

Multiple nuclear loci reveal patterns of incomplete lineage sorting and complex species history within western mouse lemurs (*Microcebus*)

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Abstract

Mouse lemurs (genus *Microcebus*) are nocturnal primates endemic to the island of Madagascar. Until recently, they were classified as two species, one from eastern and one from western Madagascar. Previously published analyses of morphometric and mitochondrial DNA data show strong support for the recognition of more than eight species, however. Here, we test the eight-species hypothesis with DNA data derived from four independently segregating nuclear loci. We find many areas of congruence between the mitochondrial and nuclear data, but incomplete lineage sorting and low mutation rates limit the phylogenetic resolution of the nuclear data. Even so, the nuclear loci unanimously find evidence for three deeply diverged lineages within the mouse lemur radiation: one that is congruent with the mtDNA “southern clade”, another that is congruent with the mtDNA “northern clade”, and one monospecific branch comprised of the species *Microcebus ravelobensis*. The latter result in particular emphasizes the need for careful biological study of this species.

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1. Introduction

It is well understood that the task of recognizing and defining species boundaries is complex and controversial (Baum, 1992; de Queiroz and Donoghue, 1988; Dobzhansky, 1935; Donoghue, 1985; Hull, 1997; Mayr, 1942). The various criteria that have been proposed for recognizing species can be summarized into three general categories: physical (e.g., morphological distinction), historical (e.g., phylogenetic), and biological (e.g., reproductive isolation). This exercise can be especially complex for organisms showing only subtle morphological variation. In such circumstances, wherein investigators find it difficult to distinguish species identity based on morphological features, organisms are often referred to as “cryptic species” (e.g.,

Gomez et al., 2002; Henry, 1994; Kiefer et al., 2002; Schiffer et al., 2004). Mouse lemurs can potentially be said to represent a cryptic species radiation. They are the world's smallest living primates, with average adult body size ranging from 30 to 72 g (Rasoloarison et al., 2000), and are strictly nocturnal. Thus, one could have predicted that mouse lemurs have an elaborate repertoire of olfactory and auditory communication signals (Braune et al., 2005)—as has been demonstrated for numerous other nocturnal primates (Ambrose, 2003; Nietsch and Kopp, 1998; Zimmerman, 1995; Zimmerman et al., 1988). It is not surprising therefore that morphological features might be only subtly variable in mouse lemurs, making them difficult to distinguish with human eyes.

The genus *Microcebus* was considered monotypic by most authorities, containing only the species *murinus* (Schwartz, 1931), from the time of its original description in 1795 (Geoffroy Saint-Hilaire, 1795) until the 1970s. Upon increased research activity and broader geographic

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sampling of mouse lemur populations, several experts reached the conclusion that there were actually at least two distinct forms (Martin, 1972; Petter et al., 1977; Tattersall, 1982): *murinus*, a long-eared gray animal from the dry western regions of Madagascar and *rufus*, a short-eared reddish animal from the humid regions of the east. The two-species ecogeographic classification remained stable until the early 1990s, after which time mouse lemurs increasingly have become the target of systematic investigation (Kappeler et al., 2005; Louis et al., 2006; Rasoloarison et al., 2000; Schmid and Kappeler, 1994; Yoder et al., 2000; Zimmermann et al., 1998). Among these studies, Rasoloarison and colleagues (2000) described three new species from the western regions of Madagascar, and resurrected two others from synonymy, bringing the total count of recognized species with *M. rufus* in the east to eight at the time. These novel species designations were based on a combination of natural history observations, distribution data, and detailed morphometric analysis.

Coincidentally, Yoder and colleagues (Yoder et al., 2000) tested the species designations of Rasoloarison with mitochondrial DNA (mtDNA) data. Combined analysis of three mitochondrial data partitions (HV1 of the control region, cytochrome *b*, and cytochrome oxidase II) consistently yielded reciprocally monophyletic clades that are congruent with the various species recognized in the Rasoloarison et al. study. Since the Yoder paper was published, additional mouse lemur studies have implemented an approach that relies on mtDNA alone, without rigorous morphometrics, for estimating phylogenetic relationships using molecular data. These studies (Kappeler et al., 2005; Louis et al., 2006) use the resulting gene tree topology to reconstruct the historical relationships among mouse lemurs and to describe new species.

1.1. The need for a multilocus perspective

In western mouse lemurs, both morphological and mtDNA data have shown perfectly congruent support for high levels of species diversity. Moreover, the substantial mtDNA data set was used to produce a set of relationships for the hypothesized species. Even so, many investigators have emphasized the importance of analyzing multilocus data sets for evolutionary and phylogeographic analyses (Avice and Wollenberg, 1997; Hare, 2001; Hoelzer, 1997; McCracken and Sorenson, 2005; Zhang and Hewitt, 2003). Although mtDNA has been favored for initial phylogenetic analyses (Moore, 1995), especially in mammals, mitochondrial genes, acting as an individual locus, can produce only a single gene tree estimate and one estimate of lineage divergence, which may or may not accurately reflect species boundaries and/or the species phylogeny (Maddison, 1997; Neigel and Avice, 1986; Nichols, 2001). Essentially, a multilocus strategy provides independent estimates of genealogical history, and congruence among estimates provides strong evidence of actual species divergence. However, loci that segregate independently of one

another will have independent histories, as individual gene histories are affected by variation in population size, rates of lineage diversification, and random lineage sorting (Wakeley, 2003). These genealogical discrepancies can result in trees with topologically incongruent relationships among species (Maddison, 1997).

Lineage sorting is the process by which gene lineages become fixed within a species such that all alleles within that species sort to a single ancestral allele within the species. If lineage sorting is complete in both of two sister species, then any sampling of alleles will produce a reciprocally monophyletic gene tree (Avice, 1989; Maddison, 1997; Neigel and Avice, 1986; Pamilo and Nei, 1988). Under neutral theory, wherein genetic drift is assumed to be the predominant force determining allele fixation rates, polymorphisms that existed in an ancestral population are expected to persist in daughter populations for about $4N$ (where N is the effective population size of the daughter populations (Nei, 1987)). Thus, historical population size is the key factor that determines whether a species pair is reciprocally monophyletic at most loci after a fixed time interval. When population size is very large, the span of time until allelic fixation will be protracted, and time to reciprocal monophyly accordingly slow. The converse is also true. When populations are small (such as with a population bottleneck), time to reciprocal monophyly will be rapid.

