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Cryptic Patterns of Speciation in Cryptic Primates: Microendemic Mouse Lemurs and the Multispecies Coalescent

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1 **Cryptic Patterns of Speciation in Cryptic Primates:**

2 **Microendemic Mouse Lemurs and the Multispecies Coalescent**

3 Running title: Cryptic speciation in mouse lemurs

4
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63

64 Data Availability Statement

65 - Raw sequencing data is available at NCBI BioProject PRJNA560399.

66 - All other data is available from the Dryad Digital Repository at

67 <https://datadryad.org/stash/share/>-

68 q8Ez_lXqi7LiDsBHJTAgM1JpdPOCGep0nU5gZ_EH9k

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69 **ABSTRACT**

70 Mouse lemurs (*Microcebus*) are a radiation of morphologically cryptic primates
71 distributed throughout Madagascar for which the number of recognized species has exploded in
72 the past two decades. This taxonomic revision has prompted understandable concern that there
73 has been substantial oversplitting in the mouse lemur clade. Here, we investigate mouse lemur
74 diversity in a region in northeastern Madagascar with high levels of microendemism and
75 predicted habitat loss. We analyzed RADseq data with multispecies coalescent (MSC) species
76 delimitation methods for two pairs of sister lineages that include three named species and an
77 undescribed lineage previously identified to have divergent mtDNA. Marked differences in
78 effective population sizes, levels of gene flow, patterns of isolation-by-distance, and species
79 delimitation results were found among the two pairs of lineages. Whereas all tests support the
80 recognition of the presently undescribed lineage as a separate species, the species-level
81 distinction of two previously described species, *M. mittermeieri* and *M. lehilahytsara* is not
82 supported – a result that is particularly striking when using the genealogical discordance index
83 (*gdi*). Non-sister lineages occur sympatrically in two of the localities sampled for this study,
84 despite an estimated divergence time of less than 1 Ma. This suggests rapid evolution of
85 reproductive isolation in the focal lineages, and in the mouse lemur clade generally. The
86 divergence time estimates reported here are based on the MSC calibrated with pedigree-based
87 mutation rates and are considerably more recent than previously published fossil-calibrated

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88 relaxed-clock estimates. We discuss the possible explanations for this discrepancy, noting that
89 there are theoretical justifications for preferring the MSC estimates in this case.

90

91 Keywords: effective population size, cryptic species, multispecies coalescent, species
92 delimitation, speciation, microendemism

93 **INTRODUCTION**

94 Mouse lemurs (*Microcebus* spp.) are small, nocturnal primates that are widespread in the
95 forests of Madagascar (Mittermeier et al. 2010), one of the world's most biodiverse environments
96 (Myers et al. 2000; Goodman and Benstead 2005; Estrada et al. 2017). Mouse lemur diversity
97 was long overlooked (Zimmermann and Radespiel 2014) until the introduction of genetic
98 analyses made it feasible to identify diverging lineages comprised of lemurs with similar
99 phenotypes and ecological niches. This genetic perspective has led to the description of many
100 new species, with 24 species recognized at present. In one such study, Radespiel et al. (2008)
101 surveyed the forests of the Makira region (Fig. 1) in northeastern Madagascar and found
102 evidence for three divergent mitochondrial lineages occurring in sympatry. One of these was
103 identified as *M. mittermeieri* (Louis et al. 2006), while the second was newly described as *M.*
104 *macarthurii*. A third lineage, provisionally called *M. sp. #3*, was hypothesized to represent a new
105 species closely related to *M. macarthurii* but was not formally named because the data were
106 limited to mtDNA sequence data from one individual. Furthermore, two other species occur in
107 the region, *M. lehilahytsara* (Roos and Kappeler in Kappeler et al. 2005) at higher elevations,
108 and *M. simmonsi* (Louis et al. 2006) in lowland forests in the south (Fig. 1).

