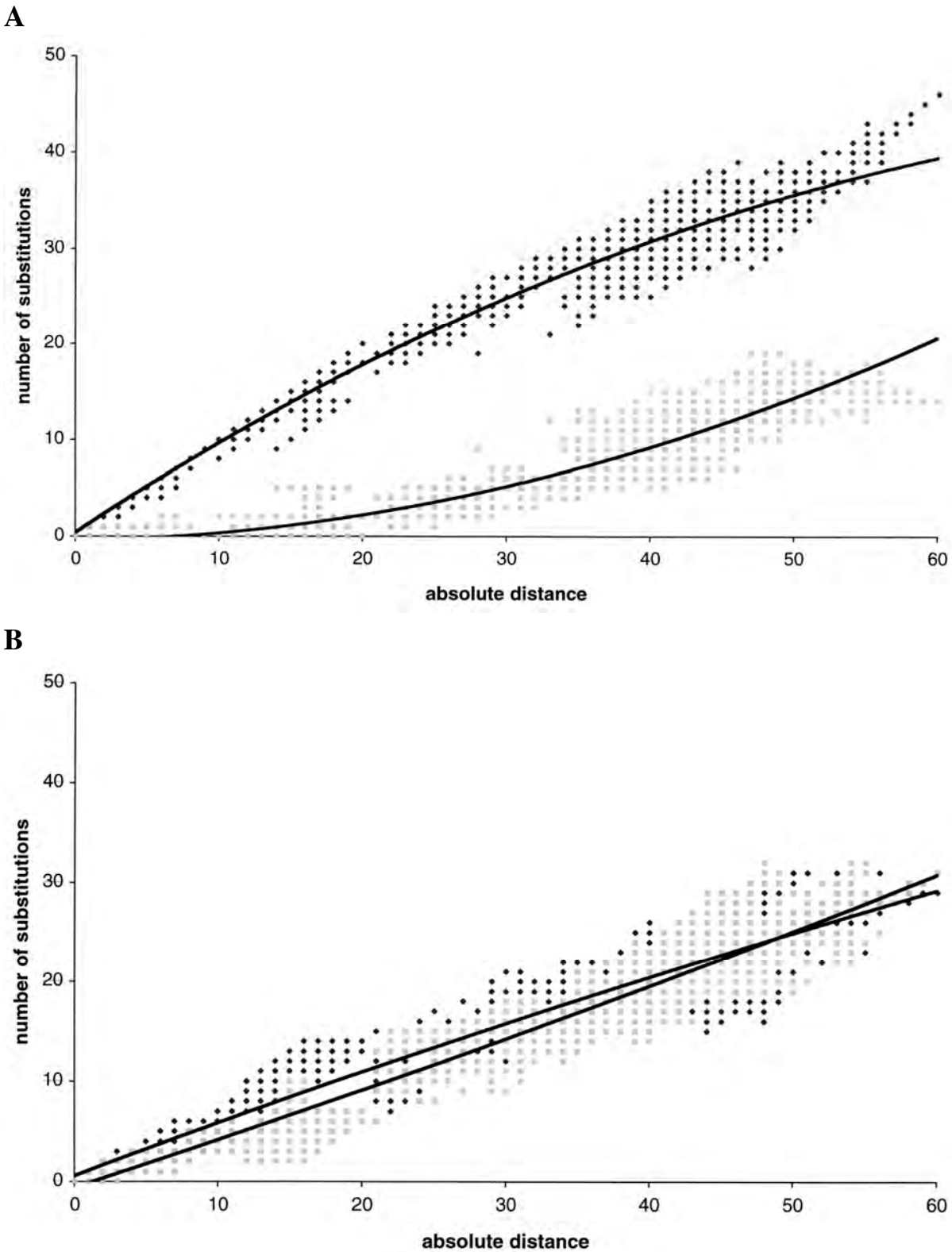


Fig. 8.9 **A** Number of TI (dark diamonds) and TV (pale squares) against the absolute distances of all four tRNA genes. **B** Number of substitutions in loops (dark diamonds) and stems (pale squares) against the absolute distances of all four tRNA genes.

Weighted Analyses

Analyses including TV only and analyses excluding all substitutions at third positions of protein-coding genes have been carried out. Analyses excluding all substitutions at third codon positions and including TV only at first positions of protein-coding genes have also been conducted. For efficient use of computing time, those weighted analyses were carried out on the SP data set, which includes only 50 individuals.

In all weighted analyses, the four lemur families Cheirogaleidae, Indridae, Lemuridae and Lepilemuridae again form a group that is strongly supported by bootstrap analysis (99–100%). There is no agreement on the phylogenetic relationships among those four lemur families, regardless of weighting or analytical method (Table 8.6). Unweighted analyses and maximum parsimony analyses using TV alone weakly support a sister group relationship of Indridae to the subclade containing Cheirogaleidae, Lemuridae and Lepilemuridae (BP=52–67%). However, in all other weighted analyses, Indridae and Lemuridae together form a subclade (BP=56–69%). Character weighting obviously does not improve the resolution power among the four problematic lemur families.

Table 8.6 Comparison of clades found in the phylogenetic trees revealed from differently weighted data.

Characters [a]	all		no3		tv		no3tv1	
Analysis [b]	MP BP	NJ BP	MP BP	NJ BP	MP BP	NJ BP	MP BP	NJ BP
Ch-In [c]								
Ch-Lm								
Ch-Lp					x			
In-Lm			56	57		69		64
In-Lp								
Lm-Lp	x	x						
Ch-In-Lm			x					
Ch-In-Lp								
Ch-Lm-Lp	52	67			52			
In-Lm-Lp				x		x	x	55
Ch-In-Lm-Lp	100	100	99	99	100	100	100	100

[a] Characters used for this analysis: all = all characters; tv = transversions only; no3 = no third positions in protein-coding sequences; no3tv1 = no third positions and transversions only at first positions in protein-coding sequences. The analyses were done with the SP data set (2 individuals per species, 48 lemurs) with two galagos used as the outgroup.

[b] Analysis: MP = maximum parsimony strict consensus tree; NJ = neighbor-joining tree; x = clade found in the tree, but no bootstrap support; number = bootstrap support for this clade

[c] Lemur families: Ch = Cheirogaleidae; Da = Daubentoniidae; In = Indridae; Lm = Lemuridae; Lp = Lepilemuridae

Using Daubentonia *as the Outgroup*

The use of galagos as the outgroup specified in all the analyses presented is potentially problematic due to the large phylogenetic distance between Lorisiformes and Lemuriformes. However, the results of the entire analytical suite are not significantly different with regard to relationships among the four lemur families if *Daubentonia* is chosen as the outgroup, with the galagos deleted from the data set (Table 8.7).

With this alternative rooting, the weighted analyses show a sister relationship of Indridae and Lemuridae in both maximum parsimony and neighbor-joining analyses (BP=52–74%). In weighted and unweighted maximum parsimony analyses, the three families Cheirogaleidae, Indridae and Lemuridae form a clade which is supported by BP analyses with up to 85% when excluding third positions from the data set. However, none of the neighbor-joining analyses shows this clade. Rooting the differently weighted data sets with *Daubentonia* does not change the relationships or degree of resolution among lemurs.

Outgroup Selection

To test the monophyly of the Malagasy lemurs, DNA sequences from other primate and nonprimate taxa have been added to the data set. Those additional sequences were obtained from GenBank (Table 8.8).

Table 8.7 Comparison of clades found in the phylogenetic trees revealed from differently weighted data. *Daubentonia* is used as the outgroup.

Characters [a]	all		no3		tv		no3tv1	
Analysis [b]	MP BP	NJ BP	MP BP	NJ BP	MP BP	NJ BP	MP BP	NJ BP
Ch-In [c]								
Ch-Lm								
Ch-Lp						x		
In-Lm	x		74	52	x	63	59	60
In-Lp								
Lm-Lp		x						
Ch-In-Lm	57		85		x		62	
Ch-In-Lp								
Ch-Lm-Lp		x						
In-Lm-Lp				x				x

[a] Characters used for this analysis: all = all characters; tv = transversions only; no3 = no third positions in protein-coding sequences; no3tv1 = no third positions and transversions only at first positions in protein-coding sequences. The analyses were done with the SP data set (2 individuals per species, 48 lemurs) with two *Daubentonia* used as the outgroup.

[b] Analysis: MP = maximum parsimony strict consensus tree; NJ = neighbor-joining tree; x = clade found in the tree, but no bootstrap support; number = bootstrap support for this clade

[c] Lemur families: Ch = Cheirogaleidae; In = Indridae; Lm = Lemuridae; Lp = Lepilemuridae

Table 8.8 Taxa, order, authors and GenBank accession numbers for the additional sequences obtained from GenBank.

Taxon	Order	Authors	GenBank #
Colobus guereza	Primates	Wang et al. 1997	U92950
Papio hamadryas	Primates	Arnason et al. 1998	Y18001
Hylobates lar	Primates	Arnason et al. 1996	X99256
Homo sapiens	Primates	Anderson et al. 1981	V00662
Pongo pygmaeus	Primates	Horai et al. 1992	D38115
whale (*Balaenoptera physalus*)	Cetacea	Arnason et al. 1991	X61145
cow (*Bos taurus*)	Cetartiodactyla	Anderson et al. 1982	J01394
cat (*Felis catus*)	Carnivora	Lopez et al. 1996	U20753
seal (*Halichoerus grypus*)	Carnivora	Arnason & Gullberg 1993	X72004
horse (*Equus caballus*)	Perissodactyla	Xu & Arnason 1994	X79547
rhinoceros (*Ceratotherium simum*)	Perissodactyla	Xu & Arnason 1997	Y07726
armadillo (*Dasypus novemcinctus*)	Edentata	Arnason et al. 1997	Y11832

Different sets of analyses using cow, cat, horse or armadillo as the outgroup have been carried out. Finally, analyses with an outgroup including cow, whale, cat, seal, horse, rhinoceros and armadillo have been conducted. *Homo*, *Pongo*, *Hylobates*, *Papio* and *Colobus* have been added to the data set as representatives of the simian primates, and the two galagos are included to represent the Lorisiformes. To reduce the amount of computing time, those differently rooted analyses were carried out on the SP data set, which includes only 48 lemurs. Table 8.9 summarises the supported clades for all the different outgroup analyses.

Table 8.9 Comparison of clades found in the phylogenetic trees revealed from data sets with different outgroups.

Outgroup [a]	div.		cow		cat		horse		armadillo	
Analysis [b]	MP	NJ	MP	NJ	MP	NJ	MP	NJ	MP	NJ
Ch-In [c]										
Ch-Lm										
Ch-Lp										
In-Lm										
In-Lp										
Lm-Lp	x	x		x	x	x	x	x	x	x
Ch-In-Lm										
Ch-In-Lp										
Ch-Lm-Lp	57	66	x	58	61	x	x	68	x	63
In-Lm-Lp										
Ch-In-Lm-Lp	100	100	100	100	100	100	100	100	100	100
CILL-Da	87	98	58	52	95	90	83	98	84	97
CILL-Da-Ga	52	53			65	76	65	85		
Ga-si			52	88						
CILL-Da-si									58	x

[a] Outgroups used for this analysis: div. = cow, whale, cat, seal, horse, rhinoceros and armadillo

[b] Analysis: MP = maximum parsimony strict consensus tree; NJ = neighbor-joining tree; x = clade found in the tree, but no bootstrap support; number = bootstrap support for this clade

[c] Lemur families: Ch = Cheirogaleidae; Da = Daubentoniidae; In = Indridae; Lm = Lemuridae; Lp = Lepilemuridae; CILL = Ch, In, Lm, Lp; si = simian primates (*Homo*, *Pongo*, *Hylobates*, *Papio*, *Colobus*)

In nearly all outgroup analyses, the two lemur families Lemuridae and Lepilemuridae form a subclade, which groups as sister to the Cheirogaleidae (Table 8.9). This arrangement, however, has only weak bootstrap support (<68%). In all analyses, the clade containing the four lemur families Cheirogaleidae, Indridae, Lemuridae and Lepilemuridae is supported with bootstrap values of 100%. Daubentonia consistently groups as sister to the other lemur families, regardless of the outgroup chosen. The monophyly of lemurs is generally strongly supported by bootstrap analysis (83–98%). Only when cow is chosen as the outgroup does the bootstrap support for the lemur clade drop down to 52–58%. Depending on the outgroup chosen, the galagos group with lemurs, with simian primates or basal to all primates.

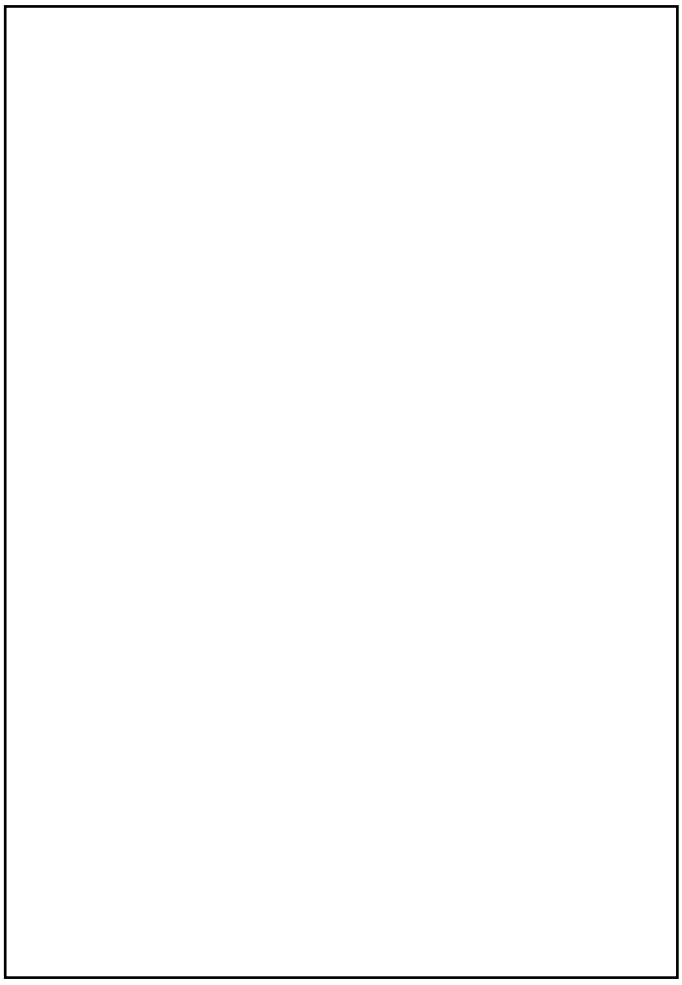


Fig. 8.10 *Lemur catta* in the Berenty Private Reserve, southern Madagascar (August 1998).