Due to the effects of incomplete lineage sorting, systematic methods can become inappropriate and uninformative at the boundaries of intra- and inter-specific divergence (Doyle, 1992; Posada and Crandall, 2001). At this level, phylogenies are inadequate in discerning relationships between taxonomic groups. When lineage sorting is incomplete, a dichotomous branching pattern is no longer applicable (Morrison, 2005), as insufficient numbers of lineages have sorted and ancestral polymorphisms are retained. Nuclear DNA has a relatively larger effective population size than mtDNA; therefore, the point in the phylogeny when a single lineage has reached fixation at a nuclear locus will theoretically be at a higher taxonomic level (e.g., at or above the species level) than for mitochondrial loci. In this circumstance, depicting the pattern of evolution as reticulated, or net-like, may better reflect the relationships among individual alleles, as alternative interconnections between point mutations may be necessary. It is at this level that haplotype network methodologies become more appropriate for examining species-level relationships, as they allow for phylogenetic uncertainty without losing information relating to allele frequencies, relationships, and geographic distributions.

In this study, we test the hypotheses of species identity and historical relationships among mouse lemurs that have been previously derived from the analysis of mtDNA alone. We use multiple nuclear introns sequenced for the identical set of mouse lemur individuals sampled by Rasoloarison et al. and Yoder et al. studies. Each nuclear marker is analyzed as a minimum spanning network to examine the

relationships of alleles within and between species that have yet to reach reciprocal monophyly, and to better test for species boundaries by allowing for reticulate relationships among alleles. Each locus is also analyzed using phylogenetic methods to estimate the species history, both individually and as a concatenated sequence (i.e., the combined data approach). A combined approach has been favored by many to fully utilize genetic information in deriving phylogenetic estimates (Rokas and Carroll, 2006), despite the potential ill effects of incomplete lineage sorting and independent evolution among genomes. Thus, for this study, we include both separate and combined analyses to allow for comparisons of trees derived from both approaches and also to determine the impact that combining data has on phylogenetic inference.

2. Materials and methods

2.1. DNA isolation and sequencing

The 118 *Microcebus* samples used in this study were extracted previously from tissue as described in Yoder et al. (2000). In that study, the clade designated as *M. rufus II* has since been renamed as *M. simmonsii*; both names are given here for clarification. Each sample was amplified using the polymerase chain reaction using both novel and previously published primers for four nuclear markers (Table 1): adenosine receptor A3 exon 2 (ADORA), alpha enolase intron 8, alpha fibrinogen intron 4, and von Willebrand Factor intron 7. The targeted introns are each found on different chromosomes in the human genome. Given that mouse lemurs and humans share homologous chromosomes regions and that the discrepancy in numbers of chromosomes between the two is due to fragmentation (66 versus 46; Kolnicki, 1999), we believe it likely that these introns should also be located on separate chromosomes in *Microcebus* and therefore evolving independently. The intron primers were EPICs (exon priming intron crossing), in which priming sites are located in two conserved exon regions that flank the intron of interest. Introns were chosen from previously published work and from the intron database, ExInt (<http://sege.ntu.edu.sg/wester/exint>). Published mouse, rat, and human sequences were aligned with the priming sequence to estimate if the exon priming sites would be conserved in *Microcebus*. The four markers were chosen from 38 candidates under the criteria that they reliably amplified *Microcebus* DNA, their length was between 300 and 1000 bp, and that there be nucleotide diversity greater than 2% among multiple species of *Microcebus*.

Amplified DNA was prepared using PCR purification kits (Qiagen) and cycle-sequenced using ABI Big Dye. DNA sequence was generated using a MJ Research Base Station and ABI 3100 sequencer, and accessioned in GenBank (Table 2). Sequences were initially edited and aligned in Sequencher 4.1 (Gene Codes). Sequences that contained a pattern of double peaks at individual nucleotide sites, a possible indication of heterozygosity, were identified and

Table 1
Nuclear markers used in this study

Locus	Intron/ Exon #	Length (bp)	PI char. ^a	Chromosome location ^b	Annealing T°/[MgCl ₂] ^c	Sequence	Reference	Model
Adenosine receptor A3 (ADORA3)	E2	350	10 (10)	1p21–13	58/2.5	TGGACTTCAAAGTCTCCCGATGACCCGAGC CCAAGCTCCCAAGTCATCTGGTCAAA	Murphy et al. (2001)	GTR I G
Alpha enolase (ENO)	I8	848	106 (109)	1p36.3	62/1.25	TGGACTTCAAAGTCTCCCGATGACCCGAGC CCAAGCTCCCAAGTCATCTGGTCAAA	Friesen and Anderson (1997)	GTR G
Alpha fibrinogen (FIBA)	I4	609	63 (83)	4q28	59/1.5	FibaF-AAAGCGCAAAGTCATAGAAAAG FibaR-CTAAAGCCCTACTGCATGACCCCT	Heckman (this study)	HKY G
von Willebrand Factor (VWF)	I11	812	75 (77)	12p13.3	59.5/2.2	vwf10-GAGCTGGATGTCTGGCCATCCATGGCAAC vwf8-GAGTGCCCTGTCACTGGTCACTCCCACTTCAA	Mancuso et al. (1989)	GTR I G

^a Phylogenetically informative characters within *Microcebus* (with outgroup).

^b Chromosome location in humans. *Microcebus* have 2N = 66.

^c MgCl₂ concentration mM/μL.

Table 2

Species	Locality	Accession numbers						
		Enolase	Fibrinogen	von Willebrand Factor	ADORA	HVI	COII	Cytochrome b
<i>M. berthae</i>	Kirindy	EF052285– EF052296	DQ003345–DQ003351	EF052409– EF052414	EF052509– EF052516	AF285463–AF285466	AF285504–AF285507	AF285540– AF285543
<i>M. rufus I</i>	Ranamafana	EF052318– EF052328	DQ003386–DQ003401	EF052442– EF052457	EF052543– EF052559	AF285467–AF285474	AF285508–AF285514	AF285544– AF285551
<i>M. myoxinus</i>	Aboalimena, Bemaraha	EF052297– EF052315	DQ003360–DQ003383	EF052423– EF052439	EF052523– EF052540	AF285458–AF285462	AF285499–AF285503	AF285554– AF285556
<i>M. sambiranensis</i>	Manongoarivo	EF052346– EF052348	DQ003413–DQ003420	EF052467– EF052472	EF052572– EF052577	AF285477–AF285479	AF285518–AF285520	AF285554– AF285556
<i>M. rufus II (simmonsii)</i>	Tampolo	EF052316– EF052317	DQ003384–DQ003385	EF052440– EF052441	EF052541– EF052542	AF285475–AF285476	AF285516–AF285517	AF285552– AF285553
<i>M. tavaratra</i>	Ankarana	EF052349– EF052357	DQ003421–DQ003429	EF052473– EF052481	EF052578– EF052586	AF285455–AF285457	AF285497–AF285498	AF285533– AF285534
<i>M. ravelobensis</i>	Ankarafantsika	EF052329– EF052345	DQ003402–DQ003412	EF052458– EF052466	EF052560– EF052571	AF285452–AF285455	AF285493–AF285496	AF285529– AF285532
<i>M. murinus</i>	Andranomena, Vohimena, Kirindy and Manamby	EF052368– EF052408	DQ003430–DQ003474; DQ003476–DQ003479	EF052482– EF052508	EF052587– EF052619	AF285480–AF285489	AF285521–AF285527; AF321177–AF321179	AF285557– AF285566
<i>M. griseorufus</i>	Beza Mahafaly	EF052358– EF052367	DQ003352–DQ003359	EF052415– EF052422	EF052517– EF052522	AF285490–AF285491	AF321180–AF321181	AF285567– AF285568