109 Given that many previous taxonomic descriptions of mouse lemurs have relied strongly, if
110 not entirely, on mtDNA sequence divergence, there has been criticism that mouse lemurs (and

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111 lemurs more generally) may have been oversplit (Tattersall 2007; Markolf et al. 2011). Species
112 delimitation using only mtDNA is now widely regarded as problematic, given that the
113 mitochondrial genome represents a single non-recombining locus whose gene tree may not
114 represent the underlying species tree (e.g., Pamilo and Nei 1988; Maddison 1997). Mitochondria
115 are also maternally inherited and therefore susceptible to effects of male-biased dispersal (e.g.,
116 Dávalos and Russell 2014), which is prevalent in mouse lemurs (reviewed in Radespiel 2016).
117 Moreover, previous attempts to resolve mouse lemur relationships using nuclear sequences have
118 been complicated by high gene tree discordance, consistent with strong incomplete lineage
119 sorting (e.g., Heckman et al. 2007; Weisrock et al. 2010). These issues can be overcome with
120 genomic approaches, which provide power for simultaneously resolving phylogenetic
121 relationships and estimating demographic parameters such as divergence times, effective
122 population sizes, and rates of gene flow — even among closely related species (e.g., Palkopoulou
123 et al. 2018; Pedersen et al. 2018).

124 Given that cryptic species are by definition difficult to identify based on phenotypic
125 characters (Bickford et al. 2007), recently developed methods for genomic species delimitation
126 have advanced our ability to recognize and quantify their species diversity. In the past decade,
127 both theory and methods for species delimitation have seen substantial progress, especially those
128 which leverage the multispecies coalescent (MSC) model (Pamilo and Nei 1988; Rannala and
129 Yang 2003). MSC-based species delimitation methods have been increasingly applied to

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130 genomic data (e.g. Carstens and Dewey 2010; Yang and Rannala 2010; Grummer et al. 2014;
131 Dincă et al. 2019; Hundsdoerfer et al. 2019), though they have also been considered
132 controversial (Edwards and Knowles 2014; Sukumaran and Knowles 2017; Barley et al. 2018).
133 The controversy largely relates to the idea that strong population structure can be mistaken for
134 species boundaries, which may lead to oversplitting (Jackson et al. 2017; Sukumaran and
135 Knowles 2017; Luo et al. 2018; Leaché et al. 2019; Chambers and Hillis 2020). To overcome this
136 potential weakness, Jackson et al. (2017) proposed a heuristic criterion, the genealogical
137 divergence index (*gdi*), with Leaché et al. (2019) further suggesting that *gdi* helps to differentiate
138 between population structure and species-level divergence. In parallel, sophisticated statistical
139 approaches have been developed that can detect the presence and magnitude of gene flow during
140 or after speciation (Gronau et al. 2011; Dalquen et al. 2017; Wen et al. 2018). Taken together,
141 these analytical developments are crucial to our ability to recognize the patterns that characterize
142 the speciation process, despite the challenge of identifying species without universally agreed
143 upon criteria (de Queiroz 2007).

144 In this study, we use a structured framework starting with phylogenetic placement of
145 lineages and culminating with the MSC to delimit species, estimate divergence times, identify
146 post-divergence gene flow, and to estimate both current and ancestral effective population sizes
147 (Fig. S1). We take advantage of increased geographic, population-level, and genomic sampling
148 to comparatively examine speciation dynamics for two pairs of closely related lineages in the

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149 region (described below as Clades I and II) and perform MSC species delimitation methods with
150 Restriction-site Associated DNA sequencing (RADseq) data to infer divergence times, effective
151 population sizes, and rates of gene flow between these lineages. We also provide a novel whole-
152 genome assembly for the previously undescribed lineage and compare inferences of effective
153 population size (N_e) through time from whole-genome versus RADseq data. We find notably
154 different species delimitation results for the lineages in the two mouse lemur clades and believe
155 that the comprehensive analytic framework here used can be applied more generally to allow
156 investigators to test hypotheses of population- versus species-level differentiation.

157 **MATERIALS AND METHODS**

158 *Summary of Analyses*

159 We generated RADseq data for 63 individuals from 6 lineages, of which 48 were from
160 the two focal clades and passed quality control. First, we used maximum likelihood approaches
161 to infer relationships among lineages and to provide a framework for subsequent species
162 delimitation analyses (Fig. 2A and C). To delimit species, we performed clustering (Fig. 2B) and
163 PCA analyses (Fig. 3A-C), as well as formal MSC species delimitation analyses using SNAPP
164 and BPP. We also used the recently developed genealogical divergence index *gdi* based on BPP
165 parameter estimates (Fig. 3D) and performed an isolation-by-distance analysis (Fig. 4). To
166 determine to what extent ongoing and ancestral gene flow may have contributed to current

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167 patterns of divergence, we used G-PhoCS and D-statistics (Fig. 5). Finally, we generated and
168 assembled whole-genome sequencing data for a single *M. sp. #3* individual. The whole-genome
169 sequences of *M. sp. #3* reported here for the first time and *M. mittermeieri* from a previous study
170 (Hunnicuttt et al., 2020) were used to infer N_e through time with Multiple Sequentially Markovian
171 Coalescent (MSMC) analysis and compared with estimates from G-PhoCS (Fig. 6). Below, we
172 describe the methods in more detail, while further details can be found in the Supplementary
173 Material.