8.4 Discussion

***Taxonomy of* Lepilemur**

The relationships among the five *Lepilemur* individuals remain consistent in all analyses. The subclade of one *L. edwardsi* individual and *L. septentrionalis* has strong bootstrap support (96–99%), as does the subclade of the two other *L. edwardsi* individuals and *L. ruficaudatus* (100%). Branch lengths in the maximum likelihood phylogram indicate that both clades and the two taxa within each clade are all deeply separated. Pairwise comparisons among the three *Lepilemur* species give values of 142 to 275 bp, which lie in the range of comparisons between other well-recognised species of *Eulemur* (138–201 bp, Chapter 5), *Hapalemur* (218–291 bp, Chapter 4), *Microcebus* (216–282, Chapter 6) or *Propithecus* (216–222 bp, Chapter 7).

Unfortunately, only 3 of the 7 *Lepilemur* species could be included in this study. However, based on tree topology and pairwise distances, the molecular data presented in this study clearly support specific status for *L. edwardsi*, *L. ruficaudatus* and *L. septentrionalis*. This finding is in agreement with previous studies on chromosomes (Rumpler & Albignac 1978; Ishak et al. 1992).

The three *L. edwardsi* individuals consistently fail to resolve into one monophyletic clade. Pairwise distances, used as a gross measure of divergence, show that one individual of *L. edwardsi* #3 is very different from the other two *L. edwardsi* examined (Table 8.4). This high degree of divergence (261–265 bp) clearly lies in the range of comparisons between other species of *Lepilemur* (142–275 bp), *Eulemur*, *Hapalemur*, *Microcebus* or *Propithecus*. Branch lengths in the maximum likelihood phylogram confirm the deep divergence among those *L. edwardsi* individuals (Fig. 8.4).

Two of the *L. edwardsi* samples (#1 and #2) used in this study are from Anjamena in northwestern Madagascar. The third *L. edwardsi* sample (#3) analysed was collected further north at Ampijoroa. The Betsiboka river lies between those two localities (Fig. 8.1). This large river is a known isolating barrier to gene flow for other lemur subspecies such as *Eulemur fulvus* (Chapter 3), *Hapalemur griseus* (Chapter 4) or *Propithecus verreauxi* (Chapter 6). The very high level of genetic differentiation among *L. edwardsi* individuals, combined with geographic distribution, suggests that two species exist in the range of the currently recognised single species *L. edwardsi*.

Composition of the Five Lemur Families

The monophyly of each of the five lemur families is supported in all analyses. The genera of each family form a clade which is supported by bootstrap values of 96–100% (Figs. 8.2 - 8.4). In the maximum likelihood phylogram, each of the families represents a monophyletic unit discrete from the others (Fig. 8.4). The genetical data presented here thus confirm the affiliation of each genus to its family as is generally accepted in the literature.

The family Lemuridae includes the genera *Eulemur*, *Hapalemur*, *Lemur* and *Varecia*. The data presented here thus clearly group *Varecia* with the other lemurid genera, which is in agreement with most previous phylogenetic studies. Only one study of morphological and behavioural characters excluded *Varecia* from the family Lemuridae (Stanger-Hall 1997). There is no support for a sister-group relationship between *Hapalemur* and *Lepilemur*, as has been suggested on the basis of morphological data (Tattersall & Schwartz 1974).

Lepilemur is indeed deeply separated from all other lemur genera in the data set. The DNA sequence data presented here thus strongly support the family status of this single genus. As expected, *Allocebus*, *Cheirogaleus*, *Microcebus* and *Mirza* together form a clade which represents the family Cheirogaleidae. The family Indridae is represented by a deeply separated clade containing the two genera *Avahi* and *Propithecus*.

Phylogenetic Relationships among Lemur Families

One goal of the present study was to clarify nodal relationships among the five lemur families. According to the results, Cheirogaleidae, Indridae, Lemuridae and Lepilemuridae consistently form a sister group to Daubentoniidae. The early divergence of *Daubentonia* among lemurs is strongly supported by bootstrap analyses in maximum parsimony and neighbor-joining methods (100%), as well as by the long branches in the maximum likelihood phylogram (Figs. 8.2 - 8.4).

Neighbor-joining and maximum parsimony analyses differ in the arrangement of Cheirogaleidae, Indridae, Lemuridae and Lepilemuridae (Figs. 8.2 and 8.3). However, these alternative arrangements are not supported by bootstrap analyses (<51%). The short branch lengths of the maximum likelihood phylogram separating these four lemur families confirm these results (Fig. 8.4). More proximal outgroup rooting, using *Daubentonia* as the outgroup, does not enhance resolution among the lemur families. Weighting the data set by excluding all transitions or by excluding the third codon positions also does not improve the resolution.

Furthermore, when the third codon positions are excluded and only transversions are considered at first positions, the four lemur families still remain poorly resolved.

The mtDNA sequence data presented here failed to yield clear resolution of phylogenetic relationships among the four lemur families Cheirogaleidae, Indridae, Lemuridae and Lepilemuridae. It can generally be excluded that this is due to the genes chosen for sequencing. The overall substitution rates of the whole data set, of the two types of genes (protein-coding and tRNAs), of each of the codons in the protein-coding genes and of the stems and loops in tRNA genes have been examined. Those investigations showed that the tRNA genes in general and in protein-coding genes the second codon positions and the transversions at first positions should contribute valuable information for phylogenetic reconstruction among distantly related taxa (Figs. 8.6 - 8.9).

It has been suggested that adding more taxa to the data set can improve the phylogenetic resolution. However, as the data set presented here already includes most lemur taxa (12 of 14 genera and 25 of 33 species), there are only a few taxa left to be added. It is unlikely that the addition of the few missing taxa would dramatically enhance the resolution of this topology. However, additional characters may improve the phylogenetic resolution among taxa. While this is a large mtDNA data set consisting of approximately 2400 bp, it would be useful to specifically include sequence data from the nuclear genome. DNA sequences from single-copy nuclear genes would provide additional loci and thus independent diagnosis for evolutionary relationships in this group.

Under the assumption that the DNA sequence data set presented here provides a good picture of lemur phylogeny, the lack of resolution among lemur families requires explanation. The families Cheirogaleidae, Indridae, Lemuridae and Lepilemuridae may, indeed, have diverged from each other within a short period of time, as is suggested by tree topologies.

Monophyly of Lemurs

One aim of this study was to examine the phylogenetic position of the family Daubentoniidae in relation to other lemurs or prosimians. The relationship of *Daubentonia* to other strepsirrhines has remained a mystery. It has been variously placed as sister to the Indridae (Yoder 1994), sister to the Cheirogaleidae (Stanger-Hall 1997), sister to a clade containing Lemuridae and Indridae (Yoder et al. 1996), as the earliest-diverging of the lemurs (Dene et al. 1980; Rumpler et al. 1989; Yoder 1994; Del Pero et al. 1995; Porter et al. 1995; Yoder et al.

1996), as a basal offshoot of the strepsirrhine clade (Adkins & Honeycutt 1994) or unresolved with respect to other lemur families (Stanger-Hall & Cunningham 1998).

There is no common agreement as to which taxa represent the sister group of the primates. In the present study, several analyses using different outgroups representing different mammalian orders (Carnivora, Cetartiodactyla, Edentata, Perissodactyla) have been carried out to avoid bias. Regardless of which outgroup was applied, *Daubentonia* consistently groups as sister to a clade containing the other four lemur families. This sister-group relationship is supported in maximum parsimony and neighbor-joining analyses with 52–98% bootstrap values (or 83–98% if the analyses with cow as the outgroup are excluded).

The mtDNA sequence data presented here thus strongly support the monophyly of the Malagasy lemurs, as proposed by Martin (1990) following a review of evidence available at that time. There was no congruence among the different outgroup analyses with respect to the phylogenetic relationships among simian primates, galagos and lemurs. Most analyses (Table 8.9) group the galagos sister to the lemurs, which supports the Strepsirrhini as a monophyletic group (52–85% bootstrap support). However, analyses using the cow as outgroup support a clade including galagos and simians, making the lemurs the first taxonomic unit to diverge among primates (52–88% bootstrap support). When the armadillo is used as the outgroup, by contrast, galagos group sister to a clade including lemurs and simians (<58% bootstrap support).

The search for a possible sister group of the primates as a means of defining ancestral states and polarise characters by outgroup comparison has often been controversial. The different outgroup analyses carried out in this study demonstrate the major influence the outgroup can have on the tree topology. When the cow was the specified outgroup, the results changed remarkably. The bootstrap support for the monophyly of lemurs dropped from 83–98% down to 52–58% and the monophyly of Strepsirrhini was lost because of the sister-group relationship of galagos with the simian primates. However, the main goal of the present study was to test the monophyly of the Malagasy lemurs. The monophyly of lemurs was always supported, regardless of the outgroup or analytical method applied.

9. General Conclusions

This chapter is to be published in a modified form.

9.1 Lemur Evolution and Systematics

Subspecies Designations

One goal of this study was to test whether mitochondrial DNA sequence data can be used as a decisive basis for subspecies designations in lemurs. In the following 7 examples phylogenetic structure among populations within a species was examined and in most cases successfully differentiated.

1) Analyses of the *Eulemur fulvus* sequence data resolve 6 clades which are strongly supported by both bootstrap and jackknife values. Pairwise distances and branch lengths clearly support these 6 clades, but these do not correspond to the 7 subspecies currently accepted. Paraphyly among 4 *E.f. fulvus* and all 3 *E.f. mayottensis* individuals indicates that *E.f. mayottensis* does not deserve subspecies status. None of the 3 taxa involved in the second clade containing 4 *E.f. albifrons*, 3 *E.f. fulvus* and 2 *E.f. sanfordi* individuals forms a monophyletic subclade. Therefore, this sequencing data set does not permit distinction between *E.f. albifrons* and *E.f. sanfordi*. The genetic analyses indicate that there are two distinct forms of *E.f. rufus* (clades 3 and 4). The 2 individuals of *E.f. albocollaris* form the fifth clade and the 2 individuals of *E.f. collaris* represent the sixth clade.

2) Tree topology clearly resolves *E. macaco macaco* and *E.m. flavifrons* into 2 different clades. Pairwise distances between those 2 subspecies are in the same range as between other lemur subspecies. The results thus support the subspecies status of *E.m. macaco* and *E.m. flavifrons*.

3) Based on tree topology and pairwise distances, the molecular data presented in this study do not support 2 monophyletic lineages for *Hapalemur griseus griseus* and *H.g. alaotrensis*. Molecular data thus suggest combination of *H.g. griseus* and *H.g. alaotrensis* into a single subspecies. In contrast, when looking at tree topology and pairwise distances, it becomes obvious that considerable genetic differentiation exists among the small sample of *H.g. occidentalis* individuals examined here. This level of phylogenetic structure among *H.g. occidentalis* individuals is equivalent to subspecific differentiation in related lemurid genera.

4) All analyses resolve the 5 *Varecia variegata rubra* individuals into a monophyletic clade. However, the 3 *V.v. variegata* individuals fail to form a single subclade. The fairly large genetic distances among *V.v. variegata* individuals, together with tree topology, indicate that more phylogenetic structure may exist in this taxon than current taxonomy would depict.

5) Within *Microcebus murinus*, 3 different subclades can be recognised on the basis of tree topology, branch lengths, and pairwise distances. Genetic differentiation among the 3 subclades within *M. murinus* reaches levels found in other lemur subspecies.

6) The samples for *Cheirogaleus medius* also demonstrate stronger than expected molecular divergences, suggesting that phylogeographic differentiation may exist in this taxon as well.

7) Based on tree topology and pairwise distances, the molecular data presented in this study do not support monophyletic lineages for *Propithecus verreauxi verreauxi*, *P.v. deckeni* or *P.v. coronatus*. The genetic data instead would support the combination of *P.v. verreauxi*, *P.v. deckeni* and *P.v. coronatus* into a single subspecies.

Using the mitochondrial DNA sequences presented in this study, it is possible to distinguish 6 *Eulemur fulvus*, 2 *E. macaco*, 3 *Hapalemur griseus*, 3 *Varecia variegata*, 3 *Microcebus murinus* and 3 *Cheirogaleus medius* clades. Mitochondrial DNA sequence data would thus allow identification at the subspecific level for individuals of unknown origin. Alongside pelage, this is hence the most decisive feature for diagnosis of subspecies designations in lemurs thus far reported.

