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# Species discovery and validation in a cryptic radiation of endangered primates: coalescent-based species delimitation in Madagascar's mouse lemurs

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## Abstract

**Implementation of the coalescent model in a Bayesian framework is an emerging strength in genetically based species delimitation studies. By providing an objective measure of species diagnosis, these methods represent a quantitative enhancement to the analysis of multilocus data, and complement more traditional methods based on phenotypic and ecological characteristics. Recognized as two species 20 years ago, mouse lemurs (genus *Microcebus*) now comprise more than 20 species, largely diagnosed from mtDNA sequence data. With each new species description, enthusiasm has been tempered with scientific scepticism. Here, we present a statistically justified and unbiased Bayesian approach towards mouse lemur species delimitation. We perform validation tests using multilocus sequence data and two methodologies: (i) reverse-jump Markov chain Monte Carlo sampling to assess the likelihood of different models defined *a priori* by a guide tree, and (ii) a Bayes factor delimitation test that compares different species-tree models without a guide tree. We assess the sensitivity of these methods using randomized individual assignments, which has been used in BPP studies, but not with Bayes factor delimitation tests. Our results validate previously diagnosed taxa, as well as new species hypotheses, resulting in support for three new mouse lemur species. As the challenge of multiple researchers using differing criteria to describe diversity is not unique to *Microcebus*, the methods used here have significant potential for clarifying diversity in other taxonomic groups. We echo previous studies in advocating that multiple lines of evidence, including use of the coalescent model, should be trusted to delimit new species.**

*Keywords*: Bayes factor, Bayesian, BPP, *Microcebus*, nuclear

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## Introduction

Bayesian methods for species delimitation have been gaining in power and ease of use since they were first introduced (Yang & Rannala 2010). These methods have tremendous appeal for numerous reasons, among the most fundamental being that they are grounded in time-tested coalescent theory, are largely objective rather than subjective (thus taking the 'art' out of species delimitation) and are applicable across the tree of life (Yang & Rannala 2010; Fujita *et al.* 2012). As long as investigators can generate sufficiently informative genetic data from multiple independently evolving loci, these methods can be employed to test for the divergence of lineages, even when phenotypes lend no

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suggestion of divergence (i.e. in cryptic species). Further, the statistical nature of these methods allow for the comparison of results across studies, even when there is no overlap in the sets of loci used.

A rich literature has developed in which simulation experiments have validated these statistical properties, demonstrating that under most scenarios, rates of false positive (i.e. oversplitting) and false negative (i.e. undersplitting) are low (Leache & Fujita 2010; Zhang *et al.* 2011, 2014; Camargo *et al.* 2012). These methods have been shown to be robust to the effects of low-level gene flow (Zhang *et al.* 2011; Camargo *et al.* 2012), as would be typical of reinforced hybrid zones between diverging species. Moreover, they are also robust to deviation in the assumed underlying species tree, a perhaps not too uncommon situation for many organismal groups (Zhang *et al.* 2014). Not surprisingly, investigators interested in objectively and reliably determining the extent of species diversity within an organismal radiation are increasingly drawn to Bayesian analyses to test species delimitation hypotheses (e.g. Leache & Fujita 2010; Camargo *et al.* 2012; Ruane *et al.* 2014).

Species delimitation in Madagascar's mouse lemurs, genus *Microcebus*, has been particularly active in recent years, based on a wide range of data and methodological approaches. Presently, we are in the stage of species delimitation that represents a transition from what Carstens *et al.* (2013) might refer to as 'discovery' to one of 'validation'. Originally classified as a single species, primatologists came to favour a two-species taxonomy that included *M. murinus*, a long-eared grey mouse lemur from the southern and western regions of Madagascar, and *M. rufus*, a short-eared reddish mouse lemur from the east (Martin 1972). This taxonomic stability was first challenged with the description of a third, smaller, species, *M. berthae*, from the dry deciduous forests of western Madagascar (Schmid & Kappeler 1994). In the interim, a fourth and much larger species, *M. ravelobensis*, was identified in northwestern Madagascar (Zimmermann *et al.* 1998). The pace of taxonomic discovery then accelerated with the introduction of genetic data, leading to the description of five new species based on combined evidence from morphology (Rasoloarison *et al.* 2000) and mtDNA (Yoder *et al.* 2000). Overall, across two decades, *Microcebus* has expanded to at least 20 described species (Kappeler *et al.* 2005; Andriantompohavana *et al.* 2006; Louis *et al.* 2006, 2008; Olivieri *et al.* 2007; Radespiel *et al.* 2008, 2012; Rasoloarison *et al.* 2013), with many of these diagnosed primarily, if not entirely, from mtDNA. Recent scepticism has stemmed from comparisons between *Microcebus* and the closely related dwarf lemurs, *Cheirogaleus*, due to a discrepancy in described diversity of dwarf lemurs vs. their mouse lemur counterparts (Thiele *et al.* 2013).

As the discovery phase of mouse lemur species diversity continues to unfold, much of mouse lemur systematics remains to be addressed with robust validation approaches (Carstens *et al.* 2013). While not all mouse lemur species are in question, many are only supported by limited genetic evidence (see Markolf *et al.* 2011), or have not been rigorously tested with modern species delimitation tools. As such, with many new species described by a range of investigators, using a range of discovery criteria, it is difficult to compare the level of confidence placed on new descriptions. The use of multilocus genetic data and powerful 'coalescent-aware' computational methods (Yang & Rannala 2014) provides an opportunity to bring mouse lemur systematics into a rigorous statistical framework, with species hypotheses refuted or validated as independently evolving population-level lineages, as would be recognized under a general lineage concept of species (de Queiroz 1998, 2007). This effort is not merely a taxonomic exercise. For example, accurate estimates of mouse lemur species boundaries are necessary for the identification of proper conservation units (e.g. Schwitzer *et al.* 2014) and studies of the evolutionary processes driving diversity within Madagascar (e.g. Wilme *et al.* 2006).

In this study, we aimed to develop a rigorous species delimitation framework for a species radiation that diverged both rapidly and relatively recently (Yang & Yoder 2003). We focus on the resolution of novel mouse lemur diversity, but the potential remains for this framework to be applied to any organismal group for which multilocus genetic data can be collected. We build our analyses around a number of hypothesized species lineages identified from a combination of genetic and geographic evidence. One of our hypotheses stems from a newly sampled population of mouse lemurs from the Tsinjoarivo rainforest in eastern Madagascar, a population of uncertain taxonomic placement, representing either an extension of an existing taxon or an undiagnosed lineage. We also test the validity of lineage hypotheses that were previously identified in Weisrock *et al.* (2010), but have not been subject to further analysis. These include putative lineages within *M. murinus*, the potential for cryptic diversity within *M. griseorufus*, the distinctiveness of a population from Montagne d'Ambre in northern Madagascar and the potential for divergence between mainland and island populations of *M. simmonsi*.

For all scenarios we perform validation tests of species delimitation hypotheses using multilocus DNA sequence data and two Bayesian methodologies: (i) a test using BPP (Yang & Rannala 2010), which employs reverse-jump Markov chain Monte Carlo (rjMCMC) sampling to explore the likelihood of data under models with different numbers of lineages defined *a priori*

by a guide tree, and (ii) a Bayes factor delimitation (BFD) test (Grummer *et al.* 2014) that compares different species-tree models without the need for an *a priori* guide tree. While BPP no longer requires a guide tree (Yang & Rannala 2014), the simultaneous testing of multiple species hypotheses in diverse clades can still present a computational challenge. Therefore, we developed an approach that performs BPP tests as a series of smaller sister-lineage comparisons, and in cases of species-tree ambiguity, tests multiple sister-lineage hypotheses. As a complement to these tests, we also perform a parallel set of analyses using a randomization of individuals across hypothesized lineages. Studies using BPP often assess the potential for spurious identification of diverging species through such analyses (e.g. Ruane *et al.* 2014). However, this approach has not been extended to BFD tests. We make this step by comparing the marginal likelihoods of test and randomized assignments to assess the potential for oversensitivity in Bayes factor support for hypothesized diverging lineages.

Collectively, we use these results to assess the validity of a number of mouse lemur species hypotheses and contribute to a more complete resolution of their diversity. As the challenge of multiple researchers using differing criteria to describe diversity in rapid radiations is not unique to mouse lemur, the methods described here also hold significant potential for clarifying diversity in other taxonomic groups.

## Methods

### *Novel population sampling*

New mouse lemur sampling consisted of 16 individuals that were nondestructively sampled using ear clippings during fieldwork (November 2006 and December 2007) by MMB at two sites within the Tsinjoarivo forest, located approximately 80 km to the southeast of Antananarivo (Fig. 1). Of these, eight individuals (five females, three males) were sampled from a site in the western half of Tsinjoarivo (19°41′15″S, 47°46′25″E) in a more fragmented region of the forest that is topographically continuous to the central high plateau (1660 m). Another eight individuals (three females and five males) were sampled from a site in the eastern half of Tsinjoarivo (19°43′15″S, 47°51′25″E) that is comprised of more primary and less disturbed forest at a lower elevation (1396 m). The Onive and Mangoro rivers define the southern and eastern barriers of these collection sites, respectively.