subsequently cloned using the TA cloning system (Invitrogen). For each individual a minimum of 8–12 colonies were picked and amplified; a subset of 6–10 colonies of the appropriate size were sequenced to determine individual haplotypes. These quantities were necessary to expose and correct for instances of *Taq* amplification error (Bracho et al., 1998). Aligned haplotypes were exported from Sequencher 4.1 into MacClade (Maddison and Maddison, 2003) as a NEXUS file for further editing.

2.2. Network analyses

Gene genealogies were inferred using a parsimony-based network construction method. The network was created using a pairwise distance matrix of absolute number of differences in Arlequin v. 2.000 (Schneider et al., 2000) for all individuals for each marker. All possible minimum spanning trees were combined into a single minimum spanning network, allowing for reticulate relationships among alleles (Excoffier and Smouse, 1994; Fig. 1a–c).

2.3. Phylogenetic analyses

Analyses were performed for each individual marker (Fig. 1d–f) and combined data sets (Figs. 2–4). Each marker was tested for recombination events using Bootscanning (Salminen et al., 1995), MaxChi (Maynard Smith, 1992; Posada and Crandall, 2001), and RDP as implemented in the software suite RDP version 2.0 (Martin and Rybicki, 2000). The haplotypes shared by more than one individual (i.e., redundant haplotypes) were included only once in each analysis. *Cheirogaleus major*, a fellow member of the family Cheirogaleidae, was included as an outgroup. Indels were either treated as missing data, or if larger than 3–4 bp, excised from the data set. While phylogenies for all four markers were estimated, only the trees derived from the three introns are presented here. The ADORA exon provided few informative characters, and therefore resulted in trees with very weak topological support. This marker is included in this study only in combined concatenated analyses.

The incongruence length difference (ILD) test was used to give a general assessment of topological congruence of pairwise comparisons of three nuclear intron sequence partitions. This was performed in PAUP* as a partition homogeneity test using a heuristic search with 1000 replicates. This test was performed twice with differing sampling strategies: (1) 55 individuals and (2) 14 individuals (1–5 individuals/species).

Combined analyses were performed using two methods: (1) analyzing concatenated sequences (Fig. 2) and (2) combining trees using consensus methods. In the former, two analyses were performed: a combined nuclear analysis and a combined nuclear and mitochondrial analysis using sequences from GenBank for three mtDNA markers (Table 2). The number of sequences included in this analysis is reduced to reflect the original sampling

in the combined mtDNA study (48 individuals). For the consensus method, tree topology was drawn with one individual per species in MacClade and a majority rule consensus was constructed in PAUP* 4.0b10 (Swofford, 2003).

Trees were constructed using Bayesian inference (Larget and Simon, 1999), implemented in Mr. Bayes 3.1.2 (Huelssenbeck and Ronquist, 2001). Models of evolution were selected for use in analyses using Modeltest 3.06 (Posada and Crandall, 1998) and chosen based on the Akaike information criterion (AIC; Akaike, 1974). A mixed model approach was used for combined analyses; in the data matrix each gene region was partitioned to allow for the optimal model for that marker to be implemented. Four Metropolis coupled MCMC chains were run for ten million generations with trees sampled every 1000 generations. Tracer software 1.3 (Rambaut and Drummond, 2004) was used to examine stationarity of log posteriors to estimate a burn in that was then discarded.

3. Results

3.1. Individual nuclear markers-networks

A total of 250 alleles were identified by this study from the three introns regions of interest. Sequences were generated for 102, 63, and 85 haplotypes from a total of 99, 113, and 93 individuals for the enolase, fibrinogen, and von Willebrand Factor introns, respectively. Further, 26, 27, and 11 heterozygotes were recovered by cloning from these respective gene regions. A minimum spanning network of alleles was created for each nuclear intron. Each locus differed in the number of alleles shared between and within species. Also, closely related alleles in each intron displayed differing divergence patterns (e.g., radiating, linear) and degrees of reticulation. In general, alleles were unique to hypothesized species rather than shared among multiple species. The exception to this general observation occurs in the species *M. berthae*, *M. myoxinus*, and *M. "rufus 1"* which tend to share alleles for each of the introns tested.

The haplotype network produced from the enolase intron (Fig. 1a) reveals four haplotype clusters that are separated by long interlinking branches. First, *M. berthae*, *M. rufus I* and *II*, *M. myoxinus*, and *M. sambiranensis* form a single cluster of alleles in which a large proportion of alleles are derived from a single haplotype that is shared by individuals from *M. rufus I* and *M. myoxinus*. *M. griseorufus* and *M. murinus* also formed a cluster in which *M. griseorufus* form a separate subcluster. Moreover, the remaining two clusters are composed of *M. tavaratra* and *M. ravelobensis*, respectively.