174 *Study Sites and Sampling*

175 *Microcebus* samples were obtained by taking ~2 mm² ear biopsies of captured (and
176 thereafter released) individuals between 2008 and 2017 at seven humid evergreen forest sites
177 (50-979 m a.s.l.) in the Analanjirofo and Sava regions of northeastern Madagascar (Fig. 1;
178 Table S1). Additional samples were used from Riamalandy, Zahamena National Park (NP),
179 Betampona Strict Nature Reserve (SNR) and Tampolo (Louis et al. 2006; Weisrock et al. 2010;
180 Louis and Lei 2016) (Fig. 1). With this sampling strategy, we expected to include all mouse
181 lemur species thought to occur in the region (from north to south): *M. mittermeieri*, *M.*
182 *macarthurii*, *M. sp. #3*, *M. lehilahytsara*, and *M. simmonsii* (Fig. 1). *Microcebus murinus*,
183 which occurs in western and southeastern Madagascar, was used as an outgroup.

184 *Sequencing Data, Genotyping and Genome Assembly*

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185 We generated RADseq libraries using the *Sbf*I restriction enzyme, following three
186 protocols (Supplementary Methods, Table S1). Sequences were aligned to the *M. sp. #3*
187 nuclear genome generated by this study, and to the published *M. murinus* mitochondrial genome
188 (LeCompte et al. 2016). We used two genotyping approaches to ensure robustness of our results.
189 First, we estimated genotype likelihoods (GL) with ANGSD v0.92 (Nielsen et al. 2012;
190 Korneliussen et al. 2014), which retains information about uncertainty in base calls, thereby
191 alleviating some issues commonly associated with RADseq data such as unevenness in
192 sequencing depth and allele dropout (Lozier 2014; Pedersen et al. 2018; Warmuth and Ellegren
193 2019). Second, we called genotypes with GATK v4.0.7.0 (DePristo et al. 2011), and filtered
194 GATK genotypes following the "FS6" filter of O'Leary et al. (2018; their Table 2). We
195 furthermore used three mtDNA fragments [Cytochrome Oxidase II (COII), Cytochrome B
196 (cytB), and d-loop] that were amplified and Sanger sequenced.

197 The genome of the *M. sp. #3* individual sampled in Mananara-Nord NP (Table S3) was
198 sequenced with a single 500bp insert library on a single lane of an Illumina HiSeq 3000 with
199 paired-end 150bp reads. We used MaSuRCA v3.2.2 (Zimin et al. 2013) for contig assembly and
200 SSPACE (Boetzer et al. 2011) for scaffolding. Scaffolds potentially containing mitochondrial or
201 X-chromosome sequence data were removed in downstream analyses.

202 *Phylogenetic Analyses*

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203 We used three phylogenetic approaches to infer relationships among lineages: (1)
204 maximum likelihood using RAxML v8.2.11 (Stamatakis 2014), (2) SVDquartets, an MSC
205 method that uses phylogenetic invariants, implemented in PAUP v4a163 (Chifman and Kubatko
206 2014), and (3) SNAPP, a full-likelihood MSC method for biallelic data that does not require joint
207 gene tree estimation (v1.3.0; Bryant et al. 2012). Analyses with RAxML and SVDquartets used
208 all available individuals, whereas SNAPP analyses were performed with subsets of 12 and 22
209 individuals for computational feasibility (see Supplementary Methods).

210 *Species Delimitation*

211 *Clustering approaches and summary statistics.* — Clustering analyses were performed
212 using corresponding methods based on ANGSD genotype likelihoods [clustering in NgsAdmix
213 v32 (Skotte et al. 2013) and PCA in ngsTools va4d338d (Fumagalli et al. 2014)] and on GATK-
214 called genotypes [clustering in ADMIXTURE v1.3.0 (Alexander et al. 2009) and PCA using the
215 glPca() function in adegenet v2.1.1 (Jombart and Ahmed 2011)]. These analyses were run for
216 Clade I and Clade II together and separately.