### *Genetic sampling*

Sequence data from the Tsinjoarivo population were generated for six loci: two mitochondrial (*cox2* and *cytb*)

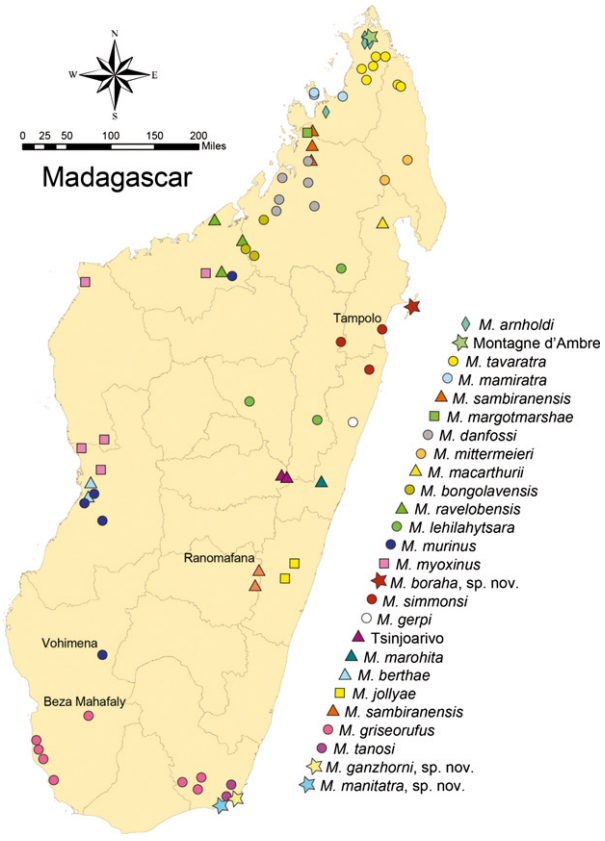


**Fig. 1** A map of Madagascar showing the distribution of described *Microcebus* species according to localities used in Yoder *et al.* (2000), Louis *et al.* (2006, 2008), Olivieri *et al.* (2007), Radespiel *et al.* (2012) and Weisrock *et al.* (2010). Also included is the newly sampled Tsinjoarivo population. Not all localities presented here are those used in this study. All coordinate information for localities used here are available in the study's Dryad accession (doi:10.5061/dryad.h6s5j). Locality names specifically mentioned in the text are designated on the map.

and four nuclear (*adora*, *eno*, *fiba* and *vwf*). These loci are the same used in previous studies of mouse lemur phylogeography and species delimitation (Heckman *et al.* 2007; Weisrock *et al.* 2010), and we combined these new data with the data set of Weisrock *et al.* (2010), allowing us to 'recycle' these data for the purpose of species validation. PCR primer information for all loci can be found in these previous studies, and full descriptions of DNA extraction, PCR and sequencing methods for this study are presented in Supporting information.

To edit and align forward and reverse sequences for each locus into a single sequence, we used GENEIOUS PRO v5.4.6 (Kearse *et al.* 2012). Many nuclear sequences contained multiple heterozygous sites, and some produced sequences that were uninterpretable due to insertion–deletion (indel) polymorphisms. We used a Bayesian approach to phase nuclear alleles for many of these

sequences (when indel polymorphism was not a factor) in PHASE v2.1.1 (Stephens *et al.* 2001; Stephens & Scheet 2005). We used TOPO® TA cloning kits to resolve phased alleles from nuclear sequences exhibiting patterns of indel polymorphism and from those in which PHASE did not produce strongly supported allele phasing.

### *Gene tree reconstruction*

For each of our five unlinked loci (four nuclear and concatenated mtDNA), we combined the newly generated sequence data from Tsinjoarivo individuals with the mtDNA and nuclear data sets used in Weisrock *et al.* (2010). In addition, we also included mtDNA data from a recently described species, *M. gerpi* (Radespiel *et al.* 2012), whose range is located approximately 150 km from the Tsinjoarivo sampling sites (*cox2*: GenBank accessions JQ083607–JQ083612; *cytb*: JQ083613–JQ083618). Each data set was analysed to identify nonredundant haplotypes using MESQUITE v2.75 (Maddison & Maddison 2011). The *cox2* and *cytb* loci were concatenated for each individual prior to haplotype identification and analysed as a concatenated matrix.

Gene trees were estimated for each nuclear locus and the concatenated mtDNA data set using MRBAYES v3.2.1 (Ronquist *et al.* 2012). The best-fit model of evolution for each locus was found using Bayesian Information Criteria implemented in PARTITIONFINDER v1.01 (Lanfear *et al.* 2012), including an exploration of different codon partitions for the mtDNA data. Each nuclear locus was analysed as a single partition with a single selected model of evolution (Table 1). Full details regarding the MRBAYES analyses are provided in Supporting information.

### *Population structure analyses*

For this study, assessments of genus-wide population structure rely on the STRUCTURE results of Weisrock *et al.* (2010). However, to further facilitate the species discovery process, we performed additional population structure analyses using STRUCTURE v2.3.4 (Pritchard *et al.* 2000) to assess the placement of individuals sampled from Tsinjoarivo, and to further explore the potential for population structure within *M. simmonsi*. Complete details of STRUCTURE analyses performed for this study are presented in Supporting information.

### *Species discovery*

We used multiple approaches to (i) identify the potential species assignment of the newly sampled Tsinjoarivo individuals, and (ii) further explore mouse lemur species delimitation across its distribution. First, we evaluated the phylogenetic placement of mtDNA haplotypes to identify sets of populations forming monophyletic groups that may reflect independent lineages. Estimates of phylogenetic relationships within *Microcebus* have been notoriously difficult to resolve with multilocus data (Weisrock *et al.* 2012), and we treat these mtDNA-based units purely as hypotheses to guide species delimitation inference. Second, we used the results of nuclear population structure to identify genetically distinct clusters across *Microcebus*. Finally, we supplemented our mtDNA gene tree and nuclear STRUCTURE-based decisions of testable species delimitation hypotheses with qualitative geographic information, choosing to test for signatures of lineage divergence among various combinations of putative sister species that are distributed in the same general area of Madagascar.

**Table 1** Locus information for six loci (four nuclear, two mitochondrial) used in analyses. Number of variable sites from Weisrock *et al.* (2010)

| Locus | Alignment length (bp) | Variable sites | Best-fit model† | Annealing temperature |
|---|---|---|---|---|
| *cox2* | 684 | 176 | SYM+I+r, HKY+I+r, HKY+r | 55 °C |
| *cytb* | 1140 | 396 | SYM+I+r, HKY+I+r, GTR+I+r | 55 °C |
| *adora* | 384 | 34 | K80 + r | 58 °C |
| *eno* | 914 | 150 | HKY+I+r | 69 °C |
| *fiba* | 632 | 74 | HKY+I+r | 59 °C |
| *vwf* | 812 | 117 | HKY+I+r | 56.5 °C |

†Models are presented for the first, second and third codon positions, respectively, for mtDNA genes.

### *Species validation – BPP*

We used a coalescent model-based approach implemented in the program BPP v2.2 (Yang & Rannala 2010) to test species delimitation hypotheses. The most recent version of BPP (v3; Yang & Rannala 2014) no longer requires a guide tree to identify putative species-tree relationships among hypothesized lineages. However, the use of a guide tree still serves a valuable role in constraining rjMCMC sampling, particularly when dealing with diverse clades containing numerous species hypotheses. Given our lack of solid hypotheses for species-tree relationships, we chose to perform BPP analyses on a series of restricted data sets representing hypothesized splits between two taxa (Table 2). The majority of these tests were performed on a simple two-taxon guide tree focusing on the splitting or lumping of two

**Table 2** Species delimitation hypotheses tested in this study

| Species delimitation hypothesis | mtDNA gene tree | Nuclear STRUCTURE clustering | Geographic distribution |
|---|---|---|---|
| (1) Tsinjoarivo + *M. lehilahytsara* | X | X | X |
| (1) Tsinjoarivo + *M. mittermeieri* | | X | |
| (1) Tsinjoarivo + *M. marohita* | | | X |
| (2) *M. simmonsi:* Ile Ste. Marie + Tampolo | X | X | X |
| (3) *M. sambiranensis* + Montagne d'Ambre | X | X | X |
| (4) *M. berthae* + *M. rufus* | X | | |
| (4) *M. berthae* + *M. myoxinus* | | | |
| (4) *M. myoxinus* + *M. rufus* | | | |
| (5) *M. griseorufus* + Beza Mahafaly | X | X | X |
| (6) *M. murinus* + Mandena | X | X | X |
| (7) *M. murinus* (excluding Mandena) + Bemanasy | X | X | X |
| (8) *M. murinus* (excluding Bemanasy & Mandena) + Vohimena | X | X | X |

Numbers in parentheses refer to numbered descriptions of species hypotheses listed in the results. Columns indicate which type of species discovery evidence is being considered, with an X indicating the evidence for a particular hypothesis.

hypothesized lineages. In the case of *M. berthae*, *M. rufus* and *M. myoxinus*, a three-taxon tree was used; however, we performed tests using all three possible topologies to account for species-tree uncertainty.