Of all the intron sequences, the fibrinogen locus has the most shared alleles, both within and among species. In this network (Fig. 1b), three clusters form that are separated by long branches. Within one of these clusters, 27 individuals of *M. berthae*, *M. rufus I*, and *M. myoxinus*

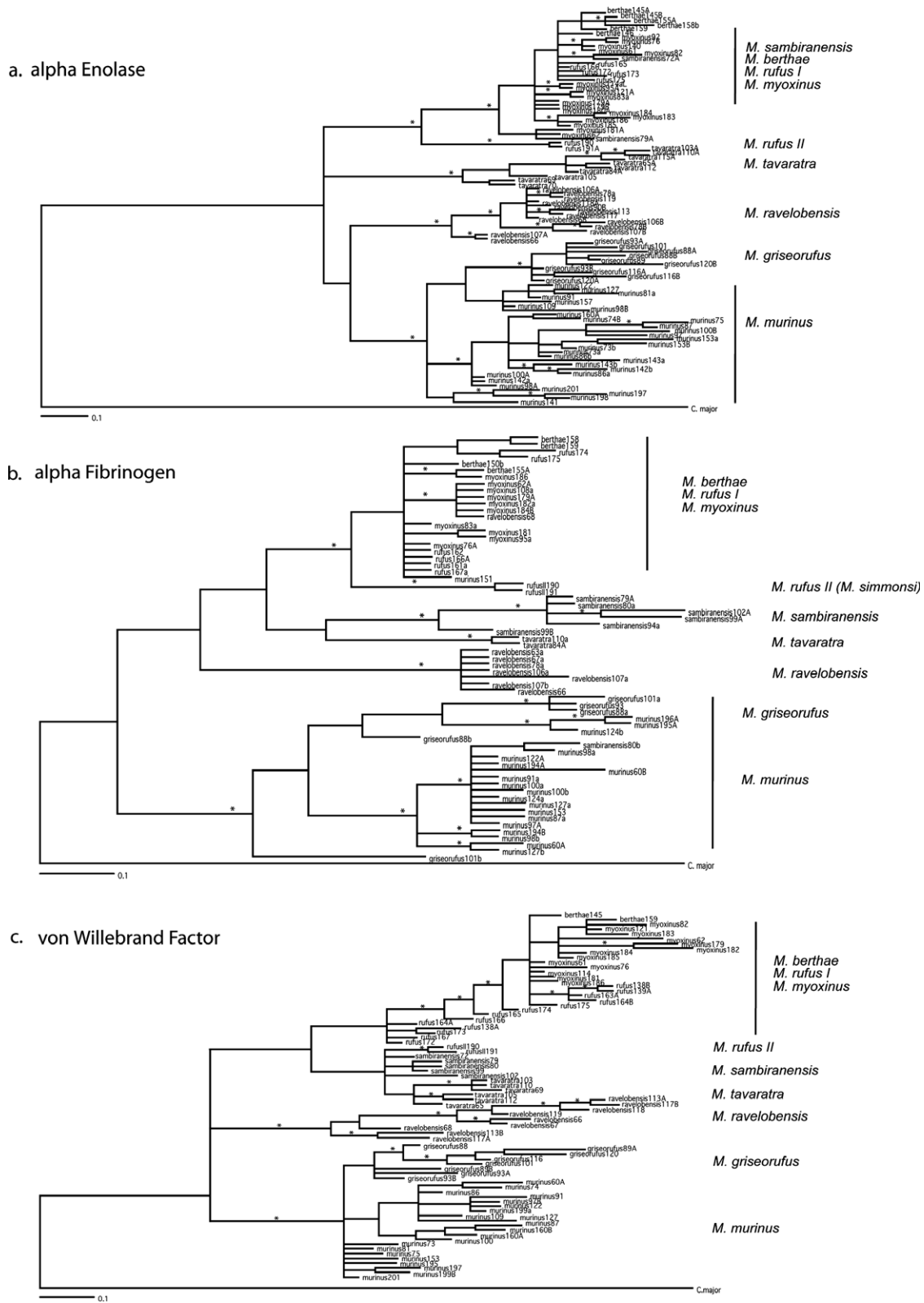


Fig. 1. Phylogenies and minimum spanning networks of *Microcebus*. Minimum spanning networks (a–c) and phylogenies (d–f) constructed from three introns: (a/d) alpha enolase (848 bp), (b/e) alpha fibrinogen (609 bp), and von Willebrand factor (792 bp). Minimum spanning networks were constructed using absolute distance pairwise comparisons in Arlequin. Each circle or square represents one haplotype. Numbers along branches are the estimated number of nucleotide changes necessary to go from one haplotype to another. Numbers within the shapes are the total number of individuals sharing that haplotype. In the absence of numbers, the number of steps or number of individuals with the haplotype is one. The tree was estimated using bayesian methods, implemented in Mr. Bayes with a model of sequences evolution optimized using Modeltest, as given in Table 1. An asterisks on a branch indicates posterior probability of 95% or greater.