In the present study 2 *Eulemur fulvus*, 1 *Hapalemur griseus* and 3 *Propithecus verreauxi* subspecies included in current taxonomy could not be recognised. However, the mtDNA sequence data permitted distinction of additional subspecies – 1 *Eulemur fulvus*, 1 *Hapalemur griseus*, 1 *Varecia variegata*, 3 *Microcebus murinus* and 2 *Cheirogaleus medius* – which have not been previously distinguished. In nocturnal taxa (e.g. *Microcebus* or *Cheirogaleus*), where pelage variation is very limited, genetic data might be the only decisive feature available to classify a subspecies. In diurnal or cathemeral taxa (e.g. *Eulemur fulvus* and *Propithecus verreauxi*) the considerable variation in pelage pattern and colour is only weakly diagnostic, so genetic data can provide an unbiased foundation for subspecies designations.

Species Designations

A further aim of this study was to use mtDNA sequence data to resolve phylogenetic relationships among species of a genus and to confirm species status of taxa whose taxonomic rank is still under discussion. The following 10 species-level topics have been investigated:

1) It has been suggested that *Eulemur fulvus albocollaris* and *E.f. collaris* deserve species status. In the present mtDNA sequence data set, genetic distances and tree topology support a recent divergence of *E.f albocollaris* and *E.f. collaris* that has not yet reached levels found in other closely related species.

2) The data failed to yield clear resolution of phylogenetic relationships among the 5 *Eulemur* species. All analyses unambiguously linked *E. fulvus*, *E. mongoz*, and *E. rubriventer* into one clade with strong bootstrap or jackknife support. However, the analyses differ in the arrangement of *E. fulvus*, *E. mongoz* and *E. rubriventer* relative to one another and in the branching order of *E. coronatus*, *E. macaco* and the *E. fulvus*/*E. mongoz*/ *E. rubriventer* clade.

3) The relationships among *Hapalemur* species remain consistent in all analyses. The resulting topologies indicate a close relationship between *H. aureus* and *H. griseus* and also that *H. simus* is deeply separated from the other 2 species. Branch lengths in the maximum likelihood phylogram and the high pairwise distances confirm the deep divergence of the 3 *Hapalemur* species. These molecular data thus strongly support specific status for *H. aureus*.

4) This study indicates a close relationship between *Lemur catta* and *Hapalemur*. In maximum parsimony and neighbor-joining analyses, *L. catta* is paraphyletic within the genus *Hapalemur*. In the maximum likelihood phylogram a very short branch separates *L. catta* from the clade formed by all 3 *Hapalemur* species. Pairwise distances between *L. catta* and each of the 3 *Hapalemur* species clearly lie in the range of comparisons between *Hapalemur* species and do not attain the range observed between other lemur genera. The molecular data presented in this study would thus suggest possible unification of *L. catta* and *Hapalemur* into a single genus.

5) Within the genus *Microcebus*, a subclade is formed by *M. ravelobensis* and *M. rufus*, with *M. murinus* forming the sister taxon to those 2 taxa. The maximum likelihood phylogram shows 3 well-separated *Microcebus* clades. Pairwise distances among all 3 *Microcebus* species are very similar in magnitude. Consequently, the molecular data support the species-level distinction of the recently discovered *M. ravelobensis*.

6) Within the sample of *M. rufus*, there are 3 subclades which differ remarkably from each other. Pairwise distances among those subclades are not as high as between *M. murinus, M. rufus* and *M. ravelobensis* but reach the level of differentiation among the 5 well-accepted *Eulemur* species. The 3 subclades within *M. rufus* are also separated by relatively long branches in maximum likelihood analyses, which additionally indicates that speciation may already be complete.

7) The molecular data strongly support the early divergence of *Propithecus diadema* among sifakas. *P. verreauxi coquereli* and *P. tattersalli* together form the sister group to a clade containing *P.v. verreauxi*, *P.v. deckeni* and *P.v. coronatus.*

8) *P. tattersalli* is clearly resolved within *P. verreauxi*. In the maximum likelihood phylogram, only a very short branch separates *P. tattersalli* from the *P.v. coquereli* clade. Furthermore, genetic distances between *P. tattersalli* and *P.v. coquereli* suggest a very recent divergence. There is no support for the recognition of *P. tattersalli* as a separate species.

9) Pairwise distances between the *P.v. coquereli*/*P. tattersalli* clade and the *P.v. verreauxi*/*deckeni*/*coronatus* clade nearly reach the level of differentiation found among the five well-accepted *Eulemur* species. The deep divergence between those two clades indicates that they may represent two species of *Propithecus.*

10) The subclade of one *Lepilemur edwardsi* individual and *L. septentrionalis* consistently groups as sister to a clade containing the other two *L. edwardsi* individuals and *L. ruficaudatus*. Branch lengths in the maximum likelihood phylogram indicate that both clades and the 2 taxa within each clade are deeply separated from each other. Based on tree topology and pairwise distances, the molecular data presented in this study thus clearly support specific status for *L. edwardsi*, *L. ruficaudatus* and *L. septentrionalis*. The very high level of genetic differentiation among *L. edwardsi* individuals suggests that 2 species are included in this taxon.

Based on mtDNA sequence data, it is always possible to distinguish among the different lemur species. The clades containing individuals of a single species consistently have complete bootstrap or jackknife support (100%). In the maximum likelihood phylogram, long branches separate the species from each other.

This study includes 25 of the 33 currently recognised lemur species. While the molecular data set presented here does not support species status of *Propithecus tattersalli*, the results suggest as yet undescribed species may exist within what are currently the single species

of *Microcebus rufus* and *Lepilemur edwardsi*. The data further indicate that *P.v. coquereli* might also be properly elevated to a full species.

The results from this study clearly resolved phylogenetic relationships among species within a genus. The only exception involves the 5 *Eulemur* species, whose branching order could not be determined. It can generally be excluded that this is due to the genes chosen for sequencing, as the same kind of data were able to resolve phylogenetic relationships among other species of related lemur genera. Based on the molecular phylogeny, it seems likely that the species of the genus *Eulemur* radiated very rapidly.

Phylogenetic Relationships among Genera and Families

The present study includes 12 of the 14 extant lemur genera. The mtDNA sequences were used to assess generic status and family status and to resolve phylogenetic relationships among lemur genera and families. Six problematic issues in lemur evolution have been examined:

1) The molecular data resolve a monophyletic genus *Eulemur*, which forms the sister group to a clade containing *Lemur catta* and *Hapalemur*. *Varecia* is deeply separated from all other lemurid taxa. The maximum likelihood phylogram confirms the deep divergences among *Varecia*, *Eulemur* and *L. catta/Hapalemur*. Pairwise distances and tree topology strongly support the classification of *Eulemur* and *Varecia* as genera distinct from *L. catta.*

2) *Mirza* and *Microcebus* consistently form a sister group relationship to *Allocebus*. *Cheirogaleus* constitutes the earliest diverging genus. Based on tree topology and high genetic divergence it follows that *Mirza coquereli*, whose taxonomic rank is still a subject of discussion, deserves generic status.

3) As previously reported, *Avahi* constitutes the earliest diverging taxon compared to *Propithecus* species. In the maximum likelihood phylogram, *Avahi* and *Propithecus* are separated by relatively long branches, indicating a long history of divergence.

4) The genetic data confirm the affiliation of each genus to its family as depicted in the current taxonomy. All analyses clearly verify the monophyly of each of the lemur families. Each clade containing the taxa of a single family receives strong bootstrap support (96–100%). In the maximum likelihood phylogram, the families are widely divergent from each other.

5) The mtDNA sequence data failed to resolve phylogenetic relationships among the four lemur families Cheirogaleidae, Indridae, Lemuridae and Lepilemuridae. However,

Cheirogaleidae, Indridae, Lemuridae and Lepilemuridae together consistently form a sister group to Daubentoniidae. The early divergence of *Daubentonia* among lemurs is strongly supported by bootstrap analyses in maximum parsimony and neighbor-joining methods (100%), as well as by the long branches in the maximum likelihood phylogram.

6) Several analyses using different outgroup representatives of the different mammalian orders were performed to test the monophyly of Malagasy lemurs. Regardless of the outgroup chosen, *Daubentonia* consistently groups as sister to a clade containing the other four lemur families. The molecular data thus strongly support the monophyly of the Malagasy lemurs.

The mtDNA sequence data presented here allow the generic status of different lemur taxa to be accurately assessed. At the genus level, each clade is supported by the highest (100%) bootstrap and jackknife support possible in both maximum parsimony and neighbor-joining analyses. Branch lengths in the maximum likelihood phylogram confirm the deep divergence of each genus from other lemur genera. The only exception involves the genera *Lemur* and *Hapalemur*, as discussed above.

All analyses resolved the phylogenetic relationships among genera within each family. However, the branching order among the clades representing the families Cheirogaleidae, Indridae, Lemuridae and Lepilemuridae could not be determined. Different weighting of the data did not improve the resolution. The molecular phylogeny can be interpreted as indicating that those 4 lemur families separated from each other within a relatively short period of time.

9.2. Classification of Lemurs

A classification should convey the optimum synthesis of information, and in most cases this is best achieved through combining both phyletic and phenetic considerations. As many different types of characters as possible should be included to achieve an unbiased picture of the taxonomy. The classification suggested in Tables 9.1 and 9.2 is based only on the results of the molecular sequence data presented here and therefore represents a partial contribution to this issue.

Table 9.1 Taxonomy of the extant lemur families Cheirogaleidae, Indridae and Daubentoniidae, as suggested if mtDNA sequence data alone are considered. [a]

Family	Genus		Species		Subspecies	
Cheirogaleidae	*Microcebus*		*murinus*		ssp. 1	
					ssp. 2	
					ssp. 3	
			rufus		-	
			cf. *rufus*		-	
			cf. *rufus*		-	
			myoxinus	?	-	
			ravelobensis		-	
	Mirza		*coquereli*		-	
	Allocebus		*trichotis*		-	
	Cheirogaleus		*major*		*major*	?
					crossleyi	?
			medius		ssp. 1	
					ssp. 2	
					ssp. 3	
	Phaner	?	*furcifer*	?	*furcifer*	?
					pallescens	?
					parienti	?
					electromontis	?
Indridae	*Avahi*		*laniger*		-	
			occidentalis	?	-	
	Propithecus		*verreauxi*		-	
			coquereli		*coquereli*	
					tattersalli	
			diadema		*diadema*	?
					candidus	?
					perrieri	?
					edwardsi	?
					holomelas	?
	Indri	?	*indri*	?	-	
Daubentoniidae	*Daubentonia*		*madagascariensis*		-	

[a] Taxa not included in the study are indicated with '?'. Taxa not described thus far are shaded.

It must be stressed that this classification is strictly based on molecular data from this study alone. It does not include any other type of data, which would be critical for a definitive synthesis. However, molecular data have proven to be quite useful in the taxonomic and phylogenetic evaluation of lemurs. This is true both in this study (see above) and in the investigations conducted by others. In the genus *Lepilemur*, the species have been diagnosed mainly by chromosomal features, as morphological features did not provide distinctive characters (for review, see Martin 1995). In contrast, in the family Cheirogaleidae chromosomal studies fail to provide any features which can be used to distinguish among species, whereas

Table 9.2 Taxonomy of the extant lemur families Lemuridae and Lepilemuridae, as suggested if mtDNA sequence data alone are considered. [a]

Family	**Genus**	**Species**		**Subspecies**	
Lepilemuridae	*Lepilemur*	*mustelinus*	?	-	
		microdon	?	-	
		leucopus	?	-	
		ruficaudatus		-	
		edwardsi		-	
		cf. *edwardsi*		-	
		dorsalis	?	-	
		septentrionalis		-	
Lemuridae	*Lemur*	*griseus*		*griseus*	
				occidentalis	
				cf. *occidentalis*	
				meridionalis	?
		aureus		-	
		simus		-	
		catta		-	
	Eulemur	*fulvus*		*fulvus*	
				rufus	
				cf. *rufus*	
				albifrons	
				albocollaris	
				collaris	
		macaco		*macaco*	
				flavifrons	
		coronatus		-	
		rubriventer		-	
		mongoz		-	
	Varecia	*variegata*		*variegata*	
				cf. *variegata*	
				rubra	

[a] Taxa not included in this study are indicated with '?'. Taxa not described thus far are shaded.

morphological characters allow the species to be accurately diagnosed. These examples demonstrate that the designation of genera, species or subspecies should always be performed with multiple data types drawn from multiple disciplines (e.g. morphology, pelage coloration, karyotypes, behaviour, DNA sequence data, distribution).

The mtDNA sequence data taxonomy suggested here (Tables 9.1 and 9.2) differs in the following points from the current taxonomy (see Tables 1.1 and 1.2):

- *Cheirogaleus medius* includes 3 subspecies (currently 1 species)
- *Microcebus rufus* contains 3 species (currently 1 species)
- *Microcebus murinus* includes 3 subspecies (currently 1 species)
- *Propithecus verreauxi coronatus* does not deserve subspecies rank
- *Propithecus verreauxi deckeni* does not deserve subspecies rank
- *Propithecus tattersalli* does not deserve species rank
- *Propithecus verreauxi coquereli* deserves species rank
- *Lepilemur edwardsi* contains 2 species (currently 1 species)
- *Lemur* and *Hapalemur* are considered as only one genus
- *Hapalemur griseus alaotrensis* does not deserve subspecies rank
- *Hapalemur griseus occidentalis* contains 2 subspecies (currently 1 subspecies)
- *Eulemur fulvus mayottensis* does not deserve subspecies rank
- *Eulemur fulvus sanfordi* does not deserve subspecies rank
- *Eulemur rufus rufus* contains 2 subspecies (currently 1 subspecies)
- *Varecia variegata* includes 2 subspecies (currently 1 subspecies)

In the present study, genetic distances are used after monophyly in a cladistic sense has been determined. A phylogenetic tree illustrates inferred evolutionary relationships among a group of organisms. Cladistics is a method of systematics that is used to construct classifications based on strict monophyletic groups. If a taxon is nested within another taxon, a cladistic approach to classification requires combination of the two paraphyletic taxa (although this is not necessarily the case with a classical approach to classification – Martin 2000). Group monophyly is hence of great importance for taxonomic considerations. However, additional information can be obtained using the relative divergences of taxa within a group. While this is certainly not a globally applicable procedure, it does allow taxonomic scaling when applied within a group of

related taxa. Groups are first examined to ascertain monophyly, then monophyletic clades are characterised as to their relative divergence levels within and among other individuals. Current taxonomic consistency is examined alongside group monophyly and genetic distances to determine appropriate taxonomic distinctiveness.