BPP is a Bayesian method and requires the designation of prior distributions for both ancestral population size ($\theta$) and the age of the root of the species tree ($\tau$). We tested species delimitation hypotheses covering a range of prior scenarios, including small $\theta$ and large $\tau$, where gene tree discordance is expected to be limited, and large $\theta$ and small $\tau$, where gene tree discordance is expected to be extensive. A total of six combinations of $\theta$ and $\tau$ prior distributions were used (Table S2, Supporting information). BPP tests were performed for both the nuclear data and the combined mtDNA and nuclear data. In the latter, a heredity scalar of 0.25 was used for the mtDNA data. In all analyses, all available sequence data (gene copies) were used for each hypothesized lineage being tested (i.e. no subsampling of data). For all BPP tests we performed an accompanying test using a randomized individual-to-species mapping file to evaluate the performance of BPP when the expected outcome is no speciation between hypothesized lineages. Additional details of BPP analyses, including our attempts to use BPP v3 in the absence of a guide tree, are included in Supporting information.

### *Species validation – Bayes factor delimitation*

Bayes factor delimitation tests were performed following the framework of Grummer *et al.* (2014). This approach uses a Bayesian coalescent-based reconstruction of species trees for a range of delimitation models that involve the lumping and splitting of hypothesized species. Marginal likelihood estimates (MLEs), measured as log likelihoods, are calculated from the Bayesian posterior distributions. MLEs for all models are ranked, and Bayes factors are then calculated as two times the difference in MLE between the best-fitting and alternative models (denoted as 2*ln*Bf). Measures of 2*ln*Bf in the range of 0–2 are generally interpreted as indicating no difference in support for two models, while a 2*ln*Bf > 10 indicates 'decisive' support in favour of the best-fitting model over its alternative (Kass & Raftery 1995). Grummer *et al.* (2014) recognized distinct lineages with a 2*ln*Bf > 10 and we follow these guidelines in this study.

Bayes factor delimitation includes species-tree estimation as part of the process for generating MLEs, and as such, does not require an input guide tree. Consequently, our BFD work was not restricted to the analysis of two- or three-taxon data sets, and instead allowed for the testing of delimitation models that accounted for multiple hypothesized splits. We performed BFD on two separate partitions of the *Microcebus* clade, one containing populations assigned to the species *M. griseorufus* and *M. murinus* and a second containing all other *Microcebus* species and populations, hereafter referred to as the 14-species clade. In both sets of analyses we included *M. ravelobensis* as an outgroup species. Within each clade we tested multiple models of lineage divergence, ranging from a full divergence model in which all hypothesized lineages were treated as separate tips in the species tree, to a fully collapsed model where all hypothesized lineages were collapsed. In total, 34 models (1–34) were tested for the 14-species clade and 16 models (A–P) were tested for the *M. griseorufus* and *M. murinus* hypotheses. Computational limits prevented us from using all available gene copies; therefore, we pruned our data to a random subsample of gene copies

from each hypothesized species (Table S3, Supporting information).

For each model of lineage divergence we performed a Bayesian reconstruction of the species tree using *BEAST v1.8.0 (Drummond *et al.* 2012). *BEAST analyses were performed using only nuclear data, and using nuclear and mtDNA data. For each model, we also analysed our four-locus nuclear data using randomized assignments of individuals to tips within hypothesized sets of diverging sister species. For example, for Model 4, tip assignments for Tsinjoarivo and *M. lehilahytsara* individuals were randomized across these two hypothesized lineages, while all others remained the same. Using this approach, we assessed the sensitivity of BFD validation tests in species delimitation.

The best-fit model of substitution was applied to each locus as described above, with the exception that the mtDNA data were treated as a single partition with a GTR+I+r substitution model. Full details of *BEAST usage for species-tree reconstruction and MLE calculations are provided in Supporting information.

## Results

### *New data generation and species discovery*

DNA sequence data were generated from the *cox2* and *cytb* mtDNA loci from all 16 sampled Tsinjoarivo individuals (Table S1, Supporting information). The same was true for all nuclear loci except for *eno*, for which data were sampled from 13 individuals (Table S1, Supporting information). The mtDNA data were found to best fit a four-partition scheme (Table 1), with first and second codon positions of both genes placed into two different partitions, third codon positions of *cytb* placed in a third partition, and third codon positions of the *cox2* gene placed in a fourth partition. Best-fit substitution models for all mtDNA partitions, and all nuclear loci, are presented in Table 1. All DNA sequence data sets, along with all input and results files from STRUCTURE, BPP and BFD analyses, are available in this study's Dryad accession (doi:10.5061/dryad.h6s5j).

Analysis of the concatenated mtDNA data produced a posterior distribution of trees very similar to those reconstructed in previous studies (Fig. 2; Weisrock *et al.* 2010). All previously described taxa were resolved as monophyletic, except for *M. rufus*, which had a single divergent haplotype found in multiple individuals sampled from Ranomafana and placed as the sister lineage to *M. myoxinus* [a result originally identified in (Yoder *et al.* 2000) and verified in Weisrock *et al.* (2010)]. As a relevant side note, this identification of a paraphyletic *M. rufus* mtDNA genealogy, wherein one haplotype clade from eastern Madagascar showed a sister-lineage relationship to western haplotype clades, was among the first indications that species diversity and geographic fidelity in mouse lemurs was far more complex than previously appreciated (Yoder *et al.* 2000). The four concatenated and nonredundant mtDNA haplotypes sampled from Tsinjoarivo were nested within a well-supported clade of all haplotypes sampled from *M. lehilahytsara* (Fig. 2). No mtDNA haplotypes were shared between the Tsinjoarivo population and *M. lehilahytsara*; however, Tsinjoarivo haplotypes had low levels of sequence divergence from *M. lehilahytsara* haplotypes, with an average uncorrected sequence divergence of 0.8% (Min. = 0.3%, Max. = 1.4%).

Nuclear gene haplotypes sampled from Tsinjoarivo were closely intermixed in reconstructed gene trees with haplotypes sampled from multiple species (Figs S1–S4). Across nuclear loci, there were limited discernible patterns of Tsinjoarivo haplotype relationships other than they tended to have their closest relationships with haplotypes sampled from species distributed across the eastern side of Madagascar.

Iterative rounds of nuclear-based STRUCTURE analyses placed Tsinjoarivo individuals in a genotypic cluster with *M. lehilahytsara* (Fig. 3A, Fig. S5, Supporting information), although two *M. mittermeieri* individuals were also given high posterior probability of assignment to this cluster. STRUCTURE analyses restricted to *M. lehilahytsara* and Tsinjoarivo individuals resulted in $\Delta K$ statistics that favoured a $K = 1$ model. Under a $K = 2$ model, all individuals were given equal posterior probabilities of assignment to the two clusters.

Nuclear-based STRUCTURE analyses of *M. simmonsi* individuals resulted in $\Delta K$ statistics that favoured a $K = 2$ model. Individuals sampled from the mainland and Ile Ste. Marie populations were placed in distinct genetic clusters with average posterior probabilities of 0.97 and 0.96, respectively (Fig. 3B).

### *Species hypotheses*

In this study, we refrained from testing all *Microcebus* species hypotheses, in part to limit the computational complexity and duration of our analyses, but also because the sister-species relationships of many species remain unclear. Collectively, we used the results described above, in combination with gene tree and nuclear STRUCTURE patterns from Weisrock *et al.* (2010), to identify a number of species hypotheses to be tested in this study (Table 2).