217 *MSC-based approaches.* — We used SNAPP to test if the two lineages each in Clade I and
218 II could be delimited using Bayes factors (Leaché et al. 2014), interpreting $2\ln$ Bayes factors
219 greater than six as strong evidence for a given model (Kass and Raftery 1995). We also applied
220 guided species delimitation analyses with BPP (Yang and Rannala 2010; Rannala and Yang

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221 2013) using full-length fasta files for a subset of individuals based on the species tree estimated
222 by SVDquartets and SNAPP.

223 *gdi*. — Coalescent node heights (τ) and ancestral effective population sizes (θ) estimated
224 by BPP were used to compute the genealogical divergence index (*gdi*; Jackson et al. 2017;
225 Leaché et al. 2019) for the lineages in Clade I and Clade II. We calculated *gdi* as in Leaché et al.
226 (2019), using their equation 7 ($gdi = 1 - e^{-2\tau/\theta}$), where $2\tau/\theta$ represents the population divergence
227 time between two taxa in coalescent units. θ is taken from one of the two taxa and therefore, *gdi*
228 was calculated twice for each species pair, alternating the focal taxon. We computed *gdi* using τ
229 and θ parameter estimates for each posterior BPP sample to incorporate uncertainty in the
230 estimates. Jackson et al. (2017) suggested the following interpretation of *gdi* values: the taxon
231 pair (a) is unambiguously a single species for $gdi < 0.2$, (b) is unambiguously two separate
232 species for $gdi > 0.7$, and (c) falls in an ambiguous zone for $0.2 < gdi < 0.7$.

233 *Isolation-by-distance*. — We tested for isolation-by-distance using the VCF file produced
234 by GATK with the `glibd()` function in the R package `dartR` 1.1.11 (Gruber et al. 2018).

235 *Inference of gene flow and divergence times*

236 G-PhoCS v1.3 (Gronau et al. 2011), a Bayesian MSC approach that allows for the
237 estimation of periods of gene flow (*i.e.* “migration bands”), was used to jointly infer divergence
238 times, population sizes, and rates of gene flow between specific lineages. Based on the results of

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239 exploratory models that each contained a single “migration band” between two lineages, we ran
240 a final model with a migration bands allowing gene flow from *mittermeieri* to *lehilahytsara* and
241 from *macarthurii* to *M. sp. #3*. Given the observed mitonuclear discordance between *M. sp. #3*
242 and *M. macarthurii* (see Results), we investigated gene flow between them in more detail by
243 running G-PhoCS using a dataset with only *M. sp. #3*, *M. macarthurii*, and *M. lehilahytsara*
244 individuals, wherein *M. sp. #3* was divided into the two populations detected using clustering
245 approaches.

246 The D-statistic and related formal statistics for admixture use phylogenetic invariants to
247 infer post-divergence gene flow between non-sister populations or taxa. We used the qpDstat
248 tool in admixtools v4.1 (Patterson et al. 2012) to compute four-taxon D-statistics for all possible
249 configurations in which gene flow could be tested between non-sister lineages among the five
250 ingroup lineages. We additionally tested for gene flow between *M. macarthurii* and *M. sp. #3* by
251 separately treating (1) the two distinct *M. sp. #3* populations detected by clustering approaches,
252 and (2) *M. macarthurii* individuals with and without “*M. sp. #3*-type” mtDNA (see Results). In
253 all tests, *M. murinus* was used as P4 (outgroup).

254 *Effective Population Size Through Time*

255 Studies have shown that population structure can generate spurious signals of population
256 size change (Beaumont 2004; Chikhi et al. 2010; Heller et al. 2013). For example, sequentially

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257 Markovian coalescent approaches such as MSMC (Schiffels and Durbin 2014) actually estimate
258 the inverse instantaneous coalescence rate, which is only equivalent to an effective size in
259 panmictic models (Mazet et al. 2016; Rodríguez et al. 2018). We therefore inferred and
260 compared population size histories using two methods. We estimated N_e over time with MSMC
261 for two species, using the whole-genome data of *M. sp. #3* and *M. mittermeieri* (Hunnicut et al.
262 2020) mapped to the chromosome-level genome assembly of *M. murinus* (Larsen et al. 2017).
263 These estimates were compared to inferred changes in N_e over time based on θ estimates from G-
264 PhoCS for each predefined extant or ancestral population. Although G-PhoCS was not expressly
265 developed to estimate change in N_e over time, this allowed us to explicitly examine broad
266 demographic trends, even with small population-level sampling.