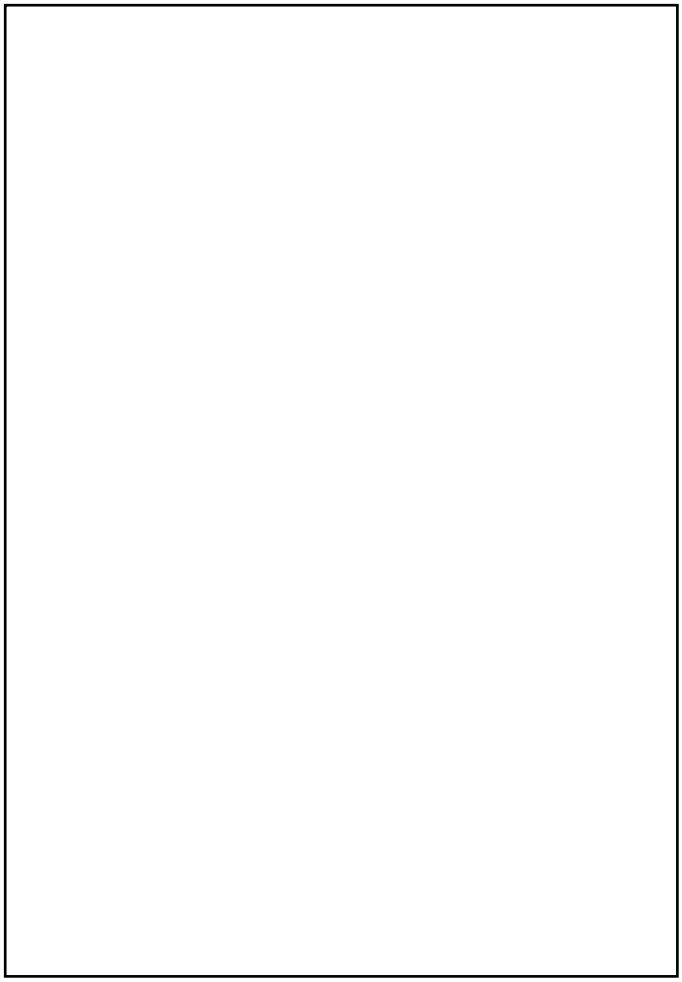


Fig. 9.1 *Varecia variegata rubra* at Apenheul Zoo (August 1999).

9.3 Zoogeographic Aspects of Lemur Evolution along the Western Coast

Madagascar is divided into 2 major zones, a relatively moist eastern region and a dry western area. Each zone provides a wide range of habitats. Based on distribution patterns of all species and subspecies for which sufficient data were available, Martin (1972) divided Madagascar into 7 major zones (Fig. 9.2). Along the western coast 3 zones (NW, W1 and W2) were defined (Martin 1972). All 3 western zones are bounded by the highlands to the east and by the Mozambique Channel to the west. NW covers the western coast north of the Betsiboka river, W1 includes the area between the Betsiboka and Tsiribihina rivers and W2 covers the western coast south of the Tsiribihina river.

In this study, individuals from 2 or all 3 western coast zones from several taxa have been included. This provides the opportunity to compare genetic differentiation among lemur taxa across the zoogeographic zones of the western coast. The rivers Tsiribihina and Betsiboka have been proposed as major physical barriers and to serve as boundaries between the western distribution zones (Fig. 9.2)(Martin 1972). The present data set allows the examination of the effects these rivers have on gene flow in a variety of lemur taxa.

Betsiboka River

Based on current taxonomy, the Betsiboka river forms the boundary between the subspecies *Eulemur fulvus fulvus* and *E.f. rufus* and between *Propithecus verreauxi coronatus* and *P.v. coquereli* (Table 9.3). However, the Betsiboka has not been indicated as a taxonomic boundary for *Eulemur mongoz*, *Hapalemur griseus*, *Lepilemur edwardsi*, *Microcebus murinus* or *Cheirogaleus medius* populations on either banks of the river.

When looking at absolute genetic distances among populations west and east of the Betsiboka river, completely different levels of genetic divergence can be observed (Table 9.4).

1) *Eulemur mongoz* individuals at Ampijoroa (east of Betsiboka river) differ by only 15 to 17 bp from their relatives at Anjamena (west of Betsiboka river). This finding is in agreement with the current taxonomy, which does not distinguish subspecies.

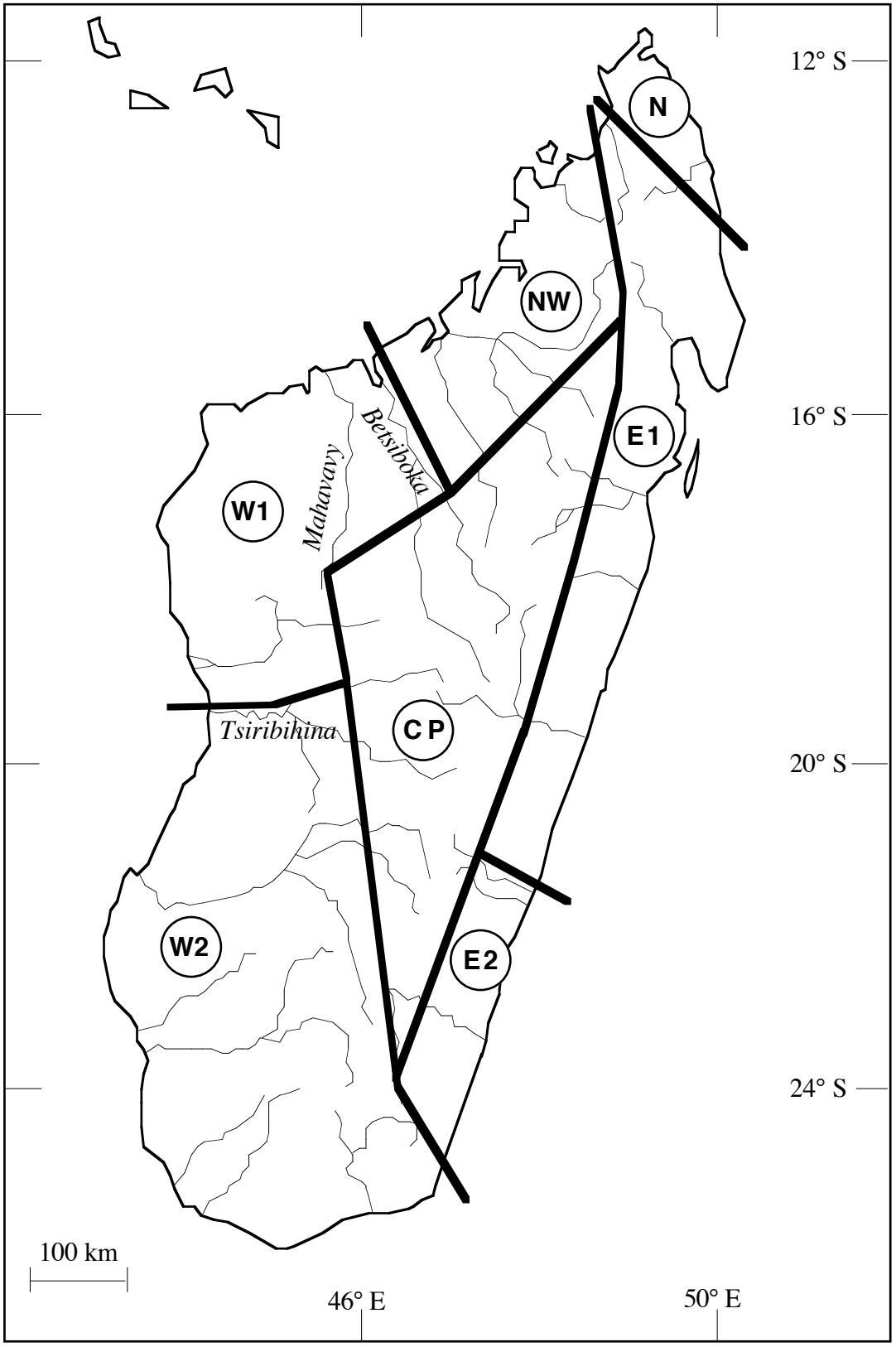


Fig. 9.2 Map of Madagascar showing the seven main areas of lemur distribution according to Martin (1972). E1 and E2 = east coast zones; W1, W2 and NW = west coast zones; N = north coast zone; CP = central plateau zone.

Table 9.3 Comparison of the current taxonomy and the taxonomy as indicated by the mtDNA sequence data set presented in this study for 4 lemur taxa at 3 geographic sites.

Taxonomy	Site	*Eulemur mongoz*	*Eulemur fulvus*	*Propithecus verreauxi*	*Lepilemur edwardsi*
current taxonomy	Ampijoroa [a]	*E. mongoz*	*E.f. fulvus*	*P.v. coquereli*	*L. edwardsi*
	Anjamena [b]		*E.f. rufus*	*P.v. coronatus*	
	Anadabomandry [c]			*P.v. deckeni*	
molecular data	Ampijoroa [a]	*E. mongoz*	*E.f. fulvus*	*P. coquereli*	*L. edwardsi*
	Anjamena [b]		*E.f. rufus*	*P. verreauxi*	*L.* cf. *edwardsi*
	Anadabomandry [c]				?

[a] east of the Betsiboka River

[b] between the Betsiboka and Mahavavy Rivers

[c] west of the Mahavavy River

2) Pairwise distances among *Eulemur fulvus fulvus* at Ampijoroa (east of Betsiboka river) and *E.f. rufus* at Anjamena (west of Betsiboka river) are 55 to 57 bp. This degree of genetic divergence lies in the range of comparisons among other lemur subspecies and thus confirms the current taxonomy.

3) Genetic distances among *Propithecus verreauxi coronatus* from Anjamena (west of Betsiboka river) and *P.v. coquereli* from Ampijoroa (east of Betsiboka river) reach 124–130 bp, which exceeds genetic differentiation normally seen among lemur subspecies. The molecular data suggest that the Betsiboka river acts as a barrier to gene flow over time and that speciation among those two populations may already be complete.

4) The highest genetic distances (261–265 bp) across the Betsiboka river can be found among *Lepilemur edwardsi* individuals from Ampijoroa and Anjamena. DNA sequence data clearly support the inference that the Betsiboka river serves as boundary between 2 different *Lepilemur* species. This is an unexpected result, as current taxonomy does not distinguish those *Lepilemur* populations even at the subspecific level.

Table 9.4 Absolute genetic distances (number of base positions) derived from comparisons between individuals of the same taxon on different sides of certain rivers.

Taxon	Tsiribihina	Tsiribihina and Mahavavy	Mahavavy	Mahavavy and Betsiboka	Betsiboka	Tsiribihina and Mahavavy and Betsiboka
Eulemur mongoz	–		8 - 10		15 - 17	
Eulemur fulvus	54 - 60		5 - 10		55 - 57	
Propithecus	20 - 24		6 - 20		124 - 130	
Lepilemur	?	142 - 146	?		261 - 265	
Hapalemur griseus	-		?	47 - 48	?	
Microcebus murinus	?		?		?	60 - 62

Mahavavy River

In general, the Mahavavy river is not considered as an isolating boundary between different taxa by current taxonomy. The only exception is provided by the subspecies *Propithecus verreauxi coronatus* and *P.v. deckeni* (Table 9.3).

The data set presented here includes individuals from Anjamena (east of the Mahavavy river) and from Anadabomandry (west of the Mahavavy river) from *Eulemur mongoz*, *E. fulvus rufus* and *Propithecus verreauxi*. Within each taxon, genetic distances between populations of those 2 localities never exceed 20 bp (Table 9.4). The molecular data set thus confirms the current taxonomy in the absence of a distinction between different subspecies of *E. mongoz* or *E. fulvus* west and east of the Mahavavy river. Likewise, the mtDNA sequence data do not support subspecific differentiation among populations of *P. verreauxi* east and west of the Mahavavy river.

Tsiribihina River

Based on current taxonomy, the Tsiribihina river separates the subspecies *Propithecus verreauxi deckeni* from *P.v. verreauxi* and the species *Lepilemur edwardsi* from *L. ruficaudatus*. In contrast, *Eulemur fulvus*, *Microcebus murinus* or *Cheirogaleus medius* populations north and south of the Tsiribihina river are not considered as taxonomically distinct units.

1) *Propithecus verreauxi deckeni* individuals from Anadabomandry (north of Tsiribihina river) differ by only 20 to 24 bp from *P.v. verreauxi* populations south of the Tsiribihina river. Molecular data do not support subspecific rank among those populations and thus indicate that the Tsiribihina river does not serve as an efficient genetic barrier in *P. verreauxi*.

2) Pairwise distances between *Eulemur fulvus rufus* individuals from Anadabomandry or Maintirano (north of Tsiribihina river) and *E.f. rufus* individuals from Morondava (south of Tsiribihina river) range from 54 to 60 bp. Unlike current taxonomy, genetic data thus clearly support a differentiation at the subspecific level among *E. fulvus* populations south and north of the Tsiribihina river.