(1) We tested the null hypothesis that the Tsinjoarivo population is placed within the same lineage as *M. lehilahytsara*, with the alternative hypothesis that they represent genetically divergent lineages. In addition, given the close genetic association with

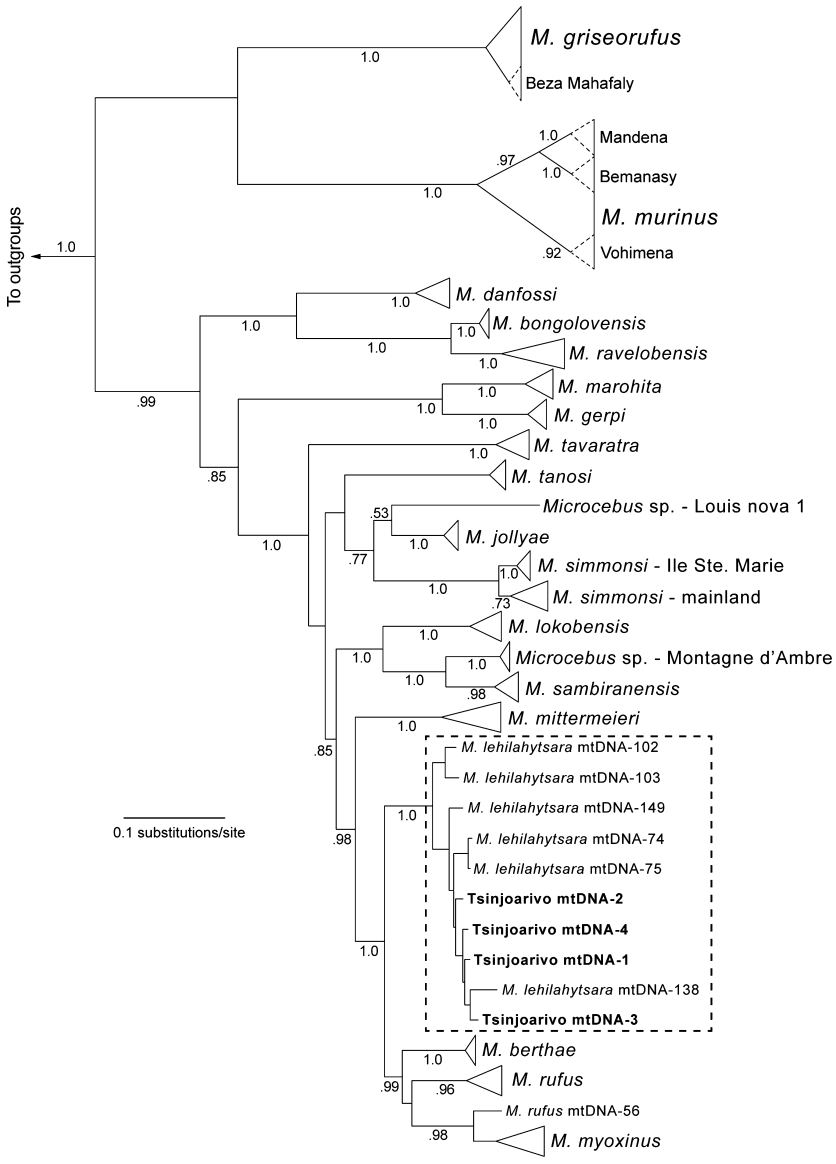


**Fig. 2** Maximum clade credibility tree generated from the posterior distribution of four replicate MRBAYES analyses of the concatenated mtDNA data. The combined posterior distribution has a mean lnL = −12615.1 (95% credibility = −12656.2 to −12574.2). Numbers on branches are posterior probabilities ≥0.5. Relationships with *Cheirogaleus* outgroups are not shown. The dashed box highlights a clade containing sampled haplotypes from Tsinjoarivo, along with haplotypes sampled from *M. lehilahytsara*.

*M. mittermeieri* indicated in the STRUCTURE results (Fig. 3A), we also tested the hypothesis that Tsinjoarivo and *M. mittermeieri* are placed within the same lineage. Finally, given the relatively close geographic proximity of Tsinjoarivo to the type locality of *M. marohita*, we test another hypothesis grouping these as a single species.

(2) Based on their reciprocal monophyly in the mtDNA gene tree (Fig. 2) and their placement in distinct nuclear genotypic clusters (Fig. 3B), we tested the hypothesis that the mainland and Ile Ste. Marie island populations of *M. simmonsi* are separate species.

(3) Identified as a potential species based on mtDNA monophyly and distinct nuclear genotypic clustering, the population of Montagne d'Ambre, along with a single individual sampled from the population of Ambanja, was tested as being a distinct species, separate from *M. sambiranensis*.

(4) We tested the hypothesis that *M. berthae*, *M. myoxinus* and *M. rufus* each represent separate species. Among these taxa, *M. rufus* is paraphyletic in the mtDNA gene tree with respect to *M. myoxinus* (Fig. 2), and all three display varying degrees of mixed assignment in nuclear STRUCTURE plots, much more so than detected in other described taxa. Consequently, we use these particular taxa as an opportunity to perform species validation of described and closely related *Microcebus* species.

(5) We tested the hypothesis that the Beza Mahafaly population is a separate species, divergent from all other *M. griseorufus* populations. Beza Mahafaly individuals are resolved as a clade in the mtDNA gene tree (Fig. 2) and form a distinct cluster in nuclear STRUCTURE plots.

(6) We tested the hypothesis that the Mandena population is a separate species, divergent from other

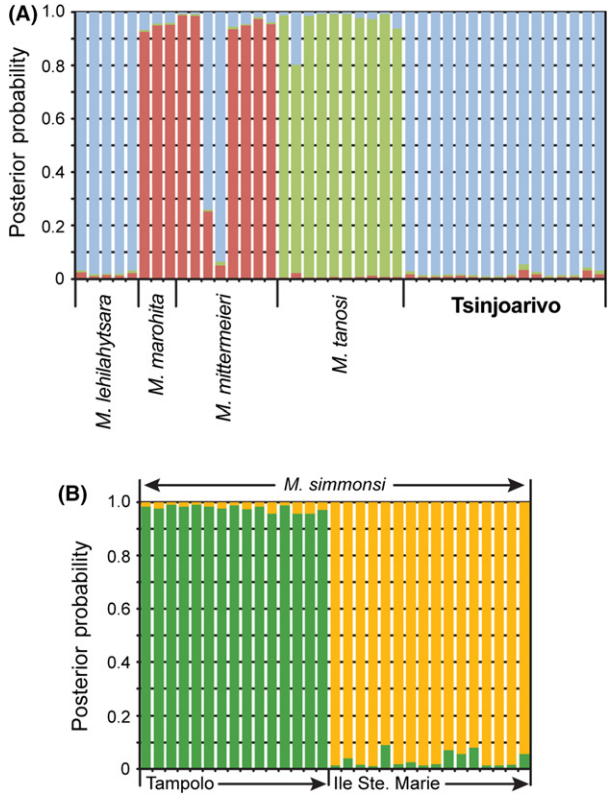


**Fig. 3** (A) Nuclear-based STRUCTURE assignment plot of individuals sampled from the Tsinjoarivo population and four described *Microcebus* species under a $K = 3$ model, which was favoured using the $\Delta K$ statistic. Vertical bars indicate the proportion of assignment to a particular genetic cluster. All individuals used in this analysis were grouped in a single genetic cluster in an earlier set of analyses that included greater numbers of *Microcebus* species. Results from these broader analyses are available in this study's Dryad accession. (B) Nuclear-based STRUCTURE assignment plot of individuals sampled from *M. simmonsi* under a $K = 2$ model, which was favoured using the $\Delta K$ statistic.

*M. murinus* populations. The Mandena population is resolved as a highly supported clade in the mtDNA tree and is assigned to its own cluster in nuclear STRUCTURE plots.

(7) In addition, we tested the hypothesis that the Bemanasy population is a separate species, divergent from other *M. murinus* populations. The Bemanasy population is also resolved as a well-supported clade in the mtDNA tree (Fig. 2) and is assigned to its own cluster in nuclear STRUCTURE plots. In these particular validation analyses we excluded sampling from the Mandena population to limit the potential for this information to bias the results.

(8) Finally, we tested the null hypothesis that the Vohimena population represents a distinct species, divergent from other *M. murinus* populations (excluding Bemanasy and Mandena). Vohimena was resolved as a mtDNA clade (Fig. 2), but nuclear STRUCTURE analyses placed it in an admixed cluster containing multiple other *M. murinus* populations. We performed this test as an exercise in exploring the sensitivity of our coalescent-based tests in hypotheses limited to evidence from the mtDNA gene tree, but where nuclear analyses do not support distinctiveness among populations.

### *Validation tests – BPP*

Here, we present the results from BPP analyses of the nuclear data. Results from analyses that included the mtDNA data were overwhelmingly consistent with the nuclear-based results and can be found in the Dryad accession. Across nearly all combinations of prior settings, replicate BPP tests rejected the null hypotheses that the Tsinjoarivo population is part of the same lineage as either *M. lehilahytsara*, *M. mittermeieri* or *M. marohita* (Fig. 4). In almost all cases, the posterior probabilities (PPs) for a divergence event between the Tsinjoarivo population and its potential sister lineage were >0.95. The one exception to this pattern was found in a single replicate analysis of one prior combination (1, 1000; 1, 100), where divergence between Tsinjoarivo and *M. lehilahytsara* received lower support (PP = 0.423). Randomized tip labelling resulted in very low PPs (≤0.184) for all divergence events and across all prior settings (Fig. 4). Divergence time estimates for these three possible splits were lowest between Tsinjoarivo and *M. lehilahytsara* (Max $\tau = 0.0007$) and largest between Tsinjoarivo and *M. marohita* (Max $\tau = 0.0037$), where $\tau$ is the expected number of substitutions per site since divergence.