267 *Mutation Rate and Generation Time*

268 We used empirical estimates of mutation rate and generation time to convert coalescent
269 units from BPP, G-PhoCS and MSMC analyses into absolute times and population sizes. We
270 incorporated uncertainty by drawing from mutation rate and generation time distributions for
271 each sampled generation of the MCMC chains in BPP and G-PhoCS (MSMC parameter estimates
272 were converted using the point estimates). For the mutation rate, we used a gamma distribution
273 based on the mean (1.236×10^{-8}) and variance (0.107×10^{-8}) of seven pedigree-based mutation
274 rate estimates for primates (see Campbell et al. 2019, Table S1). For the generation time, we
275 used a lognormal distribution with a mean of $\ln(3.5)$ and standard deviation of $\ln(1.16)$ based on

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276 estimates of 4.5 years calculated from survival data (Zohdy et al. 2014; Yoder et al. 2016) from
277 *M. rufus*, and 2.5 years from average parent age based on capture-mark-recapture and parentage
278 data in the wild (Radespiel et al. 2019) for *M. murinus*.

279 RESULTS

280 *RADseq Data and Whole-Genome Assembly*

281 We used three library generation protocols, two sequencing lengths, and a combination of
282 single and paired-end sequencing, yielding data for all 63 individuals in the study and
283 demonstrating the utility of cross-laboratory RAD sequencing, as previously shown in other taxa
284 (e.g., Gonen et al. 2015). From more than 447 million raw reads (Table S1), over 394 million
285 passed quality filters, with approximately 182 million successfully aligning to the *M. sp. #3*
286 reference genome. We obtained an average of 120,000 loci per individual with coverage ranging
287 from ~1 to ~22x (Table S1).

288 We assembled approximately 2.5 Gb of nuclear genome sequence data for *M. sp. #3* with a
289 contig N50 around 36 Kb (Table S3). While the final assembly was fragmented, as expected
290 for a single Illumina library genome, only 6.4% of mammalian BUSCOs (Benchmarking
291 Universal Single-Copy Orthologs, Simão et al. 2015) were found to be missing. The genome
292 sequence and associated gene annotations can be accessed through NCBI (Bioproject
293 PRJNA512515).

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294 *Phylogenetic Relationships*

295 RAxML and SVDquartets recovered well-supported nDNA clades for *M. simmonsii*, *M.*
296 *macarthurii*, and *M. sp. #3*, the latter two as sister taxa with 100% bootstrap support (Fig. 2;
297 Fig. S2). SNAPP also supported *M. sp. #3* as sister taxon to *M. macarthurii* (referred to as
298 Clade I) and placed *M. lehilahytsara* as sister taxon to *M. mittermeieri* (referred to as Clade II)
299 (Fig. S2). However, *M. lehilahytsara* was not monophyletic in RAxML analyses of nDNA
300 (Fig. 2C) or mtDNA (Fig. 2A), and a SVDquartets analysis of nDNA placed one *M.*
301 *lehilahytsara* individual from Ambavala as sister to all other *M. lehilahytsara* and *M.*
302 *mittermeieri*, and only weakly supported a monophyletic *M. mittermeieri* (Fig. S2A).

303 Although mtDNA analyses placed several individuals from Anjiahely in a well-supported
304 clade with *M. sp. #3*, individuals from Ambavala (see Fig. 1), Mananara-Nord NP, and
305 Antanambe (Fig. 2A; see lower gray box), nuclear RADseq data placed them unambiguously
306 within the *M. macarthurii* clade (Fig. 2B, C). This suggests that individuals from Anjiahely are
307 in fact *M. macarthurii*, but carry two divergent mtDNA lineages, and that true *M. sp. #3* are only
308 found between Ambavala and Antanambe (Fig. 1). The cause of this mitonuclear discordance
309 for *macarthurii* in Anjiahely was investigated further (see the section “Interspecific Gene
310 Flow”).