Further Genetic Differentiation along the Western Coast

For some taxa, only a few locality samples could be obtained. For several taxa, these limited samples did not come from sites across all 3 western coast zones. However, even if more

than one river lies between two populations investigated, genetic divergences can provide valuable information on gene flow along the western coast.

1) For *Lepilemur* no sample from the area between the Mahavavy and the Tsiribihina river could be obtained. However, the data set includes one *L. ruficaudatus* sample. While the exact locality of this sample is unknown, the distribution area of *L. ruficaudatus* is south of the Tsiribihina river. Pairwise distance values of comparisons between this *L. ruficaudatus* and *L. edwardsi* from Anjamena are 142 to 146 bp (Table 9.4). Genetic divergence thus supports the specific level differentiation of the current taxonomy. Based on the taxon sampling in this study, it is not possible to test whether the Tsiribihina river (as suggested by current taxonomy) or the Mahavavy river acts as the genetic barrier. However, as the Mahavavy river has failed to impede gene flow among all other lemur populations investigated in this study, it is more likely that the Tsiribihina river forms the isolating barrier between *L. edwardsi* and *L. ruficaudatus*.

2) One sample of *Hapalemur griseus occidentalis* sequenced in this study was collected at the Tsiombikibo forest (west of the Mahavavy river) while the other 2 samples originate from Ambato (north of the Betsiboka river). Absolute pairwise distances among those two localities range from 47 to 48 bp (Table 9.4). Genetic divergence thus clearly lies in the range of other lemur subspecies. This finding is inconsistent with current taxonomy, which recognises only one subspecies of *H. griseus* along the western coast. Between the Tsiombikibo forest and Ambato lie the Mahavavy and Betsiboka rivers. Because the Mahavavy river does not act as an efficient barrier to gene flow in other lemur taxa, it is more likely that those 2 *H. griseus* subspecies are isolated by the Betsiboka river.

3) *Microcebus murinus* is distributed along the whole western coast. The data set presented here includes samples from Ampijoroa (east of the Betsiboka river) and Kirindy (south of the Tsiribihina river). Pairwise comparisons among individuals of those two populations yield distance values from 60 to 62 bp. Such high genetic divergence supports the existence of 2 different *M. murinus* subspecies along the western coast which, however, are not reflected in current taxonomy. As 3 large rivers lie between those 2 sample localities, it is not possible to determine the exact genetic barrier between those 2 subspecies.

4) *Cheirogaleus medius* also occurs along the western coast. The samples investigated in this study suggest that 3 different subspecies might exist within this taxon. However, as nothing is known regarding the origin of the captive animals used in this study it is not possible to

examine any putative geographic isolating boundaries. But in the light of information on other lemur taxa along the western coast, either the Betsiboka or the Tsiribihina river might act as genetical barriers in *C. medius*.

Evolution of Lemurs along the Western Coast

The current data set clearly shows that the Betsiboka river is acting as an isolating barrier between populations of lemurs in north-western Madagascar. Furthermore, the Tsiribihina river acts as a barrier to gene flow between northern and southern populations of lemurs in central western Madagascar. The Mahavavy river does not appear to constitute a genetic isolating feature for lemur populations.

Along the western coast, 3 zones (NW, W1 and W2) have been defined which are presumed to be delimited from each other by the Betsiboka (NW–W1) and Tsiribihina (W1–W2) rivers (Martin 1972, 1995). The present molecular data set clearly supports the subdivision of Madagascar's west into these 3 major zones. Genetic divergence among taxa along the western coast show that both the Betsiboka and the Tsiribihina river act as efficient barriers to gene flow. This finding confirms that those 2 rivers are major physical barriers and serve as effective boundaries among the western distribution zones.

Depending on the taxa under consideration, the level of genetic divergence among populations separated by the Betsiboka or Tsiribihina rivers is very different. Based on the present study, the Tsiribihina river does not act as a barrier to gene flow for *Propithecus verreauxi*. In *Eulemur fulvus*, however, the Tsiribihina river separates the distribution areas of two subspecies and the same river genetically isolates *Lepilemur* at the specific level. The Betsiboka river also separates populations at all taxonomic levels. Genetic divergence is highest among *Lepilemur* populations on both sides of the river, but also *Propithecus* populations reach genetic distances at the species level. *E. fulvus* is separated at the subspecific level by the Betsiboka river, while in *E. mongoz* no genetical differentiation exists.

When looking at the pattern of genetic divergences along the western coast, the complexity of lemur evolution becomes obvious. If the high genetic divergences observed in *Lepilemur* species are any indication, these taxa have been restricted to the 3 different zones in the west for some considerable time. *Propithecus* were separated by the Betsiboka river long enough to differentiate at the specific level, while the Tsiribihina river has not served as a genetic barrier to gene flow. Genetic differentiation among the 3 *Eulemur fulvus* populations in

the 3 major zones reaches the subspecific level. *Eulemur mongoz* is the only taxon for which the Betsiboka river did not serve as a barrier to gene flow. The large degree of genetic differentiation of *Lepilemur* compared to other lemur taxa at the western coast indicates that *Lepilemur* radiated first over the 3 zones and then was followed by the other taxa. The low genetic divergence among *E. mongoz* populations west and east of the Betsiboka river indicates either that *E. mongoz* has only recently extended its distribution area by crossing the Betsiboka river or that *E. mongoz* is the only taxon for which this river does not act as an efficient barrier. Such different levels of separation were in fact predicted by Martin (1972), who pointed out that because of the sequential dynamics of evolution among Malagasy lemurs we are confronted with an extremely complex situation.

Thus far, there is no evidence for separation between the lemur taxa examined through an isolating effect of the Mahavavy river, which originates in the Bongolava Massif. The Bongolava Massif may serve as the contact zone between the different subregions (Thalmann & Rakotoarison 1994).

9.4 Additional Zoogeographic Considerations in Lemur Evolution

Central Plateau

The central plateau generally acts as an efficient barrier to gene flow between lemur taxa. Only two exceptions have been found in this study.

1) *E.f. fulvus* individuals from both north-western and eastern Madagascar have been sequenced. Pairwise distances among populations of the western and eastern coast do not exceed 6 bp. This small degree of genetic divergence supports the inference that both populations represent the same subspecies.

2) The genetic analyses indicate that there are 2 distinct subspecies of *E.f. rufus*. One occurs in the northwest, while the other occurs on the eastern coast and south of the Tsiribihina river on the western coast. Genetic distances between the western and eastern populations of the latter (5 bp) clearly lie in the range of differentiation within a subspecies.

Lemurs on the Comores

Most lemurs are endemic to the island of Madagascar. The only 2 exceptions are *Eulemur fulvus* and *E. mongoz*, which also occur on the Comoro Islands. *E. fulvus* occurs on Mayotte and *E. mongoz* is found on Anjouan and Mohéli. Both species are thought to have been taken to the Comores from Madagascar by humans. However, only the *E. fulvus* population on the Comores is recognised as a subspecies endemic to Mayotte (*E.f. mayottensis*). It is unclear whether or not Malagasy and Comorian populations of *E. mongoz* are distinct lineages. The mtDNA sequence data allow examination of the level of genetic differentiation among Malagasy and Comorian populations of *E. fulvus* and *E. mongoz*.

1) The 4 *E.f. fulvus* and 3 *E.f. mayottensis* individuals analysed in this study do not form distinct monophyletic lineages. Such paraphyly indicates that *E.f. mayottensis* does not deserve its current subspecific status. Furthermore, genetic distances between the Malagasy *E.f. fulvus* and Comorian *E.f. mayottensis* populations are not equivalent to those between subspecies.

2) The current study includes samples of 10 mongoose lemurs from 4 populations. The 3 Malagasy populations — Anadabomandry, Anjamena and Ampijoroa — are separated by large rivers (Mahavavy and Betsiboka). Three samples were obtained from unrelated captive animals, but only one was unquestionably descended from a Comorian founder. However, it is assumed that the captive population is derived mainly from animals of Comorian origin. The

3 captive animals form a clade with bootstrap support of 100%. This indicates that they all share a very similar mtDNA sequence, suggesting provenance from the same population and thus supporting all 3 having Comorian or closely related ancestors. Pairwise distances between all four investigated mongoose lemur populations are approximately the same (8–19 bp). There is no increase in genetic distance between the Malagasy and captive populations (9–19 bp) and distances are smaller than the range observed at subspecific level in other *Eulemur* taxa.

The level of genetic divergence indicates that *E. fulvus* and *E. mongoz* populations on the Comores are recently derived from their relatives in northwest Madagascar. Genetic data thus support the hypothesis that both species have been introduced to the Comores by humans at some time within the past several hundred years. There is no support for distinct subspecies of either species on the Comoro Islands.

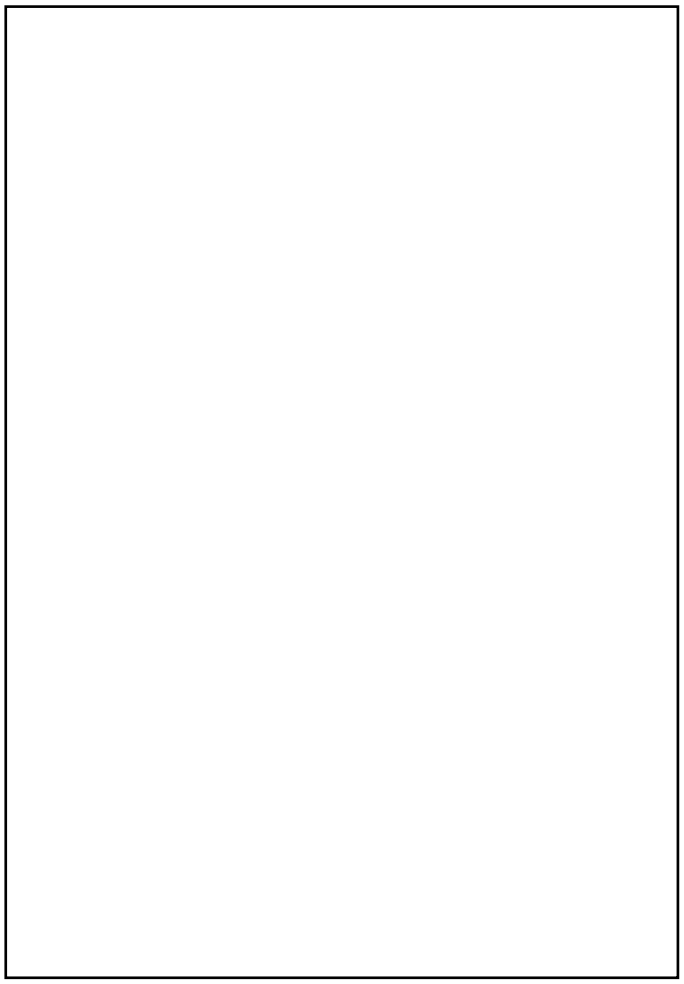


Fig. 9.3 *Eulemur mongoz* male (left) and female (right) from Parc Zoologique et Botanique de Mulhouse (July 1999).

9.5 Outlook

Adding Samples to the Data Set

The current data set can be improved by adding samples from taxa which were not included in this study. Furthermore, many samples of individuals with unknown geographic origin were included. There are major concerns about the use of zoo specimens of unknown or uncertain provenance in systematics. However, the difficulty in obtaining samples from wild-caught individuals necessitates the use of zoo samples as the only alternative. In future studies, it might be possible to include samples with precise locality data in order to address some of the questions not answered in the current study.

1) The mtDNA sequence data set presented here contains 12 of the 14 lemur genera. Further molecular studies should include the genus *Phaner* to test its proposed basal position among the Cheirogaleidae. The other missing genus is *Indri* of the family Indridae, which should be added to the current data set in future studies to resolve phylogenetic relationships among indrid genera.

2) This study could be enhanced by the inclusion of additional *E. fulvus* specimens from a wider array of localities to obtain a better interpretation of the current contrast between diagnosable genetic and phenotypic units. Dense locality sampling would allow confirmation of the two distinct lineages and exact distribution of *E.f. rufus*. The distribution boundaries between *E.f. fulvus* and other subspecies in the north requires additional clarification. Additional *E.f. albifrons* and *E.f. sanfordi* samples with locality data would allow verification of the finding that they might belong to the same subspecies.

3) Considerable genetic differentiation exists among the small sample of *H.g. occidentalis* individuals examined here. A more detailed examination of the western *H. griseus* populations would be valuable in an attempt to explore the possibility that more than one subspecies exists along the western coast. The eastern coast also deserves a more detailed investigation (e.g. by adding *H.g. meridionalis*).

4) Unexpectedly high levels of genetic divergence among the individuals of *V.v. variegata* were found, which indicates that more detailed examination of *V.v. variegata* might reveal additional subspecies or populational structure in this taxon. Dense locality sampling will be required to corroborate the diagnosis of distinct lineages of *V.v. variegata* that are currently undescribed.

5) Within both *Microcebus rufus* and *M. murinus*, genetic differentiation among different localities was found to exceed the range of intraspecific variation seen to occur among lemur samples thus far examined. The genus *Microcebus* clearly requires far more detailed studies in multiple disciplines to allow determination of specific and subspecific components.

6) The samples for *C. medius* also demonstrate stronger than expected molecular divergences, suggesting that phylogeographic differentiation may exist in this taxon. Future studies should examine *Cheirogaleus* populations from across the range in Madagascar to investigate the taxonomy and evolution of this nocturnal genus.

7) Genetic differentiation of *P. tattersalli* from *P.v. coquereli* is very low, suggesting that those two taxa diverged very recently. More comparative studies including *P. tattersalli* are required before a final decision about its taxonomic status can be made. To achieve a better understanding of the evolution of the genus *Propithecus*, the present data set should be supplemented by additional samples of *P. diadema* that occur along the eastern coast.