BPP tests for divergence between mainland (Tampolo) and island (Ile Ste. Marie) populations of *M. simmonsi*, and for divergence between the population of Montagne d'Ambre and *M. sambiranensis* resulted in strong rejection of the null hypothesis of no divergence, with PPs favouring a divergence model (≥0.99) under all prior settings (Fig. S6A, Supporting information). In both cases, randomized tip labelling resulted in low PPs for a divergence event (≤0.286) across all prior settings (Fig. S6A, Supporting information).

BPP tests for divergence among *M. berthae*, *M. myoxinus* and *M. rufus* consistently favoured a model of lineage splitting among all three species, regardless of variation in the guide tree (Fig. S6B, Supporting information). A divergence model for all sister-species comparisons was favoured with PPs = 1.0 under all prior settings. Randomized tip labelling tended to produce low support for divergence events in the three-taxon guide trees; however, for analyses under two of the different three-taxon guide trees, analyses that used prior

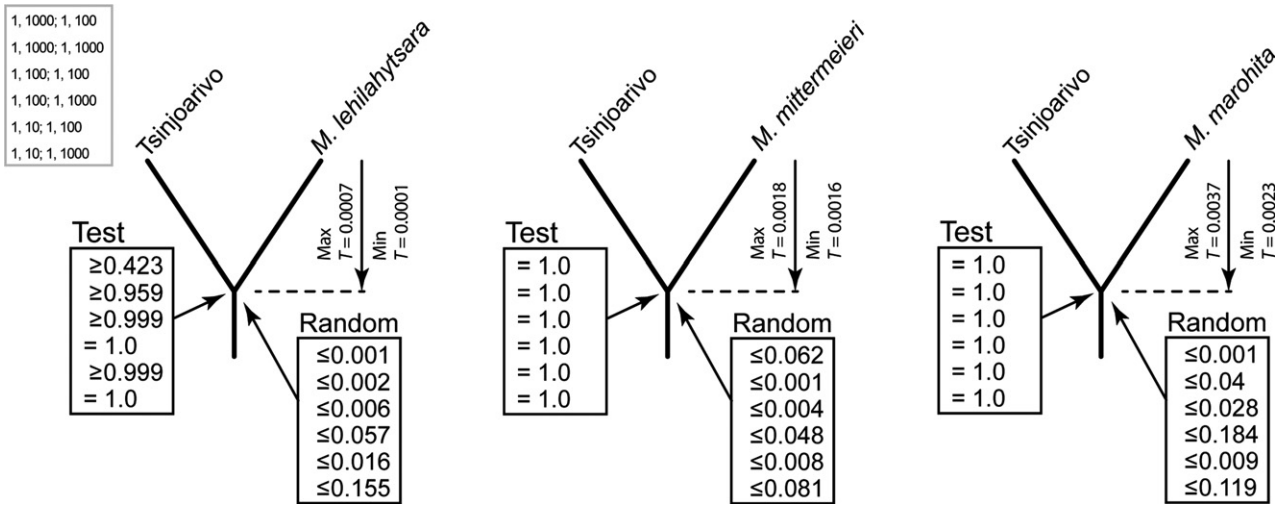


**Fig. 4** Results of nuclear-based BPP tests for species hypotheses involving the Tsinjoarivo population and three *Microcebus* species (*M. lehilahytsara*, *M. mittermeieri* and *M. marohita*). The grey-bordered box provides a reference to the six different gamma distribution prior settings used in analyses (population size; divergence time). Numbers within black-bordered boxes are posterior probabilities (PPs) for the divergence event under each prior setting and reflect the same order as the prior-settings box. PPs for each prior setting are presented as the minimum across the two different species delimitation algorithms (0 and 1) and the four replicate analyses performed for each algorithm. Results are presented for both the actual test of divergence and a test using randomized tip labels across the two lineages. Relative divergence times (*T*) are given for each splitting event and are presented as the maximum and minimum estimated mean observed across all prior settings and species delimitation algorithms.

settings assuming a small population size (1, 1000) resulted in high PPs for divergence events (e.g. PP = 1.0). These results were not consistent across replicates or delimitation algorithms, suggesting that analyses did not converge. All other prior settings resulted in low PPs for divergence events when analysed using randomized tip labels.

BPP tests for divergence between the Beza Mahafaly population and all remaining *M. griseorufus* populations favoured a model of divergence under most prior combinations, but yielded somewhat lower levels of support (PPs ≥ 0.66) under a prior combination of small population size and deep divergence (Fig. S6C, Supporting information). Randomized tip labelling produced high PPs (up to 0.957) for a model of divergence under some prior combinations.

BPP tests for divergence between *M. murinus* and the Mandena population favoured a model of divergence, with PPs = 1.0 across all prior settings (Fig. S6C, Supporting information). Tests of divergence between the Bemanasy population and *M. murinus* (excluding the Mandena population) also supported a history of divergence, with PPs ≥ 0.942 across all prior settings. In both of these cases, randomized tip labels resulted in low PP support for divergence events; however, under prior settings assuming larger population sizes (e.g. 1, 10), PPs for divergence tended to increase (e.g. PP = 0.312–0.47). BPP tests for divergence between the Vohimena population and the remaining *M. murinus* populations (excluding Mandena and Bemanasy) also indicated a history of divergence with high PPs under prior settings of relatively medium-to-large population size; however, both prior settings assuming small population size (1, 1000) resulted in some replicate analyses that favoured a null hypothesis of no divergence. Other replicate analyses under these small-population prior settings produced an alternate result of high support for a divergence event (PP = 1.0), indicating a lack of convergence in analyses under these prior settings. Analyses using randomized tip labels resulted in low PP support for divergence under prior settings for small population size, but resulted in consistently high PP support (e.g. PP = 1.0) for divergence under prior settings for large population size.

### *Validation tests – Bayes factor delimitation*

We present the results of BFD analyses for the nuclear data only; analyses that included mtDNA were largely concordant (Figs S7–S8). Further, we only report results from path sampling (PS) MLEs, as Stepping Stone (SS) MLE results were similar. A full summary of MLEs across all employed estimation methods (PS and SS), data sets (nuclear, nuclear and mtDNA, and randomized), models and their associated Bayes factors can be found in this study's Dryad accession.

Within the 14-species clade we tested a total of 34 models representing a range of species divergence hypotheses. Results for the first 16 of these models are presented in Fig. 5A, along with their combined-replicate PS MLEs, corresponding 2*ln*Bf, and PS MLEs from analyses using randomized tip assignments. The best-supported model was Model 13, which treated Tsinjoarivo and *M. lehilahytsara* as a single lineage, with divergence among all other hypothesized species (Fig. 5A). Bayes factors minimally distinguished this model from a model of full divergence (Model 16; 2*ln*Bf = 0.3) and a model that lumped *M. sambiranensis* and Montagne d'Ambre into a single lineage (Model 14; 2*ln*Bf = 3.6).

After these top three models, model support dropped precipitously, with 2*ln*Bfs ≥ 9.8 for all other models. Models that considered the mainland and Ile Ste. Marie populations of *M. simmonsi* to be a single lineage were rejected with 2*ln*Bfs ≥ 20.0. Aside from Models 8 and 14 (2*ln*Bfs = 9.8 and 3.6, respectively), all additional models that lumped the Montagne d'Ambre population with *M. sambiranensis* were rejected with 2*ln*Bfs ≥ 20. Finally, all models that placed *M. berthae* and *M. rufus* into a single lineage were rejected, with 2*ln*Bfs ≥ 20.6. While this sister-species relationship was supported (PP = 0.99) in the estimated species tree (Fig. S9, Supporting information), we also assessed two additional models placing *M. berthae* and *M. myoxinus* into a single lineage (Model 33), or placing *M. myoxinus* and *M. rufus* into a single lineage (Model 34), both of which were substantially poorer fits to the data (Fig. S10, Supporting information; 2*ln*Bfs ≥ 58.6). Similarly, models assessing alternative placements of the Tsinjoarivo population within either *M. marohita* or *M. mittermeieri* were substantially poorer fits to the data, with 2*ln*Bfs ≥ 274.4 (Fig. S10, Supporting information). For all models, analyses using randomized tip assignments produced MLEs indicating a worse fit to the data than corresponding analyses using the tested tip assignments.