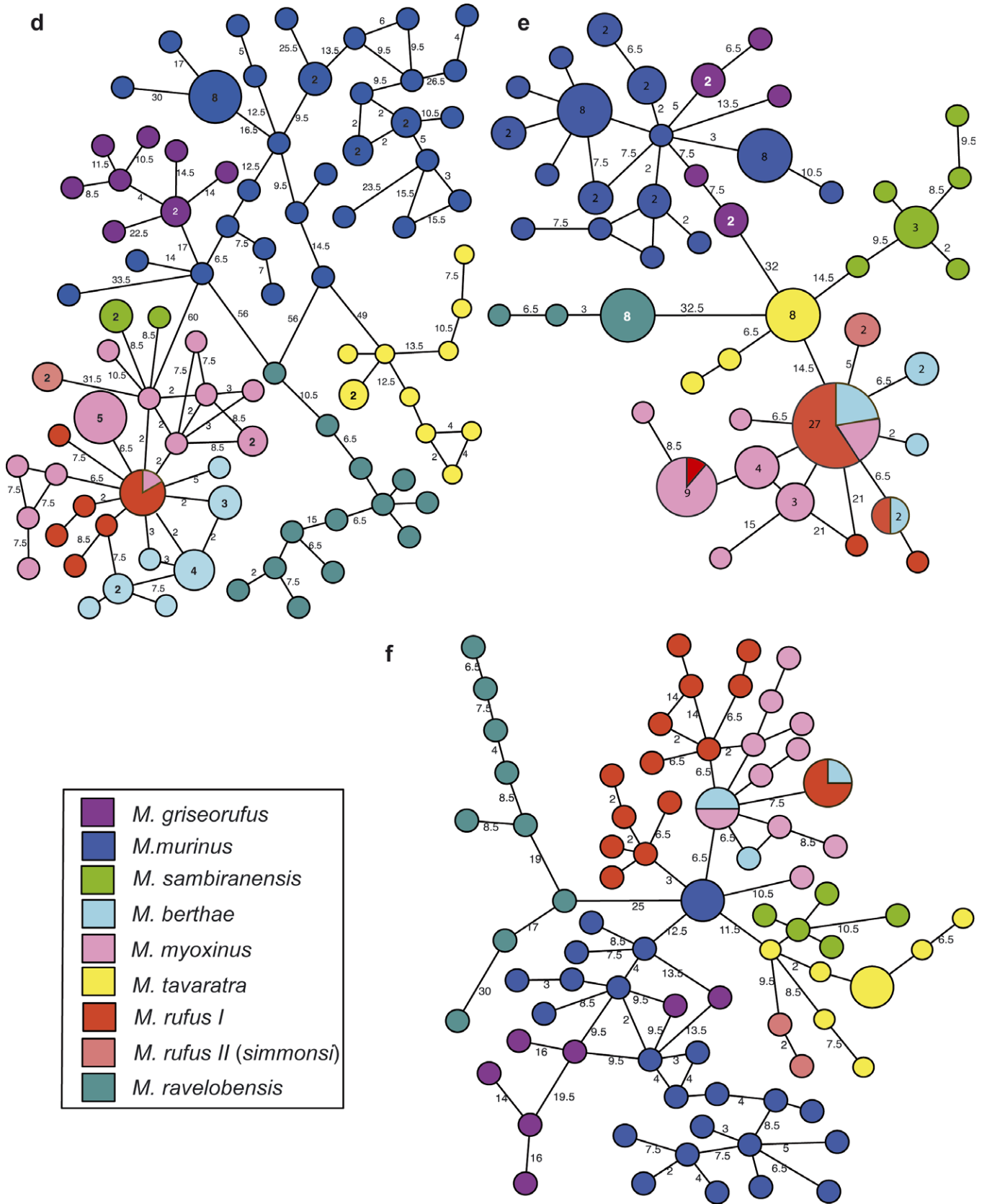


Fig. 1 (continued)

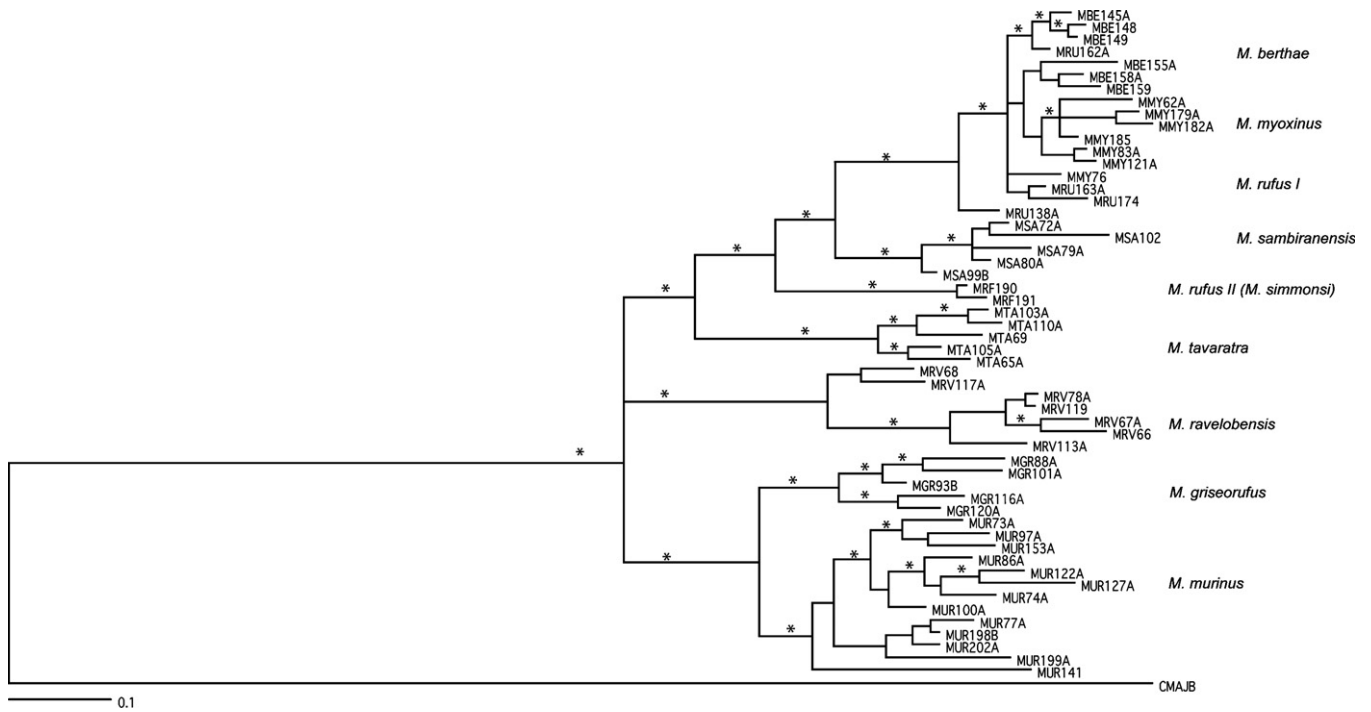


Fig. 2. Combined nuclear intron tree. Phylogeny constructed by concatenating three nuclear introns and one nuclear exon combined for a total of 2717 bp. The tree was estimated using bayesian methods, implemented in Mr. Bayes with a mixed partitioning of models of sequences evolution optimized using Modeltest, as given in Table 1. An asterisks on a branch indicates posterior probability of 95% or greater.

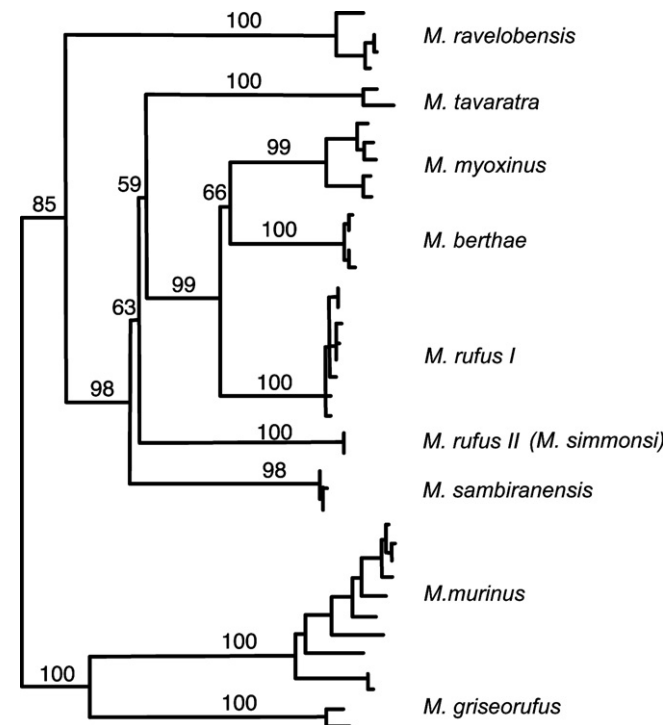


Fig. 3. Mitochondrial Phylogeny recreated from Yoder et al. (2000). The tree was constructed using three combined mitochondrial markers: cytochrome b, cytochrome oxidase I, and hypervariable region I.

share a single haplotype from which other haplotypes from those species are derived; individuals from multiple species share two of these haplotypes. In addition, a cluster containing *M. murinus* and *M. griseorufus* is observed.