311 *Species Delimitation*

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312 *Genetic structure.* — A PCA with both pairs of sister lineages (Clade I: *M. macarthurii* and
313 *M. sp. #3*; Clade II: *M. mittermeieri* and *M. lehilahytsara*) distinguished the two clades along
314 PC1, and distinguished *M. macarthurii* and *M. sp. #3* along PC2 (Fig. 3B). When restricting
315 clustering analyses to Clade I, $K = 2$ was the best-supported number of clusters with both
316 approaches, distinguishing *M. macarthurii* and *M. sp. #3* (Fig. S5; Fig. S7B). At $K = 3$, *M.*
317 *sp. #3* was divided into two clusters with individuals from Mananara-Nord NP and Antanambe
318 separated from Ambavala individuals (Fig. S7B, Fig. S10). A separate PCA analysis for
319 Clade I also distinguished these two groups along PC2 (Fig. 3C), which we hereafter refer to
320 as “southern *M. sp. #3*” (Mananara-Nord NP and Antanambe are south of the Mananara river)
321 and “northern *M. sp. #3*” (Ambavala is north of the river, and 24.0 km from Mananara-Nord NP
322 and 35.2 km from Antanambe; Fig. 1), respectively. When restricting clustering analyses to
323 Clade II, ADMIXTURE and ngsAdmix suggested optimal values of 1 and 2, respectively; at $K=2$,
324 *M. mittermeieri* and *M. lehilahytsara* were largely but not entirely separated by both approaches
325 (Fig. S5, Fig. S7C, Fig. S11). A PCA distinguished *M. mittermeieri* and *M. lehilahytsara*
326 along PC1 but with little separation (Fig. 3D).

327 *SNAPP and BPP.* — SNAPP Bayes factors strongly favored splitting Clade I into two
328 species ($2\ln\text{BF} = 17,304$ and $34,326$ for two different datasets, Table S6), as well as splitting
329 Clade II, although with a smaller difference in marginal likelihood scores ($2\ln\text{BF} = 1,828$ and

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330 993). All putative species assignments were recovered by the guided delimitation analysis with
331 BPP (Fig. S12).

332 *Genealogical divergence index (gdi)*. — For the Clade I sister pair, *gdi* was 0.727 (95%
333 HPD: 0.718-0.737) from the perspective of *M. macarthurii* (i.e. above the upper threshold for
334 species delimitation), and 0.500 (0.488-0.511) from the perspective of *M. sp. #3* (i.e. in the upper
335 ambiguous zone for species delimitation; Fig. 3A). In contrast, *gdi* values for the Clade II
336 putative species pair were much lower and even below the lower threshold for species
337 delimitation: 0.080 (0.074-0.086) from the perspective of *M. lehilahytsara*, and 0.193 (0.187-
338 0.201) from the perspective of *M. mittermeieri* (Fig. 3A).

339 *Isolation-by-distance (IBD)*. — While comparisons within and between lineages appeared
340 to follow a single isolation-by-distance pattern for *M. mittermeieri* and *M. lehilahytsara* (Clade
341 II, $r=0.693$, $p=0.002$, Fig. 4B), comparisons within versus between lineages differed strongly
342 for *M. macarthurii* and *M. sp. #3* (Clade I, Fig. 4A). Specifically, genetic distances between *M.*
343 *macarthurii* and *M. sp. #3* were much larger than within lineages and were also much larger than
344 between *M. mittermeieri* and *M. lehilahytsara*, despite similar geographic distances.

345 *Interspecific gene flow*. — G-PhoCS inferred high levels of gene flow in Clade II, from *M.*
346 *mittermeieri* to *M. lehilahytsara* [population migration rate ($2Nm$) = 1.59 (95% HPD: 1.50-1.68),
347 migrants per generation: 0.18% (95% HPD: 0.09-0.27%)], and much lower levels of gene flow in

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348 Clade I, from *M. sp. #3* to *macarthurii* [$2Nm = 0.08$ (95% HPD: 0.07-0.09), migrants per
349 generation: 0.10% (0.05-0.15%)] (Fig. 5C). G-PhoCS also inferred low levels of gene flow
350 between the two clades, most likely between ancestral populations, but the timing and direction
351 of gene flow could not be determined (Supplementary Results; Fig. S13), and D-statistics
352 testing for gene flow between the clades were not significant (Fig. S14).

353 We further investigated gene flow between *M. sp. #3* and *M. macarthurii* by taking the
354 strong population structure within *M. sp. #3* into account. D-statistics suggested that northern *M.*
355 *sp. #3* and *M. macarthurii* with “*M. sp. #3*-type” mtDNA share a slight excess of derived alleles
356 in relation to southern *M. sp. #3*, significantly deviating from 0, which indicates gene flow
357 (Fig. S15A). Using a G-PhoCS model with separate northern and southern groups for *M. sp.*
358 *#3*, we found that (1) gene flow with *M. macarthurii* took place before and after the onset of
359 divergence between northern and southern *M. sp. #3*, (2) gene flow between extant lineages
360 occurred or occurs only between northern (and not southern) *M. sp. #3* and *M. macarthurii*, and
361 (3) gene flow is asymmetric, predominantly into *M. macarthurii* (Fig. S15B).