**(A)**

| | *M. simmonsi* - Tampolo | *M. simmonsi* - Ile Ste. Marie | *M. sambiranensis* | *Microcebus* - Monatgne d'Ambre | **Tsinjoarivo** | *M. lehilahytsara* | *M. berthae* | *M. rufus* | **TEST**: path sampling MLE | **TEST**: path sampling 2*ln*Bf | **RANDOM**: path sampling MLE |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Model 1 | | | | | | | | | –8173.0 | 41.0 | n/a |
| Model 2 | ■ | ■ | | | | | | | –8166.5 | 28.0 | –8178.8 |
| Model 3 | | | ■ | ■ | | | | | –8171.6 | 38.2 | –8180.4 |
| Model 4 | | | | | ■ | ■ | | | –8172.1 | 39.2 | –8176.7 |
| Model 5 | | | | | | | ■ | ■ | –8162.5 | 20.0 | –8177.2 |
| Model 6 | ■ | ■ | ■ | ■ | | | | | –8163.8 | 22.6 | –8180.0 |
| Model 7 | ■ | ■ | | | ■ | ■ | | | –8162.8 | 20.6 | –8182.6 |
| Model 8 | ■ | ■ | | | | | ■ | ■ | –8157.4 | 9.8 | –8184.2 |
| Model 9 | | | ■ | ■ | ■ | ■ | | | –8171.1 | 37.2 | –8182.0 |
| Model 10 | | | ■ | ■ | | | ■ | ■ | –8163.8 | 22.6 | –8184.7 |
| Model 11 | | | | | ■ | ■ | ■ | ■ | –8163.8 | 22.6 | –8184.9 |
| Model 12 | ■ | ■ | ■ | ■ | ■ | ■ | | | –8163.0 | 21.0 | –8187.8 |
| Model 13 | ■ | ■ | ■ | ■ | | | ■ | ■ | –8152.5 | ★ | –8187.8 |
| Model 14 | ■ | ■ | | | ■ | ■ | ■ | ■ | –8154.3 | 3.6 | –8189.5 |
| Model 15 | | | ■ | ■ | ■ | ■ | ■ | ■ | –8167.7 | 24.4 | –8187.0 |
| Model 16 | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | –8152.6 | 0.3 | –8192.1 |

**(B)**

| | *M. murinus* | *M. murinus* - Mandena | *M. murinus* - Bemanasy | *M. murinus* - Vohimena | *M. griseorufus* | *M. griseorufus* - Beza Mahafaly | **TEST**: path sampling MLE | **TEST**: path sampling 2*ln*Bf | **RANDOM**: path sampling MLE |
|---|---|---|---|---|---|---|---|---|---|
| Model A | | | | | | | –6581.4 | 88.8 | n/a |
| Model B | | ■ | | | | | –6564.8 | 55.6 | –6584.1 |
| Model C | | | ■ | | | | –6574.1 | 74.2 | –6584.4 |
| Model D | | | | ■ | | | –6571.6 | 69.2 | –6584.7 |
| Model E | | | | | ■ | ■ | –6577.5 | 81.0 | –6584.2 |
| Model F | | ■ | ■ | | | | –6545.1 | 16.2 | –6586.0 |
| Model G | | ■ | | ■ | | | –6557.6 | 41.2 | –6586.0 |
| Model H | | ■ | | | ■ | ■ | –6563.2 | 52.4 | –6589.4 |
| Model I | | | ■ | ■ | ■ | ■ | –6567.7 | 61.4 | –6585.6 |
| Model J | | | ■ | | ■ | ■ | –6573.0 | 72.0 | –6588.1 |
| Model K | | | | ■ | ■ | ■ | –6569.3 | 64.6 | –6587.4 |
| Model L | ■ | ■ | ■ | ■ | | | –6537.8 | 1.6 | –6592.9 |
| Model M | | ■ | ■ | | ■ | ■ | –6543.0 | 12.0 | –6591.4 |
| Model N | | ■ | | ■ | ■ | ■ | –6557.8 | 41.6 | –6593.3 |
| Model O | | | ■ | ■ | ■ | ■ | –6564.8 | 55.6 | –6590.2 |
| Model P | ■ | ■ | ■ | ■ | ■ | ■ | –6537.0 | ★ | –6596.1 |

**Fig. 5** Summary of results for Bayes factor (2*ln*Bf) delimitation tests of species hypotheses using the nuclear data. Results are presented for path sampling-based marginal likelihood estimates (MLEs) and are split between tests performed on (A) the 14-species clade, and (B) the *M. griseorufus* and *M. murinus* clade. Results from 16 of the 34 models tested for the 14-species clade are presented here. Vertical dashed lines separate sets of species hypotheses within each clade. Models are represented as a horizontal set of boxes. Light grey shading of boxes within a model indicates that the component populations of a particular species hypothesis were lumped together into a single lineage. Dark shading of boxes within a model indicate that the component populations of a particular species hypothesis were treated as separate lineages. For example, in (A), Model 1 treats all *M. simmonsi* populations as a single lineage, whereas Model 2 treats the Tampolo and Ile Ste. Marie populations as separate lineages. For each model, MLEs are presented for both actual test results, and from randomized tip assignments. Stars identify the best-fit models for both clades.

BFD results for the *M. murinus* + *M. griseorufus* clade closely favoured two models: a full divergence model (Model P) for all hypothesized lineages (Fig. 5B) and a similar model treating *M. griseorufus*, including Beza Mahafaly, as a single lineage (Model L; 2*ln*Bf = 1.6). All other models, including one reflecting the current taxonomy of a single composite *M. murinus* lineage and a single composite *M. griseorufus* lineage (Model A), were rejected with 2*ln*Bfs ≥ 12. For all models, analyses using randomized tip assignments produced MLEs indicating a worse fit to the data than corresponding analyses using the tested tip assignments.

## Discussion

### *Bayesian species delimitation*

Our use of two different Bayesian coalescent-based methods provided complementary approaches to the validation, or refutation, of species hypotheses. A major benefit of using BPP is its ability to efficiently test hypotheses across a range of prior distributions for effective population size and divergence time. Support for divergence across priors provides assurance that results are not biased by incorrect assumptions. While BPP no longer requires the use of a guide tree, rjMCMC sampling coupled with sampling across species-tree space greatly increases computation time, especially when testing multiple hypotheses within diverse clades (our attempts to apply this approach to the 14-species clade resulted in an incomplete analysis after 30 days of CPU time). Consequently, our solution was to limit BPP tests to hypothesized combinations of sister-species pairs (or three-taxon guide trees in the case of *M. berthae*, *M. myoxinus* and *M. rufus*). In cases involving uncertainty for the correct sister-species pairing (e.g. Tsinjoarivo), we tested multiple hypotheses of sister-species divergence. This approach may provide a general solution to the application of BPP to diverse and unresolved clades where analyses that search across species-tree space prove to be computationally challenging or intractable. Our use of Bayes factor tests provided an ideal alternative solution to the need for a guide tree, allowing for uncertainty in the species tree, and permitting a model selection approach for choosing the overall species delimitation history that best fit our data. Using this method, we were not limited to individual tests of species hypotheses, but were able to consider multiple permutations of hypotheses simultaneously. Importantly, while these methods are complementary, both have been shown to be accurate in delimiting diverging lineages, even under divergence models with low levels of gene flow (Zhang *et al.* 2011; Camargo *et al.* 2012; Grummer *et al.* 2014).

### *Species validation in mouse lemurs*

Bayesian tests of *Microcebus* species hypotheses support a history of divergence for many of the hypothesized lineages addressed in this study. This conclusion is based on the recovery of high posterior probabilities in BPP analyses across a wide range of prior settings, and the rejection of most models relative to a nearly full model of divergence using Bayes factors. The Bayes factor results are noteworthy because, aside from similar levels of support for a small set of best-fitting models in both clades (models 13, 14 and 16 in the 14-species clade, and models P and L in the *M. murinus–M. griseorufus* clade), all remaining models produced 2*ln*Bfs of at least 9.8, indicating decisive support for the top models (Kass & Raftery 1995; Grummer *et al.* 2014). Results from Bayesian tests strongly support the distinctiveness of recognized species like *M. berthae*, *M. myoxinus* and *M. rufus*, taxa that exhibit close genetic affinities in mtDNA gene trees and nuclear STRUCTURE analyses. Interestingly, these results support the splitting of *M. simmonsi* into two separate species-level lineages and the splitting of *M. murinus* into multiple species-level lineages. They also reveal inconsistent patterns of support for the delimitation of the Tsinjoarivo population as a separate lineage. Collectively, our results indicate that species-level divergence within *Microcebus* is greater than what is recognized by the existing taxonomy, and follow a broader pattern of validating high levels of species diversity in a diverse set of taxa using coalescent-based methodologies (e.g. Leache & Fujita 2010; Harrington & Near 2012; Niemiller *et al.* 2012; Barley *et al.* 2013; Satler *et al.* 2013; Aydin *et al.* 2014; Ruane *et al.* 2014).