8) Unfortunately, only 3 of the 7 *Lepilemur* species could be included in this study. However, in the small sample studied there are strong indications for a previously undetected species. The genus *Lepilemur*, which is distributed throughout Madagascar, should receive a high priority for much more detailed investigations.

Adding More Characters to the Data Set

The segment of the mtDNA amplified and sequenced in this study includes a fragment of the COIII gene, complete sequences for the NADH-dehydrogenase subunits 3, 4L and 4 (ND3, ND4L, ND4), as well as the $tRNA^{Gly}$, $tRNA^{Arg}$, $tRNA^{His}$, $tRNA^{Ser}$, and partial $tRNA^{Leu}$ genes. This region of mtDNA allowed successful resolution of most lemur taxa at the subspecies, species and genus level. The approximately 2400 bp chosen to sequence in this study hence proved to be highly suitable for investigations of phylogenetic relationships among lemurs across all taxonomic levels.

As with data in previous investigations, the data presented here failed to yield clear resolution of phylogenetic relationships among the 5 *Eulemur* species and among 4 of the 5 lemur families. A variety of solutions have been proposed for overcoming difficult phylogenetic problems. It has been suggested that increased sampling, of either characters or taxa, can improve accuracy. In the genus *Eulemur*, all species and subspecies have been included in the current study, so adding more taxa is no option. It is unlikely that the addition of

the few missing taxa would dramatically enhance the resolution among the lemur families. However, adding more DNA sequence data may improve the phylogenetic resolution among taxa. It would be of special value to include sequence data from the nuclear genome. Nuclear DNA sequences would provide an alternative locus to the mtDNA genome allowing independent diagnosis for evolutionary relationships among lemurs.

Evolution of Lemurs

For many lemur genera, taxonomy at the species level and below has not been investigated very closely. For example, the molecular data presented here may provide the most complete overview for the genus *Eulemur*. Because most taxa in this genus are kept in various zoos all over the world, it was possible to obtain samples from all species and subspecies. Additionally, the cathemeral *Eulemur* has been investigated by many scientists in the field, making it possible to obtain additional samples from wild-caught animals. Likewise, evolutionary diversity within the genus *Microcebus* (Cheirogaleidae) appears to be comparable to diversity within the genus *Eulemur* (Lemuridae). However, *Microcebus* has far fewer described species or subspecies than *Eulemur*. These 'cryptic' taxa suggest that closer investigation of *Microcebus* and other lemur genera with wide distributions may also reveal more taxonomic structure.

In general, nocturnal forms (*Microcebus*, *Mirza*, *Cheirogaleus*, *Phaner*, *Allocebus*, *Avahi*, *Daubentonia*) have long been relatively neglected by researchers. The investigation of nocturnal species commonly makes field examinations very difficult and time-consuming. As a result, fewer species per genus and fewer subspecies per species have been recognised on average among the nocturnal lemurs than among their diurnal relatives. Many lemur taxa are not at all (*Avahi*, *Indri*) or very rarely (*Lepilemur*, *Propithecus*, *Hapalemur*, *Phaner*, *Daubentonia*) kept in captivity. Samples of those taxa can hence only be obtained directly from the wild. However, the majority of current lemur habitat in Madagascar remains difficult to access and provides harsh working conditions for such investigations.

Despite the costs and hardships consequent of further investigations, the evolution of lemurs on Madagascar provides a perfect model for the study of evolutionary biology. In each geographically isolated subregion, samples from all lemur taxa present should be collected and analysed. While this would primarily provide a complete overview of the distribution of lemurs remaining on Madagascar it would also allow further examination of their genetic diversity. The

DNA sequence data would allow determination of genetic divergences among populations from each subregion and thus inferences about the efficiency of geographic barriers (e.g. rivers or mountains) as isolating mechanisms. Evolutionary patterns from different taxa could be compared both within and across regions. This would yield critical insights into the various evolutionary patterns exhibited by the different taxa. Using a combination of nuclear DNA markers and mtDNA markers, a time frame for the radiation of lemurs could be estimated. Ultimately, the unique primate fauna of Madagascar can provide insights not only into their own unique evolutionary history but also into the mechanisms and results of evolutionary forces in primates in general. Such information is immediately critical for the short-term goal of providing the information required for conservation efforts. In the long term, such data would serve to increase our knowledge of the pattern and process of evolution in this unique endemic group.

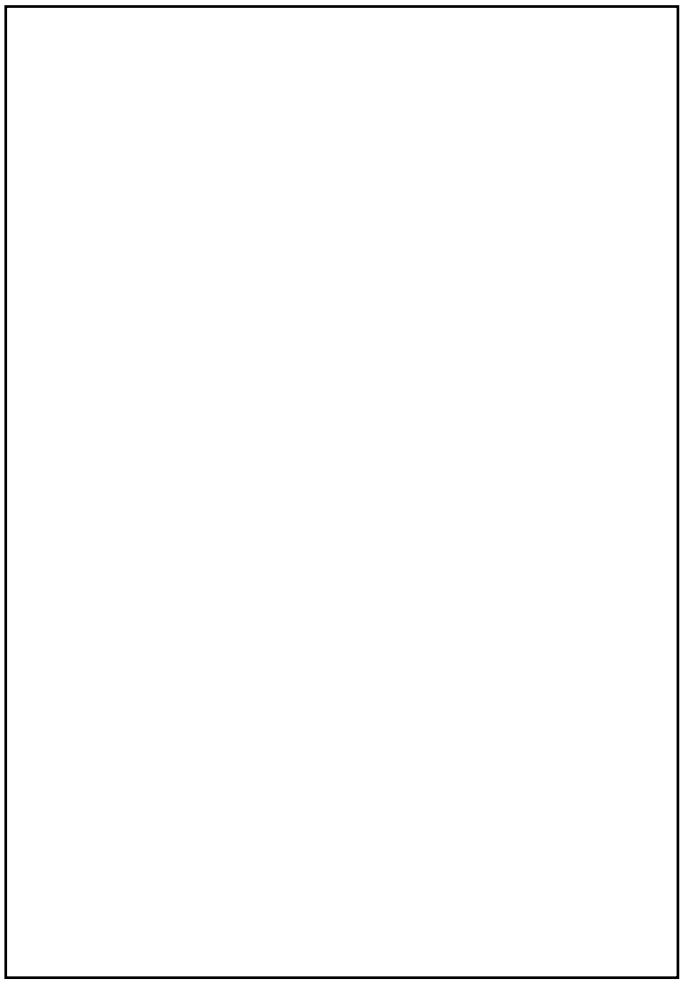


Fig. 9.4 *Eulemur macaco macaco* female (left) and male (right) kept in Strasbourg (January 1998).

10. Summary

10.1 Summary

This study investigates the systematics of the lemurs of Madagascar. Five lemur families are currently recognised, but their taxonomy, nodal relationships and composition need clarification. The families **Lepilemuridae** and **Daubentoniidae** each contain only 1 genus (*Lepilemur* and *Daubentonia*). The family **Indridae** is classified into 3 genera (*Avahi*, *Indri* and *Propithecus*). *Propithecus* includes 3 species containing up to 10 described subspecies, whose evolutionary relationships remain contentious. In particular, it is unclear whether *P. verreauxi deckeni* and *P.v. coronatus* populations are differentiated at the subspecific level. Furthermore, the taxonomic status of the recently discovered *P. tattersalli* also requires further examination. The family **Cheirogaleidae** is currently classified into 8 species in 5 genera, whose phylogenetic relationships have yet to be clarified. Taxonomic status of *Mirza coquereli*, *Allocebus trichotis* and the recently discovered *Microcebus ravelobensis* require further examination. The family **Lemuridae** includes 4 genera. The taxonomy and phylogenetic relationships between *Lemur*, *Eulemur* and *Hapalemur*, and of *Varecia* to the other lemurids continue to be hotly debated. Nodal relationships among the 5 *Eulemur* species also remain uncertain. The phylogenetic relationships among *Hapalemur* species and subspecies as well as their taxonomic status need to be verified. *Eulemur fulvus* includes 7 subspecies, whose evolutionary relationships remain a matter for debate. In particular, it is unclear whether the Malagasy and Comorian *E.f. fulvus* populations are differentiated at the subspecific level (*E.f. mayottensis*). Furthermore, it has been suggested that *E.f. collaris* and *E.f. albocollaris* are separate species.

A mitochondrial DNA sequence data set from the ND3, ND4L, ND4 genes and 5 tRNAs (Gly, Arg, His, Ser, Leu) was generated to try to clarify phylogenetic relationships among lemur families, genera, species and subspecies. To attempt this goal, a total of 131 lemurs from 12 genera, 25 species and 18 subspecies have been sequenced. Two galagos were included as outgroup taxa. The ~2400 bp sequences were analysed using maximum parsimony, neighbor-joining and maximum likelihood methods. Different weighting schemes and outgroups have been applied in trying to resolve phylogenetic relationships.

The mitochondrial DNA sequence data used in this study yield a strong phylogenetic signal. A number of clear findings emerged: The data strongly support the monophyly of the Malagasy lemurs. Regardless of which outgroup was applied or how the data set was weighted, *Daubentonia* consistently groups as sister to a clade containing the other 4 lemur families. However, the molecular data failed to yield clear resolution of phylogenetic relationships among the 4 families Cheirogaleidae, Indridae, Lemuridae and Lepilemuridae. Nevertheless, the monophyly of each of the 5 lemur families is supported in all analyses.

Lepilemur is indeed deeply separated from all other lemur genera in the data set, which strongly supports the family status (**Lepilemuridae**) of this single genus. Tree topology and pairwise genetic distances clearly confirm specific status for *L. edwardsi*, *L. ruficaudatus* and *L. septentrionalis*. Paraphyly and a high degree of genetic divergence among *L. edwardsi* individuals clearly suggests that two species exist in the range of the currently recognised species *L. edwardsi*.

In **Cheirogaleidae**, *Mirza* and *Microcebus* form a clade representing the sister group of *Allocebus*, while a clade containing *Cheirogaleus major* and *C. medius* diverges first. *M. ravelobensis* and *M. rufus* form a subclade within *Microcebus*, with *M. murinus* as its sister group. Pairwise distance comparisons and tree topology support the generic status of *Mirza coquereli* and species-level divergence of *M. ravelobensis*. Furthermore, '*M. rufus*' may well represent more than one species.

In **Indridae**, all analyses group *Avahi* as sister to the clade containing all *Propithecus*. *P. diadema* is the first species to diverge within the genus *Propithecus*. Among the remaining *Propithecus*, one subclade is formed by *P.v. coquereli* and *P. tattersalli*, while *P.v. verreauxi*, *P.v. deckeni* and *P.v. coronatus* form the second subclade. All analyses fail to resolve *P.v. coronatus* and *P.v. deckeni* into separate monophyletic lineages. Based on pairwise distance comparisons and tree topology, it is concluded that *P. tattersalli* does not represent a distinct species and that *P.v. deckeni* and *P.v. coronatus* do not deserve subspecific rank. On the other hand, these analyses indicate that *P.v. coquereli* may well represent a distinct species.

In **Lemuridae**, the results support monophyly of *Eulemur*, a basal divergence of *Varecia*, and a sister-group relationship for *Lemur*/*Hapalemur*. Based on tree topology and pairwise distance comparisons, it is concluded that *Varecia* and *Eulemur* both represent distinct genera separate from *L. catta*. *H. griseus* and *H. aureus* form a clade, but the sequence data do

not permit resolution of the trichotomy involving *H. simus*, *H. aureus*/*H. griseus* and *L. catta*. Within *Eulemur*, there is strong support for a clade containing *E. fulvus*, *E. mongoz* and *E. rubriventer*. However, analyses failed to resolve clearly relationships among those 3 species or with the more distantly related *E. coronatus* and *E. macaco*. The sequencing data support the current subspecific status of *E.m. macaco* and *E.m. flavifrons*, and that of *V.v. variegata* and *V.v. rubra*. However, tree topology and relatively high genetic distances among individual *V.v. variegata* indicate that there may be more phylogenetic structure within this taxon than is indicated by current taxonomy. The sequence data do not yield clear resolution of *H.g. griseus* from *H.g. alaotrensis*. Considerable genetic differentiation exists among the small sample of *H.g. occidentalis* individuals examined here, indicating that more than one subspecies may exist along the western coast. Analyses resolved 34 *E. fulvus* specimens into 6 clades: ((*albocollaris, collaris*) (*rufus* (*rufus* (*fulvus*/*mayottensis* (*albifrons*/*fulvus*/*sanfordi*))))). It can be concluded that *E.f. albocollaris* and *E.f. collaris* do not represent distinct species from *E. fulvus* and that Comorian brown lemurs do not deserve subspecific rank. No genetic differentiation was detected between *E.f. albifrons* and *E.f. sanfordi*; on the other hand, there are obviously two separate lineages of *E.f. rufus*.