362 *Divergence Times*

363 We estimated divergence times under the MSC model using BPP and G-PhoCS both with
364 and without interspecific gene flow (Fig. 5; Fig. S17). Results were similar across these
365 approaches, with the exception of divergence times between sister lineages in G-PhoCS models

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366 with versus without gene flow (Fig. 5). Specifically, the divergence time between *M. sp. #3*
367 and *M. macarthurii* (Clade I) without gene flow was estimated at 115 kya (95% HPD range: 52-
368 190 ka across G-PhoCS and BPP models) (Fig. 5; Fig. S17), but at 193 kya (95% HPD:
369 89-318 ka) when incorporating gene flow (Fig. 5C-D). In Clade II, this difference in
370 estimated divergence times was considerably larger: under an isolation model it was estimated to
371 be 103 kya (95% HPD: 49-171 ka; Fig. 5) and as much as 520 kya (95% HPD: 249-871 ka)
372 when modeled with gene flow (Fig. 5). Deeper nodes were not as strongly affected:
373 divergence time between Clades I and II was estimated at 687 kya (95% HPD: 337-1126 ka)
374 across G-PhoCS and BPP isolation models, and at 796 kya (95% HPD: 360-1311 ka) in a G-
375 PhoCS model with gene flow (Fig. 5D).

376 *Effective Population Sizes*

377 We found large differences in N_e among lineages, with considerably larger N_e for the
378 lineages in Clade II, *M. lehilahytsara* (mean estimate and 95% HPD range across the BPP and G-
379 PhoCS models with and without interspecific gene flow: 159 k; 58-265 k) and *M. mittermeieri*
380 (78 k; 36-140 k), than the lineages in Clade I, *M. sp. #3* (24 k; 12-38 k) and *M. macarthurii* (12
381 k; 5-19 k) (Fig. 5A-C). Wide HPD intervals for *M. mittermeieri* and *lehilahytsara* are due to
382 differences between models with and without gene flow. Using the G-PhoCS model focused on

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383 Clade I, fairly similar effective population sizes were estimated separately for northern (47 k; 17-
384 78 k), southern (23 k; 12-37 k), and ancestral (33 k; 17-53 k) *M. sp. #3* lineages (Fig. S13).

385 Using the whole-genome data for one individual of *M. sp. #3* (from the southern group)
386 and for *M. mittermeieri*, a comparison of MSMC analyses and G-PhoCS models with and without
387 gene flow (Fig. 6) showed highly similar and markedly declining estimates of population
388 sizes towards the present for *M. sp. #3* (Fig. 6A). Estimates for *M. mittermeieri* were more
389 variable across analyses but none showed a consistent decline towards the present (Fig. 6B).

390 DISCUSSION

391 We used an MSC-based framework for genomic species delimitation and identified rapid
392 and recent diversification of mouse lemurs in a relatively small area in northeastern Madagascar.
393 The same region was previously identified to harbor high levels of lemur microendemism and to
394 be vulnerable to the effects of climate change (Brown and Yoder 2015) and anthropogenic habitat
395 alteration (Schüßler et al. 2020), marking it as a region of conservation concern. Species-level
396 divergence was strongly supported for *M. sp. #3* and its sister species *M. macarthurii* (Clade I,
397 Fig. 2), but not for the pair of *M. mittermeieri* and *M. lehilahytsara* (Clade II, Fig. 2),
398 overturning our *a priori* expectation that the latter were distinct species (Olave et al., 2014). We
399 inferred that the focal species all diverged from their common ancestors within the past million
400 years and documented two cases of sympatric occurrence, each with one representative from

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401 Clade I and one from Clade II. The combined findings of recent divergence and sympatric
402 overlap suggest that reproductive isolation can evolve rapidly in mouse lemurs.

403 *Support for Separate Sister Species Differs Sharply Between the Two Clades*

404 Evidence for distinguishing *M. sp. #3* and *M. macarthurii* as separate species was strong
405 and consistent across analyses. They were reciprocally monophyletic across all phylogenetic
406 analyses of RADseq data (Fig. 2C; Fig. S2; Fig. S3), separated unambiguously in
407 clustering and PCA analyses (Fig. 2B; Fig. 3BC; Fig. S4; Fig. S6–S10), were
408 strongly supported as separate lineages using SNAPP Bayes factors (Table 1) and BPP (Fig.
409 S12), and passed the heuristic species delimitation criterion of *gdi* (Fig. 3A). A comparison of
410 genetic and geographic distances moreover showed a clear distinction between intra- and
411 interspecific genetic distances (Fig. 4). Finally, gene flow between these two lineages was
412 estimated to have occurred at very low levels (G-PhoCS migration band = 0.08; Fig. 5c).