Where does coalescent-based validation of species hypotheses fit in the current framework of lemur species diversity? This may be best addressed in the context of recent critiques of rising species diversity in lemurs, and in *Microcebus* in particular (Tattersall 2007; Markolf *et al.* 2011). The broad concern has been that the rapid expansion of recognized species is the result of a shift towards a more liberal application of the phylogenetic species concept and the arbitrary diagnosis of local patterns of population differentiation as new species (Tattersall 2007). A more targeted critique is that many species diagnoses are based solely on mtDNA, often sampled from just a few individuals, and that the analysis of this single genealogical history is prone to mistaking intraspecific structure for interspecific divergence (Markolf *et al.* 2011). A benefit to using coalescent-based species delimitation is that it provides an objective approach to evaluating genetic data (Fujita *et al.* 2012). Instead of interpreting patterns in single gene trees, gene trees from multiple loci are analysed in

a probabilistic framework to statistically test their combined fit to species trees that treat sampled populations either as separately diverging or as components of the same lineage. This approach should ameliorate concerns about arbitrary conclusions derived from genetic data because test statistics, such as PPs and Bayes factors, can be evaluated to make objective choices about which sets of populations should be diagnosed as species.

Moreover, as demonstrated in this study, oversensitivity in species delimitation (oversplitting) can be assessed by performing analyses on randomized data across two (or more) hypothesized lineages, thus creating 'simulated' panmictic populations. For our BPP analyses, this approach demonstrated that the hypothesized splits between the Vohimena population and remaining *M. murinus* populations, and between the Beza Mahafaly population and remaining *M. griseorufus* populations, are both prone to providing posterior support for a model of divergence under some prior combinations. Using a similar approach in our BFD analyses, we show that, for our best-fitting models, MLEs generated from analyses using randomized tip assignments are substantially worse relative to the actual test assignments.

While there is justification for adopting a coalescent-based species delimitation approach in lemurs, as seen recently with the delimitation of a cryptic species in the genus *Cheirogaleus* (Thiele *et al.* 2013), critiques may be levelled at the recent, and potentially reversible divergence for delimited lineages. Indeed, coalescent-based methods of species delimitation are proficient in recognizing lineage divergence even at shallow evolutionary timescales (Camargo *et al.* 2012). Regardless, this can serve as the baseline methodology for stabilizing taxonomy within species radiations, with future descriptions specifying a diagnosis via coalescent analyses (Fujita *et al.* 2012). Systematists may also choose to use coalescent-based results as part of a suite of criteria for diagnosing species via genetic data. For example, previous multilocus species delimitation studies in lemurs have used monophyly in mtDNA gene trees and resolution as a distinct nuclear cluster in population structure analyses as criteria for species diagnosis under a general lineage concept (Groeneveld *et al.* 2009; Weisrock *et al.* 2010). A combination of all three criteria could serve as a more conservative approach towards recognizing new species via genetic data. Ultimately, we echo Carstens *et al.* (2013) in advocating that evidence garnered from multiple different species delimitation methods, including those combining genetic and non-genetic forms of data (e.g. Markolf *et al.* 2013) and those utilizing different implementations of the coalescent model, should be trusted in their use to delimit new species.

### *Taxonomic conclusions in mouse lemurs*

Results from our coalescent-based analyses are inconsistent in their support of the Tsinjoarivo population as a diverging lineage. While BPP analyses support models of divergence between Tsinjoarivo and hypothesized sister lineages, BFD analyses are equivocal in treating Tsinjoarivo as a distinct lineage. This is further contrasted by a lack of monophyly for Tsinjoarivo haplotypes in the mtDNA gene tree, and a lack of evidence from STRUCTURE analyses for population structure separating Tsinjoarivo and *M. lehilahytsara*. Based on these discrepancies, there is little evidence for treating Tsinjoarivo as a diagnosed species.

We also find limited support for the validation of a hypothesized species from the population of Montagne d'Ambre. BPP analyses strongly support the delimitation of this species, but a BFD model lumping Montagne d'Ambre with *M. sambiranensis* received a 2*ln*Bf of just 3.6, a measure of 'positive', but not decisive, support (Kass & Raftery 1995). Louis *et al.* (2008) also described a population of mouse lemurs at Montagne d'Ambre as *M. arnholdi* based on diagnostic character differences with other populations in mtDNA data. Furthermore, other mtDNA-based species (e.g. *M. margotmarshae* and *M. mamiratra*) have been described from northern Madagascar that were not included in this study. The lack of shared orthologous sequence data prevents us from specifically testing these mtDNA-based hypotheses, and our results demonstrate both the importance of using a multilocus and objective species delimitation approach for the validation of species hypotheses and the potential for future research aimed at clarifying lemur diversity in this region.

In contrast to these conclusions, we find evidence for the delimitation of three cryptic species of mouse lemurs, as tested in hypotheses 2, 6 and 7 (see Results). Two of these involve the Bemanasy and Mandena populations of *M. murinus*, both of which were diagnosed as population-level lineages in Weisrock *et al.* (2010) based on mtDNA monophyly and nuclear population structure. Here, our coalescent-based tests validate these species delimitation hypotheses. Both of these new species are located in southeastern Madagascar in fragments of humid littoral forest, and are separated from the larger western *M. murinus* range by dry spiny forest occupied by *M. griseorufus*, with an ecotone that fosters some hybridization between species (Gligor *et al.* 2009; Hapke *et al.* 2011), and also restricts *M. murinus* dispersal. As noted in Weisrock *et al.* (2010), the small geographic distance separating Bemanasy and Mandena is surprising, given the support for their genetic divergence. While this study sampled just one population from each putative lineage, a comprehensive survey of

mtDNA sequence data from 218 *M. murinus* individuals across this region also revealed two divergent geographic clades corresponding to the Bemanasy and Mandena lineages, with no evidence of haplotype sharing (Hapke *et al.* 2012). As a result, we propose that each of these populations warrants recognition as distinct species (Appendix I).

The results of this study also strongly support the delimitation of the Ile Ste. Marie population of *M. simmonsi* as a distinct species from its mainland counterpart (Appendix I). These populations have not previously been delimited as separate species, although their mtDNA haplotypes have been previously resolved as reciprocally monophyletic groups (Weisrock *et al.* 2010). Here, STRUCTURE analyses of data restricted to all *M. simmonsi* individuals produced two distinct genetic clusters associated with these island and mainland groups (Fig. 3B) and Bayesian coalescent analyses validated these hypothesized lineages.

### *Integrating genetic insight with evidence from natural history*

It is becoming increasingly apparent that in mouse lemurs, like other taxonomic groups, an array of behavioural, dietary, physiological and signalling mechanisms contribute to genetic isolation and drive species divergence (e.g. Schwab 2000; Braune *et al.* 2008; Dammhahn & Kappeler 2008; Hapke *et al.* 2011; Rakotondranary & Ganzhorn 2011; Yoder *et al.* 2014, 2016). For mouse lemurs, their diminutive size, nocturnal habits and remote geographic distribution require significant effort on the part of field biologists who wish to study these enigmatic primates, which further emphasizes the role of multilocus genetic evidence in characterizing species diversity. Following Leache & Fujita (2010), we expect that additional data from physiology, behaviour and ecology will only strengthen delimitations performed under the framework described here. Future studies will continue to provide insight into the unique ecological and behavioural traits of mouse lemurs and, in turn, further inform the mechanisms that have driven and continue to maintain species boundaries in *Microcebus*. From a wider perspective, establishing a rigorous framework for delimiting species diversity, as described here for mouse lemurs, will provide clarity to current taxonomic boundaries, stem critique of taxonomic decisions and streamline future descriptions in an increasingly molecular landscape.

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D.W.W. conceived of this study. R.R. and M.B.B. carried out field sampling. S.H., M.E.F., J.B., M.A.B. and D.W.W. collected the genetic data. S.H., M.E.F., N.M.L. and D.W.W. analysed the data. S.H., R.R., P.M.K., A.D.Y. and D.W.W. wrote the manuscript. All authors read and approved the final submission.

## Data accessibility

All DNA sequence data sets, along with all input and results files from MRBAYES, STRUCTURE, BPP and Bayes factor analyses, are available in this study's Dryad accession (doi:10.5061/dryad.h6s5j).

## Appendix I: Species descriptions

Here, we provide formal descriptions of three new mouse lemur species diagnosed from nuclear and mtDNA sequence data using species discovery delimitation criteria of mtDNA gene tree monophyly, distinct nuclear population structuring and explicit hypothesis-driven validation using coalescent-based Bayesian species delimitation tests. Two species described here represent new descriptions within the *Microcebus murinus* species complex. The third represents a splitting of *M. simmonsi*, with the island population from Ile Ste. Marie described as a new species. Given the strict conditions of Bayesian species delimitation paired with additional species diagnosis requirements, we consider these descriptions to be conservative.

(i) *Microcebus manitatra*, sp. nov.

*Holotype*. RMR 218; adult female, skin, skull, skeleton and preserved tissues. Collected 25 April 2007 by R. M. Rasoloarison. Specimen stored at the Département de Biologie Animale, Université d'Antananarivo. Photographs of skin, skull and mandible of the holotype as well as a comparison with *M. murinus* (RMR 17, from Vohimena) are provided in Fig. S11 (Supporting information).