10.2 Zusammenfassung

Die vorliegende Studie untersucht die Systematik der Lemuren Madagaskars. Zur Zeit werden bei den Lemuren fünf Familien unterschieden, deren Taxonomie, phylogenetische Beziehungen und Zusammensetzung aber noch weiterer Klärung bedürfen. Die Familien **Lepilemuridae** und **Daubentoniidae** beinhalten je nur eine Gattung (*Lepilemur* und *Daubentonia*). Bei der Familie **Indridae** werden drei Gattungen unterschieden (*Avahi*, *Indri* und *Propithecus*). *Propithecus* wird in drei Arten unterteilt, von welchen bis zu zehn Unterarten beschrieben wurden, deren evolutionäre Beziehungen allerdings umstritten sind. Es ist zum Beispiel unklar, ob die Populationen von *P. verreauxi deckeni* und *P.v. coronatus* tatsächlich getrennte Unterarten sind. Des weiteren sollte die Taxonomie der kürzlich entdeckten Art *P. tattersalli* genauer untersucht werden. Die Familie **Cheirogaleidae** umfasst acht Arten und fünf Gattungen, deren phylogenetische Beziehungen aber umstritten sind. Die Taxonomie von *Mirza coquereli*, *Allocebus trichotis* und des kürzlich entdeckten *Microcebus ravelobensis* müssen genauer untersucht werden. Die Familie **Lemuridae** beinhaltet vier Gattungen. Die Taxonomie und phylogenetischen Beziehungen zwischen *Lemur*, *Eulemur* und *Hapalemur* und von *Varecia* zu anderen Lemuriden bleiben umstritten. Auch die Evolution der fünf *Eulemur* Arten ist unklar. Die verwandtschaftlichen Beziehungen zwischen den Arten und Unterarten von *Hapalemur* sowie deren Taxonomie sollten noch verifiziert werden. *Eulemur fulvus* beinhaltet sieben Unterarten, deren evolutionäre Beziehungen immer noch debattiert werden. Insbesondere ist unklar, ob die *E.f. fulvus* Populationen von Madagaskar und den Komoren sich auf dem Niveau von Unterarten unterscheiden (*E.f. mayottensis*). Des weiteren wurde auch schon vorgeschlagen, dass *E.f. collaris* und *E.f. albocollaris* verschiedene Arten sein könnten.

Es wurde ein Datensatz bestehend aus mitochondrialen DNA Sequenzen von den ND3, ND4L, ND4 Genen und fünf tRNAs (Gly, Arg, His, Ser, Leu) produziert, um die phylogenetischen Beziehungen zwischen Familien, Gattungen, Arten und Unterarten zu untersuchen. Um dieses Ziel zu erreichen, wurden insgesamt 131 Lemuren aus 12 Gattungen, 25 Arten und 18 Unterarten sequenziert. Zwei Galagos wurden als Outgroup mit einbezogen. Die aus ca. 2400 Basenpaaren bestehenden Sequenzen wurden mittels Maximum Parsimony, Neighbor-joining und Maximum Likelihood Methoden analysiert. Die Daten wurden unterschiedlich gewichtet und verschiedenste Outgroups wurden gewählt, um die phylogenetischen Beziehungen zu eruieren.

Der Datensatz mitochondrialer DNA, welcher in dieser Studie verwendet wurde, lieferte ein starkes phylogenetisches Signal. Etliche Problemstellungen konnten gelöst werden: Die Daten unterstützen deutlich die Monophylie der Lemuren Madagaskars. Egal welche Outgroup verwendet wurde oder wie die Daten gewichtet wurden, *Daubentonia* gruppiert immer als Schwestergruppe zu den anderen vier Lemurenfamilien. Aufgrund der vorliegenden Daten war es nicht möglich, die phylogenetischen Beziehungen unter den vier Familien Cheirogaleidae, Indridae, Lemuridae und Lepilemuridae zu bestimmen. Die Monophylie jeder einzelnen der fünf Lemurenfamilien ist aber mit allen Analysen nachgewiesen worden.

Wie erwartet, ist *Lepilemur* sehr stark von allen anderen untersuchten Lemurengattungen getrennt, was den Familienrang dieser einzelnen Gattung unterstützt (**Lepilemuridae**). Die Form des Stammbaums und die paarweisen genetischen Distanzen weisen deutlich darauf hin, dass es sich bei *L. edwardsi*, *L. ruficaudatus* und *L. septentrionalis* um Arten handelt. Paraphylie und ein hoher Grad an genetischer Divergenz unter den *L. edwardsi* Individuen lassen vermuten, dass eigentlich zwei Arten im Verbreitungsgebiet des zur Zeit als einer Art anerkannten *L. edwardsi* existieren.

Innerhalb der **Cheirogaleidae** gruppieren *Mirza* und *Microcebus* gemeinsam als Schwestergruppe zu *Allocebus*, während *Cheirogaleus* (*C. major* und *C. medius*) sich als erstes abgetrennt hat. Innerhalb von *Microcebus* formieren *M. ravelobensis* und *M. rufus* eine Untergruppe, zu welcher *M. murinus* die Schwestergruppe bildet. Die genetischen Daten weisen darauf hin, dass '*M. rufus*' mehrere Arten beinhaltet.

Bei den **Indridae** wird *Avahi* zu einer Gruppe gestellt, welche alle *Propithecus* enthält. *P. diadema* ist die erste Art, welche sich innerhalb von *Propithecus* abspaltet. Unter den verbleibenden *Propithecus* formieren sich *P.v. coquereli* und *P. tattersalli* zur einen Untergruppe, während *P.v. verreauxi*, *P.v. deckeni* und *P.v. coronatus* die zweite Untergruppe bilden. Keine der Analysen konnte *P.v. coronatus* und *P.v. deckeni* in zwei monophyletische Linien auflösen. Aus dem Vergleich von paarweisen genetischen Distanzen und der Form des Stammbaums muss geschlossen werden, dass *P. tattersalli* keine eigene Art repräsentiert und dass *P.v. deckeni* und *P.v. coronatus* keine eigenen Unterarten darstellen. Im Gegensatz dazu deuten die Analysen darauf hin, dass *P.v. coquereli* eine eigene Art zu sein scheint.

Bei den **Lemuridae** unterstützen die Resultate eine Monophylie von *Eulemur*, eine erste Abspaltung von *Varecia* und eine Schwestergruppen-Beziehung zwischen *Lemur*/*Hapalemur*.

Basierend auf der Form des Stammbaums und dem Vergleich von paarweisen Distanzen kann gefolgert werden, dass sowohl *Varecia* als auch *Eulemur* als Gattungen von *L. catta* zu trennen sind. *H. griseus* und *H. aureus* bilden zusammen eine Untergruppe. Die Sequenzierdaten erlaubten es aber nicht, die Trichotomie zwischen *H. aureus*/*H. griseus*, *H. simus* und *L. catta* aufzulösen. Innerhalb von *Eulemur* gibt es eine gut abgestützte Gruppierung von *E. fulvus*, *E. mongoz* und *E. rubriventer*. Die phylogenetischen Beziehungen innerhalb dieser Gruppe oder zwischen dieser Gruppe und den anderen beiden Arten *E. coronatus* und *E. macaco* können aber nicht bestimmt werden. Die Sequenzierdaten unterstützen den Unterarten-Status von *E.m. macaco* und *E.m. flavifrons* und von *V.v. variegata* und *V.v. rubra*. Aufgrund der Form des Stammbaums und der hohen genetischen Distanzen zwischen den einzelnen *V.v. variegata* Individuen kann angenommen werden, dass dieses Taxon mehr als eine Unterart beinhaltet. Die beiden Unterarten *H.g. griseus* und *H.g. alaotrensis* konnten aufgrund der Sequenzierdaten nicht unterschieden werden. Eine bemerkenswerte genetische Variabilität existiert innerhalb der kleinen Stichprobe von *H.g. occidentalis*, was darauf hinweist, dass entlang der Westküste mehr als eine Unterart existiert. Die Analysen unterteilten die 34 *E. fulvus* Individuen in sechs Gruppen: ((*albocollaris, collaris*) (*rufus* (*rufus* (*fulvus*/*mayottensis* (*albifrons*/*fulvus*/*sanfordi*))))). Aus den Daten kann geschlossen werden, dass *E.f. albocollaris* und *E.f. collaris* keine eigene Arten repräsentieren und dass *E. fulvus* von den Komoren keine eigene Unterart darstellt. Im Gegensatz zu *E.f. albifrons* und *E.f. sanfordi*, welche genetisch nicht getrennt werden konnten, finden sich innerhalb von *E.f. rufus* zwei deutlich unterschiedliche Linien.

10.3 Résumé

L'étude présente examine la méthode systématique pour l'étude des lémuriens de Madagascar. A ce jour, on distingue chez les lémuriens 5 familles différentes, dont la taxonomie, les relations phylogénétiques et la composition devrons encore être élucidées. Les familles **Lepilemuridae** et **Daubentoniidae** ne contiennent chacune seulement qu'un genre (*Lepilemur* et *Daubentonia*). La famille **Indridae** est séparée en 3 genres différents (*Avahi*, *Indri* et *Propithecus*). *Propithecus* est subdivisé en 3 espèces. Jusqu'à 10 sous-espèces ont été décrites, dont les relations évolutives sont controversées. Il n'est par exemple pas clair, si les populations de *P. verreauxi deckeni* et *P.v. coronatus* sont vraiment des sous-espèces séparées. En plus la taxonomie de l'espèce *P. tattersalli*, découverte récemment, devrait être examinée plus précisément. La famille **Cheirogaleidae** contient 8 espèces et 5 genres, dont les relations phylogénétiques sont controversées. La taxonomie de *Mirza coquereli*, *Allocebus trichotis* et de *Microcebus ravelobensis* qui ont été trouvés récemment, devra être examinée plus précisément. La famille **Lemuridae** contient 4 genres. La taxonomie et les relations phylogénétiques entre *Lemur*, *Eulemur* et *Hapalemur* et la relation entre *Varecia* et les autres lémuriens reste controversée. L'évolution des 5 espèces *Eulemur* n'est pas claire non plus. Les relations parentales des espèces et sous-espèces de *Hapalemur* aussi bien que leur taxonomie devrons être vérifiées. *Eulemur fulvus* contient 7 sous-espèces, dont les relations évolutives sont toujours débattues. Notamment ce n'est pas clair si la population d'*E.f. fulvus* de Madagascar et des Comores se différencient au niveau des sous-espèces (*E.f. mayottensis*). De plus il a été conseillé de considérer *E.f. collaris* et *E.f. albocollaris* comme des espèces différentes.

Un ensemble de données se composant de séquences ADN mitochondriale des gènes ND3, ND4L, ND4 et de 5 tRNAs (Gly, Arg, His, Ser, Leu) a été produit pour analyser les relations phylogénétiques entre les familles, les genres, les espèces et les sous-espèces. Pour atteindre ce but, 131 lémuriens, appartenant à 12 genres, 25 espèces et 18 sous-espèces, ont été analysés. Deux galagos ont été inclus comme 'outgroup'. Les séquences contenant environ 2400 paires de bases ont été analysées avec des méthodes 'maximum parsimony', 'neighbor-joining' et 'maximum likelihood'. Les combinaisons variés ont été analysées et des 'outgroups' différents ont été choisis afin de résoudre le problème des relations phylogénétiques.

Les données de l'ADN mitochondriale qui ont été utilisées dans cette étude, nous montre un signal phylogénétique intense. Des problèmes divers ont été résolus: Les données soutiennent

clairement la monophylie des lémuriens de Madagascar. Quel que soit le 'outgroup' utilisé et quel que soit l'analyse des données, *Daubentonia* reste toujours groupé comme groupe associé des autres 4 familles de lémuriens. Compte tenu des données dont on disposait il n'a pas été possible de déterminer les relations phylogénétiques des 4 familles Cheirogaleidae, Indridae, Lemuridae et Lepilemuridae. Néanmoins, la monophylie de chacune des cinq familles de lémuriens a été prouvée par toutes les analyses.

Comme prévu, *Lepilemur* est très différent des autres genres de lémuriens analysés, ce qui soutient le statut de famille pour ce genre singulier (**Lepilemuridae**). La forme de l'arbre généalogique et les variations génétiques chez les différentes paires indique clairement, que chez *L. edwardsi*, *L. ruficaudatus* et *L. septentrionalis* il s'agit bien d'espèces. La paraphylie et une divergence génétique importante entre les individus de *L. edwardsi* laissent supposer qu'il existe deux espèces dans cette répartition géographique, au lieu d'une.

Chez les **Cheirogaleidae** *Mirza* et *Microcebus* forment un groupe associé à celui de l'*Allocebus*. Le genre *Cheirogaleus* (*C. major* et *C. medius*) s'est dissocié le premier. Chez *Microcebus M. ravelobensis* et *M. rufus* forment un sous-groupe, avec *M. murinus* comme groupe associé. Les données génétiques indiquent, que '*M. rufus*' pourrait représenter plus d'une espèce.

Chez les **Indridae**, *Avahi* est associé avec un groupe, qui contient tous les *Propithecus*. *P. diadema* est la première espèce qui s'embranche chez les *Propithecus*. Parmi les *Propithecus* qui restent, *P.v. coquereli* et *P. tattersalli* forment un premier sous-groupe alors que *P.v. verreauxi*, *P.v. deckeni* et *P.v. coronatus* forment un deuxième sous-groupe. Aucune des analyses n'a pu faire bifurquer *P.v. coronatus* et *P.v. deckeni* en deux lignées monophylétiques. En comparant les variations génétiques des paires et en observant l'arbre généalogique, il faut conclure que *P. tattersalli* ne représente pas une propre espèce et que *P.v. deckeni* et *P.v. coronatus* ne méritent pas le rang de sous-espèces. Par contre les analyses indiquent que *P.v. coquereli* pourrait bien être une espèce séparée.