However, *M. griseorufus* haplotypes fail to cluster exclusively with each other, unlike in the enolase network. Again, *M. ravelobensis* forms a separate, independent group within the network.

The von Willebrand Factor intron network (Fig. 1c), like that derived from enolase, demonstrates haplotypes that are generally unique to individuals. Within the cluster containing *M. berthae*, *M. myoxinus*, and *M. rufus*, there are again shared haplotypes among species. In this network, clusters are separated by shorter branch lengths than seen in the previous two networks. The most notable distance is *M. ravelobensis* from all other species.

3.2. Individual nuclear markers-phylogenies

The phylogenies estimated from each intron vary in their support of the hypothesized species clades and resolution of deeper relationships. However, two multispecies clades are common to all trees: one is composed of *M. sambiranensis*, *M. rufus II (M. simmonsii)*, *M. tavaratra*, *M. berthae*, *M. myoxinus*, and *M. rufus I* (clade A) and the second is composed of *M. griseorufus* and *M. murinus* (clade B). *M. ravelobensis* fails to group with either clade with significant support ($\geq 95\%$ Bayesian posterior probability), and thus forms a trichotomy with clades A and B. Thus, with the exception of the unresolved position of *M. ravelobensis*, clades A and B correspond exactly to the “northern” and “southern” clades (respectively) identified by the mtDNA analysis (Yoder et al., 2000; Yoder and Heckman, 2006).



Fig. 4. Phylogeny constructed using nuclear and mtDNA. Phylogeny constructed using three nuclear introns, one nuclear exon, and three mitochondrial markers combined for a total of 5480 bp. The tree was estimated using Bayesian methods, implemented in Mr. Bayes with a mixed partitioning of models of sequences evolution optimized using Modeltest, as given in Table 1. An asterisks on a branch indicates posterior probability of 95% or greater, numbers are posterior probabilities less than 95%.

The enolase intron (Fig. 1a,d), which had the most informative sites (12.5%, relative to 10.3% in fibrinogen and 9.2% in von Willebrand Factor) and longest sequences (Table 1), yields a tree with the fewest polytomies and the most supported species clades. The only unresolved clade with multiple species is one composed of *M. berthae*, *M. rufus I*, *M. myoxinus*, and *M. sambiranensis*. *M. griseorufus* falls in a clade with *M. murinus* wherein haplotypes for the former species are paraphyletic. Deeper relationships within the tree have low support, resulting in a basal polytomy of the aforementioned clades plus *M. tavaratra* and *M. ravelobensis*.

The fibrinogen intron has an intermediate percentage of informative sites and the highest number of shared alleles among individuals, both within and among species. In this case, only three species show haplotype clades with high support: *M. sambiranensis*, *M. tavaratra*, and *M. rufus II* (*M. simmonsii*). Two multispecies clades result: the first includes *M. berthae*, *M. rufus I*, *M. myoxinus*, and *M. rufus II* (*M. simmonsii*) and the second includes *M. griseorufus* and *M. murinus*. In the latter, *M. griseorufus* haplotypes fail to

form a clade, in contrast to the enolase tree, in which a *M. griseorufus* clade falls within a paraphyletic *M. murinus*.

The von Willebrand Factor intron has the lowest percentage of informative sites among the three introns. This intron gives the least support to the species boundaries previously hypothesized; only *M. rufus II* (*M. simmonsii*) and *M. ravelobensis* clades have high statistical support. Once again *M. berthae*, *M. rufus I*, *M. myoxinus* group together in a clade in which species boundaries are unresolved. *M. sambiranensis* fails to form a clade, and instead forms a polytomy with *M. tavaratra* and *M. rufus II* (*M. simmonsii*). *M. griseorufus* and *M. murinus* form a similar paraphyletic pattern as was found using the enolase marker.

3.3. Combined analyses

The ILD test demonstrated conflicting results depending on the sampling strategy used in the analysis. When 55 individuals were included, significant incongruence was estimated for each of the three pairwise comparisons

among nuclear intron partitions ($P = 0.001$). In contrast, when 14 individuals were included in the analysis, enolase was significantly incongruent with both von Willebrand Factor and alpha fibrinogen introns ($P = 0.001$), but the latter two introns were not significantly different ($P = 0.11$) as estimated by this method.

A consensus tree method for combining gene trees provided little information about the relationships among species. Clades A and B are recovered in a 50% majority rule consensus tree analysis. Clade A, however, lacks internal resolution and forms a comb structure, due to the inconsistent placement of *M. sambiranensis* and *M. rufus II* (*M. simmonsii*). When the trees from the ADORA exon and mtDNA data sets were included slightly more structure within clade A resulted.

The concatenated sequence data set of nuclear markers, including the ADORA exon sequence, totals 2717 bp. The resulting phylogeny supports all previously hypothesized species boundaries as monophyletic groups except for *M. berthae*, *M. rufus I*, and *M. myoxinus* (Fig. 2). Interspecific relationships are well supported for with posterior probabilities greater than 95% except that *M. ravelobensis* remains as a trichotomy with clades A and B.

When the nuclear markers are combined with the mtDNA markers for a total of 5480 bp, all species boundaries form the same haplotype clades as the mitochondrial tree (Fig. 3, 4), and show similar relationships among species, excluding the position of *M. ravelobensis* and the relative positions of *M. rufus II* (*M. simmonsii*) and *M. sambiranensis*, due to poor support at those nodes. There is one topological incongruence within Clade A relative to the mtDNA tree: the position of *M. tavaratra*. This discrepancy holds true when nuclear data are weighted 2:1 and 8:1 against the mitochondrial data to make up for a 1:3 difference in informative characters.