*Type locality*. Madagascar: Toliara Province, Anosy Region, Fort-Dauphin District, Bemanasy Forest (Ambatotsirongorongo). 25°5.11′S; 46°46.52′E.

*Referred specimen*. Madagascar: Toliara Province, Anosy Region, Fort-Dauphin District, Bemanasy Forest (Ambatotsirongorongo): skin, skull, skeleton and preserved tissues for RMR 215–217. Preserved tissues for RMR 219–220.

*Phenotypic measurements of the holotype*. Standard measurements (in mm except for mass) recorded in the original field catalogue and on the skin tag of the type include the following: total length = 276; head–body length = 125; tail length = 150; hind-foot length = 33; ear length = 25; and body mass = 58 g. Selected cranial measurements (all in mm) are as follows: greatest skull length = 32.7; skull height = 15.4; palate length = 12.8;

zygomatic breadth = 20.3; nasal length = 8.4; occipital length = 4.7; canine height = 2.5; and molar length = 1.7. The skin, the skull and associated skeleton are all in high quality condition.

*Description. M. manitatra* is a relatively large mouse lemur characterized by its size (total length = 270–276 mm), long tail with dense, short fur (150 mm), relatively short hind feet (33 mm) and long ears (25–26 mm). In comparison with *M. murinus*, *M. manitatra* is smaller. The dorsal pelage of *M. manitatra* is uniformly greyish brown on the back and tail and the underside is a greyish beige with dark grey underfur. This contrasts with *M. murinus* which has a variable greyish-brown to brownish-grey back and tail with a dull reddish-brown or cinnamon mid-dorsal stripe, as well as a mixed beige and grey underside.

*Diagnosis.* This new species includes a population from the southeastern region of Madagascar that is disjunct from the broader range of *M. murinus* across western and southern Madagascar. It is also distinct from the nearby population of Bemanasy. This species forms a monophyletic group in the mtDNA gene tree, is assigned to a distinct nuclear cluster in population structure analyses and is strongly supported with coalescent-based Bayesian species delimitation tests.

*Etymology.* This species is named for the Malagasy word 'manitatra' meaning 'range expansion', which reflects the extension of this species' distribution into the southeast of Madagascar, disjunct from the primarily western distribution of *M. murinus*.

(ii) *Microcebus ganzhorni*, sp. nov.

*Holotype.* Genomic DNA is accessioned at the Duke Lemur Center (DLC) as DLC #100; adult male. Field sample was collected as an ear clip tissue sample on 8 August 1998 by Jörg U. Ganzhorn.

*Type locality*. Madagascar: Toliara Province, Anosy Region, Tôlanaro District, Mandena Forest; 24°56.9′S; 46°59.7′E.

*Phenotypic measurements of the holotype.* No measurements are currently available for *M. ganzhorni.*

*Diagnosis.* This new species includes a population from the southeastern region of Madagascar that is disjunct from the broader range of *M. murinus* across western and southern Madagascar. It is also distinct from the nearby population of Bemanasy. This species forms a monophyletic group in the mtDNA gene tree, is assigned to a distinct nuclear cluster in population structure analyses and is strongly supported with coalescent-based Bayesian species delimitation tests.

*Etymology.* This species is named for Dr. Jörg Ganzhorn, Professor at the Universität Hamburg, in honour of his substantial scientific contributions to the understanding of the biology of mouse lemurs (and all lemurs), and his efforts towards their conservation.

(iii) *Microcebus boraha*, sp. nov.

*Holotype.* RMR 115; adult female, skin, skull, skeleton and preserved tissues, collected 10 October 2003 by R. M. Rasoloarison. Specimen stored at the Département de Biologie Animale, Université d'Antananarivo. Photographs of skin, skull and mandible of the holotype as well as a comparison with *M. simmonsi* (RMR 108, from Tampolo) are provided in Fig. S12 (Supporting information).

*Type locality*. Madagascar: Toamasina Province, Analanjirofo Region, Sainte-Marie District, Ikalalao Forest; 16°55′S; 49°52′E, about 68 m above sea level.

*Referred specimen*. Madagascar; Province de Toamasina, Région d'Analanjirofo, District de Sainte-Marie, Forêt d'Ikalalao. Skin, skull, skeleton and preserved tissues for RMR 116–119. Preserved tissues for RMR 120–130.

*Phenotypic measurements of the holotype.* Standard measurements (in mm except for mass) recorded in the original field catalogue and on the skin tag of the type include the following: total length = 285; head–body length = 136; tail length = 143; hind-foot length = 34; ear length = 19; and body mass = 56.5 g. Selected cranial measurements (all in mm) are as follows: greatest skull length = 35.4; skull height = 14.4; palate length = 15.6; zygomatic breadth = 21.7; nasal length = 11.3; occipital length = 3.9; canine height = 2.0; and molar length = 2.1. The skin, the skull and associated skeleton are all in high quality condition.

*Description. M. boraha* is a relatively large mouse lemur characterized by its size (total length = 280–292 mm), long dense tuft tail (141–148 mm), notably long hind feet (34–36 mm), and short ears (19–20 mm). The dorsal pelage is reddish with a poorly defined mid-dorsal stripe. The underside is beige and slightly reddish.

*Diagnosis.* This new species includes populations from the island of Ile Ste. Marie off the east coast of Madagascar. This species forms a monophyletic group in the mtDNA gene tree, is assigned to a distinct nuclear cluster in population structure analyses and is strongly supported with coalescent-based Bayesian species delimitation tests.

*Etymology.* This species is named for the Malagasy name, Nosy Boraha, of the island of Ile Ste. Marie.

A comparison of skin samples from *M. boraha* and *M. manitatra* against six other *Microcebus* species from eastern Madagascar is provided in Fig. S13 (Supporting information). For *M. manitatra, M. tanosi, M. marohita* and *M. boraha*, the skin samples shown are holotypes. For all others, these are referred specimens.

## Supporting information

Additional supporting information may be found in the online version of this article.

**Fig. S1.** Maximum clade credibility tree generated from the Bayesian posterior distributions resulting from MRBAYES analysis of aligned *adora* haplotypes.

**Fig. S2.** Maximum clade credibility tree generated from the Bayesian posterior distributions resulting from MRBAYES analysis of the aligned *eno* haplotypes.

**Fig. S3.** Maximum clade credibility tree generated from the Bayesian posterior distributions resulting from MRBAYES analysis of the aligned *fiba* haplotypes.

**Fig. S4.** Maximum clade credibility tree generated from the Bayesian posterior distributions resulting from MRBAYES analysis of the aligned *vwf* haplotypes.

**Fig. S5.** Nuclear-based STRUCTURE assignment plots from iterative rounds of analysis starting with all sampled populations from the 14-species clade. In each round of analyses, the optimal level of K was identified using the $\Delta K$ statistic and the cluster containing the Tsinjoarivo individuals was subjected to an additional set of analyses.

**Fig. S6.** Results of BPP tests for tested hypotheses of speciation among additional *Microcebus* species and populations.

**Fig. S7.** Summary of results from Bayes factor (2*ln*Bf) delimitation tests of species hypotheses for the full (mtDNA + nuclear loci) dataset. Results are split between tests performed on (A) the 14-species clade, and (B) the *M. griseorufus* and *M. murinus* clade.

**Fig. S8.** Additional full (mtDNA + nuclear loci) dataset Bayes factor delimitation results for Models 17–34 of species delimitation hypotheses within the 14-species clade.

**Fig. S9.** Maximum clade credibility trees from Bayesian species tree reconstructions of (A) the 14-species clade analyzed under the full-divergence model (model 16), and (B) the *M. griseorufus* + *M. murinus* clade analyzed under the full divergence model (model P).

**Fig. S10.** Additional nuclear Bayes factor delimitation results for Models 17–34 of species delimitation hypotheses within the 14-species clade.

**Fig. S11.** Photos of the holotype for *Microcebus manitatra* sp. nov. Provided are photos of the preserved skin sample (top), skull and mandible (middle), and a comparison of skin samples between the *M. manitatra* holotype and a representative individual of *M. murinus* from Vohimena (bottom).

**Fig. S12.** Photos of the holotype for *Microcebus boraha* sp. nov. Provided are photos of the preserved skin sample (top), skull and mandible (middle), and a comparison of skin samples between the *M. boraha* holotype and a representative individual of *M. simmonsi* from Tampolo (bottom).

**Fig. S13.** A comparison of preserved skins from two of the newly described species from this study, *Microcebus boraha* and *M. manitatra*, and six additional species from eastern Madagascar.

**Table S1.** Full gene copy sampling for BPP-based species delimitation tests for all species or population and loci used in this study.

**Table S2.** Prior distributions used in BPP analyses.

**Table S3.** Gene copy sampling used in *BEAST-based Bayes factor delimitation analyses for each species or population and loci used.

**Appendix S1.** Expanded methods.