Chez les **Lemuridae** les résultats soutiennent la thèse d'une monophylie de *Eulemur*, une séparation à la base d'origine de *Varecia* et une relation de groupe associé entre *Lemur*/*Hapalemur*. S'appuyant sur la forme de l'arbre généalogique et la comparaison des distances génétiques, on peut conclure que *Varecia* et *Eulemur* représentent tous deux, des genres séparés de *L. catta*. *H. griseus* et *H. aureus* forment ensemble un sous-groupe. Les

données des séquences n'ont pas permis de défaire la trichotomie entre *H. aureus*/*H. griseus*, *H. simus* et *L. catta*. Chez *Eulemur* il existe de fortes évidences en faveur de la réunion de *E. fulvus*, *E. mongoz* et *E. rubriventer* dans un même groupe. Les relations phylogénétiques de ce groupe ou entre ce groupe et les deux autres espèces *E. coronatus* et *E. macaco* ne peuvent être définies. Les données de séquences soutiennent le grade de sous-espèces de *E.m. macaco* et *E.m. flavifrons* et de *V.v. variegata* et *V.v. rubra*. Basé sur la forme de l'arbre généalogique et les grands variations génétiques entre les différents individus de *V.v. variegata*, on peut supposer que ce taxon est plus complexe que le laisse supposer la taxonomie actuelle. D'après l'analyse des données des séquences ont ne peut pas distinguer les deux sous-espèces *H.g. griseus* et *H.g. alaotrensis*. Le petit échantillon de *H.g. occidentalis* présente une variabilité génétique remarquable, ce qui indique que le long de la côte ouest il existe plus d'une sous-espèce. Les analyses divisent les 34 individus de *E. fulvus* en 6 groupes: ((*albocollaris, collaris*) (*rufus* (*rufus* (*fulvus*/*mayottensis* (*albifrons*/*fulvus*/*sanfordi*))))). On peut conclure que *E.f. albocollaris* et *E.f. collaris* ne représentent pas des espèces différentes de *E. fulvus*, et que les lémurs bruns de Comores ne méritent pas un rang de sous-espèce. Contrairement à *E.f. albifrons* et *E.f. sanfordi*, qui n'ont pas pu être séparé génétiquement, il se trouve chez *E.f. rufus* deux lignées bien distinctes.

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12. Appendix

Taxa, origin, identification numbers, and GenBank accession numbers for the 131 lemurs and 2 galagos sequenced.

Taxon	Origin	ID #	GenBank #
Eulemur mongoz 1	Anjamena (Northwest)	JP169	AF224514
Eulemur mongoz 2	Anadabomandry (Northwest)	JP177	AF224515
Eulemur mongoz 3	Ampijoroa (Northwest)	JP220	AF224519
Eulemur mongoz 4	unknown	JP1	AF224512
Eulemur mongoz 5	unknown	JP49	AF224513
Eulemur mongoz 6	unknown	JP240	AF224521
Eulemur mongoz 7	Anjamena (Northwest)	JP178	AF224516
Eulemur mongoz 8	Anjamena (Northwest)	JP196	AF224517
Eulemur mongoz 9	Anadabomandry (Northwest)	JP211	AF224518
Eulemur mongoz 10	Ampijoroa (Northwest)	JP221	AF224520
Eulemur coronatus 1	unknown	JP33	AF224522
Eulemur coronatus 2	unknown	JP34	AF224523
Eulemur coronatus 3	unknown	JP121	AF224524
Eulemur rubriventer 1	unknown	JP129	AF224525
Eulemur rubriventer 2	unknown	JP130	AF224526
Eulemur rubriventer 3	Andasibe (East)	JP229	AF224527
Eulemur macaco macaco 1	unknown (North)	JP80	AF224528
Eulemur macaco macaco 2	Ambato (North)	JP82	AF224529
Eulemur macaco macaco 3	Ambato (North)	JP83	AF224530
Eulemur macaco flavifrons 1	Maromandia (North)	JP74	AF224531
Eulemur macaco flavifrons 2	Maromandia (North)	JP75	AF224532
Eulemur macaco flavifrons 3	Maromandia (North)	JP77	AF224533
Eulemur fulvus fulvus 1	unknown	JP2	AF224564
Eulemur fulvus fulvus 2	pet in Anjozorobe	JP41	AF224534
Eulemur fulvus fulvus 3	Ampijoroa (Northwest)	JP215	AF224535
Eulemur fulvus fulvus 4	Ampijoroa (Northwest)	JP218	AF224536
Eulemur fulvus fulvus 5	pet in Antsohihy (Northwest)	JP330	AF224537
Eulemur fulvus fulvus 6	pet in Antsohihy (Northwest)	JP331	AF224538
Eulemur fulvus fulvus 7	pet in Foulpointe (East)	JP336	AF224539
Eulemur fulvus fulvus 8	pet in Vatomandry (East)	JP337	AF224540
Eulemur fulvus mayottensis 1	unknown	JP72	AF224541
Eulemur fulvus mayottensis 2	Comoro Islands	JP225	AF224542
Eulemur fulvus mayottensis 3	Comoro Islands	JP226	AF224543
Eulemur fulvus rufus 1	East	JP123	AF224544
Eulemur fulvus rufus 2	Anjamena (Northwest)	JP161	AF224545
Eulemur fulvus rufus 3	Anjamena (Northwest)	JP171	AF224547
Eulemur fulvus rufus 4	Anadabomandry (Northwest)	JP176	AF224548
Eulemur fulvus rufus 5	Anadabomandry (Northwest)	JP181	AF224549
Eulemur fulvus rufus 6	Anjamena (Northwest)	JP206	AF224550
Eulemur fulvus rufus 7	Morondava (West)	JP332	AF224551
Eulemur fulvus rufus 8	Maintirano (West)	JP333	AF224552
Eulemur fulvus rufus 9	Southeast	JP338	AF224553
Eulemur fulvus rufus 10	Southeast	JP339	AF224554
Eulemur fulvus rufus 11	Southeast	JP340	AF224555
Eulemur fulvus rufus 12	Southeast	JP341	AF224556
Eulemur fulvus rufus 13	Southeast	JP342	AF224557
Eulemur fulvus albocollaris 1	Vondrozo (Southeast)	JP222	AF224558
Eulemur fulvus albocollaris 2	pet, region Mahazoarivo (Southeast)	JP145	AF224562
Eulemur fulvus collaris 1	pet in Fort Dauphin (South)	JP304	AF224559
Eulemur fulvus collaris 2	unknown	JP307	AF224560
Eulemur fulvus sanfordi 1	pet, region Vohemar	JP126	AF224561
Eulemur fulvus sanfordi 2	pet, region Anivorana-Diego Suarez (North)	JP125	AF224563

Taxon	Origin	ID #	GenBank #
Eulemur fulvus albifrons 1	unknown	JP25	AF224565
Eulemur fulvus albifrons 2	unknown	JP135	AF224566
Eulemur fulvus albifrons 3	unknown	JP134	AF224567
Eulemur fulvus albifrons 4	Andranobe Forest (Northeast)	JP323	AF224568
Lemur catta 1	unknown	JP3	AF053684
Lemur catta 2	unknown	JP27	AF224569
Lemur catta 3	unknown	JP52	AF224570
Hapalemur aureus 1	Ranomafana (Southeast)	JP143	AF224581
Hapalemur aureus 2	Ranomafana (Southeast)	JP144	AF224582
Hapalemur griseus alaotrensis 1	unknown	JP4	AF224575
Hapalemur griseus alaotrensis 2	Belempona (East)	JP139	AF224576
Hapalemur griseus alaotrensis 3	Anororo (East)	JP140	AF224577
Hapalemur griseus griseus 1	unknown	JP234	AF224571
Hapalemur griseus griseus 2	Maromiza (East)	JP346	AF224572
Hapalemur griseus griseus 3	Maromiza (East)	JP347	AF224573
Hapalemur griseus griseus 4	Maromiza (East)	JP348	AF224574
Hapalemur griseus occidentalis 1	Ambato (North)	JP31	AF224578
Hapalemur griseus cf. *occidentalis 2*	Forêt de Tsiombikibo (Northwest)	JP241	AF224579
Hapalemur griseus occidentalis 3	Ambato (North)	JP275	AF224580
Hapalemur simus 1	Karianga (Southeast)	JP127	AF224583
Hapalemur simus 2	Karianga (Southeast)	JP128	AF224584
Varecia variegata variegata 1	unknown	JP30	AF224585
Varecia variegata variegata 2	unknown	JP131	AF224586
Varecia variegata variegata 3	unknown	JP132	AF224587
Varecia variegata rubra 1	unknown	JP5	AF224588
Varecia variegata rubra 2	unknown	JP32	AF224589
Varecia variegata rubra 3	unknown	JP236	AF224590
Varecia variegata rubra 4	Andranobe Forest (Northeast)	JP324	AF224591
Varecia variegata rubra 5	Andranobe Forest (Northeast)	JP325	AF224592
Lepilemur edwardsi 1	Anjamena (Northwest)	JP163	AF224593
Lepilemur edwardsi 2	Anjamena (Northwest)	JP207	AF224594
Lepilemur edwardsi 3	Ampijoroa (Northwest)	JP259	AF224595
Lepilemur ruficaudatus	unknown	JP233	AF224596
Lepilemur septentrionalis	Ankarana, Mahamasina (North)	JP280	AF224597
Allocebus trichotis	Vohidrazana (East)	JP349	AF224620
Cheirogaleus medius 1	unknown	JP6	AF224614
Cheirogaleus medius 2	unknown	JP70	AF224615
Cheirogaleus medius 3	unknown	JP282	AF224616
Cheirogaleus major 1	Mantasoa (East)	JP137	AF224617
Cheirogaleus major 2	unknown	JP138	AF224618
Cheirogaleus major 3	Andasibe (East)	JP118	AF224619
Microcebus murinus 1	unknown	JP285	AF224624
Microcebus murinus 2	Ampijoroa (Northwest)	JP288	AF224625
Microcebus murinus 3	Ampijoroa (Northwest)	JP289	AF224626
Microcebus murinus 4	Ampijoroa (Northwest)	JP292	AF224627
Microcebus murinus 5	Mandena (South)	JP308	AF224628
Microcebus murinus 6	Kirindy (West)	JP313	AF224629
Microcebus ravelobensis 1	Ampijoroa (Northwest)	JP299	AF224630
Microcebus ravelobensis 2	Ampijoroa (Northwest)	JP301	AF224631
Microcebus ravelobensis 3	Ampijoroa (Northwest)	JP303	AF224632
Microcebus ravelboensis 4	Ampijoroa (Northwest)	JP321	AF224633
Microcebus rufus 1	Andasibe (East)	JP141	AF224634
Microcebus rufus 2	Andasibe (East)	JP142	AF224635
Microcebus rufus 3	Nosy Be (North)	JP309	AF224636
Microcebus rufus 4	unknown	JP315	AF224637
Microcebus rufus 5	Andasibe (East)	JP316	AF224638
Microcebus rufus 6	Andasibe (East)	JP317	AF224639

Taxon	Origin	ID #	GenBank #
Mirza coquereli 1	unknown	JP268	AF224621
Mirza coquereli 2	unknown	JP269	AF224622
Mirza coquereli 3	unknown	JP270	AF224623
Avahi laniger	unknown	JP345	AF224598
Propithecus diadema edwardsi	Ranomafana (Southeast)	JP343	AF224599
Propithecus tattersalli	unknown	JP344	AF224600
Propithecus verreauxi verreauxi 1	Amboasary Reserve (South)	JP237	AF224601
Propithecus verreauxi verreauxi 2	Kirindy forest (West)	JP271	AF224602
Propithecus verreauxi verreauxi 3	Berenty Private Reserve (South)	JP350	AF224603
Propithecus verreauxi verreauxi 4	Berenty Private Reserve (South)	JP351	AF224604
Propithecus verreauxi deckeni 1	Anadabomandry (Northwest)	JP172	AF224605
Propithecus verreauxi deckeni 2	Anadabomandry (Northwest)	JP208	AF224606
Propithecus verreauxi deckeni 3	Anadabomandry (Northwest)	JP209	AF224607
Propithecus verreauxi coronatus 1	Anjamena (Northwest)	JP147	AF224608
Propithecus verreauxi coronatus 2	Anjamena (Northwest)	JP154	AF224609
Propithecus verreauxi coronatus 3	Anjamena (Northwest)	JP166	AF224610
Propithecus verreauxi coquereli 1	Andrevorevo-Antsohihy (Northwest)	JP136	AF224611
Propithecus verreauxi coquereli 2	Ampijoroa (Northwest)	JP212	AF224612
Propithecus verreauxi coquereli 3	Ampijoroa (Northwest)	JP217	AF224613
Daubentonia madagascariensis 1	Andratamarina (Northeast)	JP7	AF224640
Daubentonia madagascariensis 2	Anjiamangirana (Northwest)	JP119	AF224641
Daubentonia madagascariensis 3	Anjiamangirana (Northwest)	JP120	AF224642
Otolemur crassicaudatus	unknown	JP8	AF224643
Galago senegalensis	unknown	JP53	AF224644

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14. *Curriculum vitae*

Name:		Pastorini
Vorname:		Jennifer
Geboren am:		29. Dezember 1968 in Zürich (ZH)
Heimatort:		Zürich (ZH)
Ausbildung:	1985 - 1988	Alte Kantonsschule Aarau
	1988	Matura Typus C
	1988 - 1990	Biologiegrundstudium an der Universität Zürich
	1989 - 1990	Kleines Nebenfach Biomathematik
	1990 - 1991	Grosses Nebenfach Zoologie (Genetik und Ökologie)
	1990 - 1995	Hauptfach Anthropologie
	1991 - 1994	Diplomarbeit am Anthropologischen Institut
		"*DNA-Fingerprinting für Populations- und Verwandtschaftsanalysen bei fünf Makakenarten*"
		unter der Leitung von Prof. Dr. Robert D. Martin
	1995	Diplom in Anthropologie
	1995 - 2000	Dissertation am Anthropologischen Institut
		"*Molecular Systematics of Lemurs*"
		unter der Leitung von Prof. Dr. Robert D. Martin
		und Prof. Dr. Michael R.J. Forstner
Stellung:	1994 - 2000	Assistentin am Anthropologischen Institut