4. Discussion

There is a growing realization that mtDNA gene trees should be supplemented with other loci for resolving the relationships among species (Adkins et al., 2001; Ballard et al., 2002; Matthee et al., 2001; McCracken and Sorenson, 2005). In this study, several nuclear loci were used to test species boundaries and to determine the phylogenetic relationships of mouse lemurs in the genus *Microcebus*. The results are compared with those reported in a previous molecular study, estimated solely from mtDNA evidence. The nuclear markers each provided a novel gene tree estimate of the species tree. Individually, the nuclear markers failed to reliably resolve each of the species boundaries or the phylogenetic relationships found in the combined mitochondrial tree produced in the Yoder study (2000; Fig. 3), due to the lack of topological resolution and to low statistical support. Combined, the four nuclear markers replicated six of the nine clades previously found, and relationships among species were well supported. A tree combining total genetic evidence (mtDNA and nDNA)

replicated all nine species clades as the original mtDNA tree, though topological resolution is less than the nuclear data combined alone. The tree estimates generally shared a number of topological features. For example, the genus was consistently divided into two multispecies clades. Also, the paraphyletic status of *M. rufus*, found in the mtDNA study, was confirmed using nuclear markers.

Among the trees produced in this study and the previously derived mtDNA tree, patterns of topological ambiguities were detected that spanned multiple tree depths, for example: (1) at the base of the tree, clades A, B, and *M. ravelobensis* formed a trichotomy, (2) within clade A, the relationships of multiple species were either unresolved or incongruent with those displayed on the mtDNA tree, specifically the position of *M. tavaratra*, and (3) at the tips of the trees, *M. berthae*, *M. rufus I*, *M. myoxinus* consistently formed a polyphyletic assemblage of haplotypes. Further, where species formed haplotype clades in the mitochondrial study, these clades were inconsistently reproduced in the nuclear gene trees. These discordant patterns and their implications will be discussed further below.

The position of *M. ravelobensis* in nuclear gene phylogenies was less resolved than in the mtDNA tree. Whereas this species was shown to be the basal member of the “northern” clade (Clade A) in the mtDNA study, its position in the nuclear phylogenies is either unresolved or weakly supported within either clade A or B. This pattern of trichotomy is emphasized in the minimum spanning networks as the number of changes linking the three groups is large and roughly equivalent. Thus, the genetic data indicate that it is almost certainly the most divergent single species within the western mouse lemur radiation, resulting in an unresolved relationship.

An additional example of the uncertainty in species relationships is within clade A, specifically the relative positions of *M. sambiranensis*, *M. rufus II* (*M. simmonsii*), and *M. tavaratra*. This is a region that has little statistical support in both the nuclear gene trees and in the mitochondrial phylogeny, as well. Essentially, these three species in each gene tree estimate have irregular or unsupported positions in the tree. While this pattern for the *M. rufus II* (*simmonsii*), *M. sambiranensis*, and *M. tavaratra* may be an example of homoplasy in the mtDNA, resulting from short internode length (Fig. 3; McCracken and Sorenson, 2005), the lack of support in the nuclear markers is possibly due to the limited number of phylogenetically informative characters. This issue of insufficient data is seemingly resolved, however, when the nuclear sequences are combined, resulting in a tree with well-supported internal nodes. Though it is worth noting that the ambiguity of this region of the tree may be compounded by differences in the sorting of alleles at these gene regions (e.g., *M. sambiranensis*' position for enolase). This information, in total, again highlights the need for multiple independent estimates of phylogenetic relationships. This approach should be a clear prerequisite for deriving the relationships within a lower clade level,

where nuclear markers will provide limited variability necessary for estimating interspecific relationships.

At the tips of each tree, *M. berthae*, *M. rufus I*, and *M. myoxinus* nuclear haplotypes form an unresolved polyphyletic clade, in contrast to the clades recovered in the mtDNA study. This behavior is not uncommon for closely related species, however (Hudson and Coyne, 2002; Neigel and Avise, 1986; Rosenberg, 2003; Takahata and Nei, 1985). In nuclear DNA, the mutation rate is typically slower, thereby providing fewer phylogenetically informative characters. There is evidence that lineage sorting has not completed for these three species, as they share (ancestral) polymorphisms at each locus (Fig. 1). This result was found in both phylogenetic and minimum spanning network analyses. The minimum spanning network displays how alleles are shared across interspecific boundaries with alternative interconnections between haplotypes forming a network. For example, in the fibrinogen intron network, one allele is shared by a large number of individuals from all three species. Single haplotypes diverge from this allele in a radiating pattern, indicative of a rapid divergence from a central population. It is possible, however, that the haplotypes relationships for these three species may be the result of introgression. This possibility will be discussed further below.

4.1. Combined data

There is a longstanding philosophical controversy whether or not to combine partitioned data (see Huelsenbeck et al., 1996 for review). Essentially, partitioned data can always be combined, conditionally combined, or never combined. Conditional combination is determined by testing the assumption that only partitions with equivalent phylogenetic information are combined. Therefore, congruence among gene tree estimates has been considered an important criterion in conditional data combination. However, the most commonly used test for congruence, the partition homogeneity test has little bearing on the combinability of data sets (Yoder et al., 2001), due to evidence of false positives (Cunningham, 1997), false negatives (Ramirez, 2006), and relative lack of statistical power (Barker and Lutzoni, 2002). As combining data generally provides advantages in phylogenetic estimation and the combination of mtDNA and nuclear data may provide resolution at different parts of the tree (Pereira et al., 2002), we chose to combine sequence and tree data in analyses of all three nuclear regions and also the mitochondrial gene regions. This combination was performed to fully explore the data set, while being mindful that conclusions based on the combined analyses were dependent on congruent evolutionary histories for the loci. We combined the data in two manners: by concatenating sequences and by generating consensus trees.

Combining by consensus failed to resolve the relationships between species, as ambiguities in the topologies were pervasive and resulted in an unresolved comb structure. As

suggested (Swofford, 1991), consensus methods may be used to implicate sources of topological incongruence. In this role, we recognized the incongruences among gene trees at all levels of the phylogeny, as described above. Though this pattern may also arise from combining collapsed nodes, as was more prevalent in this study. Gene regions may not be statistically well supported at all levels of the tree, due to mutation rate heterogeneity, making combination by consensus impractical. This consensus tree result highlights a number of important issues for analyzing multiple loci in a phylogenetic study. First, the unresolved topology emphasizes the classic point that gene trees are single estimates of the species tree (Maddison, 1997; Nichols, 2001). If a single nuclear locus had been included in this phylogenetic study, a vastly different set of relationships would have resulted. Second, this result suggests that using the consensus method we have yet to include a sufficient number of loci for determining the true species tree. Additional *congruent* and *resolved* gene tree estimates would be necessary before the consensus tree has topological structure that reflects the species history.

All gene sequences and taxa were included despite incongruences and ambiguities among the estimated gene trees, as highlighted in the consensus tree method. Including all data in a combined analysis (Kluge, 1989), in this case mtDNA and nDNA genetic markers, produced less supported and resolved internal nodes than tree estimates produced using the combined nuclear data set alone. The mtDNA tree and each of the nuclear gene trees have incongruent relationships among *M. sambiranensis*, *M. rufus II* (*M. simmonsii*), and *M. tavaratra*. The result suggests that because there is limited phylogenetically informative variability and/or the signal is conflicting in each individual nuclear marker, the combination of nuclear and mtDNA evidence results in a topology with no support at the same level that was problematic in the mtDNA alone tree. In this case, where the mtDNA data is ineffective at recovering a supported topology, in the mid-section of the tree, the information provided by the nDNA is overwhelmed and results in low posterior probabilities at internal nodes. In total, by combining the data, we were able to confirm that a problematic region in the tree was due to a general evolutionary process, such as a rapid radiation, rather than issues specific to the mtDNA gene tree.

In contrast, concatenating genes into a large data set resulted in greater resolution of the topology at the tips, where mtDNA evidence successfully produced haplotype clades. However, in this case, the combined nuclear sequences data set was less successful at resolving the relationships within the polyphyletic trio *M. berthae*, *M. rufus I*, and *M. myoxinus*; these relationships were resolved only when the mitochondrial data was also included. However, this was expected given the conflicting phylogenetic signal of the unsorted ancestral lineages in the nuclear sequences. This begs the question, however, of whether the phylogeny is indeed the best hierarchical representation of species divergence events.

4.2. Species boundaries

In the previous molecular study of *Microcebus* using mtDNA, hypothesized species boundaries were congruent with haplotype clades in all cases. In the present study, however, hypothesized species failed to meet this criterion. Taxonomic boundaries for all hypothesized species were supported only when both nuclear and mitochondrial DNA sequences were concatenated. In the individual nuclear gene trees, species-specific haplotypes frequently formed polyphyletic groupings. This is especially true though not exclusive to the relationships among *M. rufus*, *M. berthae*, and *M. myoxinus* haplotypes.

Potentially, there are multiple explanations for this pattern, including introgression, incomplete lineage sorting, and insufficient sampling. While none of these hypotheses can be discounted completely and further analyses are necessary, such as implementing coalescent simulations (Buckley et al., 2006), we assert that the most influential phenomenon is incomplete lineage sorting. For the *Microcebus* radiation, time since species divergence has been sufficient for mtDNA haplotypes to sort, but not for nuclear alleles. This pattern is expected due to the fourfold discrepancy in effective population size and to the fact that nuclear markers diverge more slowly (Ballard and Whitlock, 2004; Birky et al., 1989), with the result that lineage sorting can take four times longer in nDNA than in mtDNA. Thus, new alleles fixate by drift faster in the mitochondrial genome, resulting in a shorter time to reciprocal monophyly, than in the nuclear genome. As a consequence, ancestral polymorphisms are shared for significantly longer in the nuclear genome, than in the mitochondrial genome, potentially yielding examples of polytomies and relatively few examples of species boundaries forming well-supported clades. This pattern was found in the gene trees of *Microcebus*. In all estimates, there were examples of species that formed unresolved clades. Moreover, there were no patterns of introgression in the mtDNA gene tree. The introgression of alleles would delay mitochondrial haplotype fixation, unless there was a related cost in fitness or gene flow was solely a consequence of male dispersal. Mouse lemur species are highly variable in their social group composition and dispersal patterns (Schulke and Ostner, 2005). While *Microcebus murinus* has been characterized as having a female philopatric social system (Radespiel et al., 2001), this pattern cannot be generalized to the whole genus as there is gathering evidence to the contrary. For example *M. ravelobensis* has been described as having a dispersed multimale/multifemale system with a promiscuous mating strategy (Weidt et al., 2004). In addition the average relatedness of *M. berthae* females was reported to be low, though higher than in males (Dammhahn and Kappeler, 2005).

The most likely candidates for introgression are the three species that consistently form polyphyletic clades, *M. berthae*, *M. myoxinus*, and *M. rufus*. The likelihood of scenario of hybridization is further complicated by geo-

graphic distributional patterns. *M. rufus* is separated from *M. berthae* and *M. myoxinus* by a currently inhospitable central plateau, further lessening the likelihood of introgression among these three species in the recent past. Despite uncertainty of the mechanism producing the pattern of unresolved species clades, based on the analyses in this study, *M. rufus*, *M. berthae*, and *M. myoxinus* fail to meet the criteria of species concepts that use historical criteria, or a pattern of common descent, such as the Phylogenetic Species Concept (PSC; Cracraft, 1989; Donoghue, 1985). Previous studies of *Microcebus* morphology have significantly differentiated two of the three species (Atsalis et al., 1996; Rasoloarison et al., 2000), but to date, there has been no study comparing all three groups simultaneously. For these three groups to be rigorously tested for species status, additional criteria such as reproductive isolation and ecological differentiation should be investigated. Considering their cryptic morphology, one possible independent source of this information may be from *Microcebus* vocalization behavior, as multiple species demonstrate variability in sexual advertisement vocalizations at both the interspecific and intraspecific levels (Zimmermann et al., 2000). We hope the present study encourages further comparisons among these three groups.

5. Conclusions

This study has confirmed the mtDNA-based finding that mouse lemur species do not form clades congruent with western and eastern ecotypes, and is consistent with the previously described “northern” and “southern” clades. At least two clades from the eastern regions of Madagascar are placed in widely divergent locations within the mouse lemur phylogeny. The association between the two *M. rufus* clades with the other members of clade A implies a relationship between eastern and western species that was previously unexpected (see Yoder and Heckman, 2006). An explanation for this association may be found in subfossil studies, as *Microcebus* specimens have been found in central Madagascar, confirming migration corridors between east and west (Godfrey et al., 1999). These corridors are no longer present, as central Madagascar is now inhospitable grassland, though it was once a mosaic of forest and grassland (Burney, 1987, 1997). If possible, the inclusion of subfossil sequences into a *Microcebus* phylogenetic analysis would provide important insight into the biogeography and true evolutionary history of the genus.

This study has emphasized the importance of multiple markers and limitations of a mtDNA gene tree for determining an accurate species history for *Microcebus*. Notably, the relationships among the species in Clade A have been called into question, possibly due to rapid divergence, notably the position of *M. tavaratra*. Concatenating nuclear sequences into a single data set produced the most resolved phylogeny with the highest statistical support for clades. Combining nuclear and mitochondrial sequences high-

lighted the unresolved relationships estimated originally using mtDNA sequences alone. We feel this study highlights a complex divergence history in the genus *Microcebus* that cannot be estimated without the use of additional genetic markers. We hope that this study encourages caution when estimating species history for a single gene tree.

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