413 In contrast, separate species status of *M. lehilahytsara* and *M. mittermeieri* (Clade II) was
414 not supported by comprehensive analysis of the genomic data. These species were paraphyletic
415 in RAxML and SVDquartets analyses (Fig. 2A,C; Fig. S2) and not as clearly separated in
416 clustering and PCA analyses (Fig. 2B; Fig. 3BD; Fig. S5, S7–S9; Fig. S10).

417 Although the Bayes factor support from SNAPP was strong by standard guidelines (Kass and
418 Raftery 1995), the evidence was much weaker relative to species in Clade I and decreased when

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419 more individuals were included (Table S6). It is unsurprising that Bayes factors will support
420 splitting lineages with genetic structure (Sukumaran and Knowles 2017; Leaché et al. 2019) even
421 with low levels of gene flow (Barley et al. 2018). Therefore, standard guidelines for interpreting
422 Bayes factors may be of limited value for delimiting species, as informed by the lack of
423 monophyly, high levels of inferred gene flow, and failure of additional delimitation tests
424 observed here. Guided delimitation also separated *M. lehilahytsara* and *M. mittermeieri* (Fig.
425 S11), but similar criticisms of oversplitting (e.g. Barley et al. 2018) lead us to not interpret MSC
426 delimitation results as evidence of species status. Most strikingly, reciprocal *gdi* statistics for
427 Clade II were <0.2 , thus falling in the range suggested to unambiguously indicate a single
428 species (Jackson et al. 2017; Leaché et al. 2019; Fig. 3A). Finally, comparing genetic and
429 geographic distances within Clade II showed that a single isolation-by-distance pattern fits both
430 intra- and interspecific comparisons (Fig. 4). While the range of *M. lehilahytsara* expands
431 considerably further south than the populations examined here, our results strongly suggest that
432 *M. mittermeieri* and *M. lehilahytsara* are best considered a single species. Sampling gaps are
433 expected to cause false positive species delimitations rather than false negatives (Barley et al.
434 2018; Chambers and Hillis 2020; Mason et al. 2020), thus indicating that additional sampling of
435 *M. lehilahytsara* populations farther south should not affect our conclusions regarding genetic
436 continuity. As such, and given that the original description of *M. lehilahytsara* precedes that of
437 *M. mittermeieri*, we recommend that *M. mittermeieri* be synonymized as *M. lehilahytsara*.

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438 *Mitonuclear Discordance and Gene Flow*

439 Mitonuclear discordance was observed for a subset of *M. macarthurii* individuals from
440 Anjiahely. These individuals carried mtDNA similar to that of *M. sp. #3* (see Radespiel et al.
441 2008) but had nDNA indistinguishable from sympatric *M. macarthurii*. Although genealogical
442 discordance could be due to incomplete lineage sorting (e.g., Heckman et al. 2007; Weisrock et
443 al. 2010), mitochondrial introgression is supported by D-statistics (Fig. S15) and the inferred
444 low levels of gene flow from the northern *M. sp. #3* population into *M. macarthurii* by G-PhoCS
445 (Fig. S13). Besides a possible case in Sgarlata et al. (2019), mitochondrial introgression has
446 not previously been reported in mouse lemurs. We conclude that, somewhat curiously, the
447 discovery of a divergent mtDNA lineage at Anjiahely (Radespiel et al. 2008) which prompted the
448 current work was apparently the result of mtDNA introgression from an undescribed species into
449 its sister species.

450 *Population Size and Species Delimitation*

451 The comparison of effective population sizes in Clades I and II revealed marked
452 differences, which can affect species delimitation tests such as *gdi* (Leaché et al., 2019). The *gdi*
453 is calculated using population sizes and divergence times estimated under models with no gene
454 flow, and since divergence time estimates in these models were highly similar in both clades
455 (Fig. 5), differences in effective population sizes also appear to play a role in the stark
456 difference in *gdi*. Indeed, *gdi* aims to quantify the probability that two sequences from the focal

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457 taxon coalesce more recently than the divergence time between the taxa, and larger effective
458 population sizes result in slower sorting of ancestral polymorphisms (Maddison 1997).

459 Assessing “progress” in speciation by quantifying rates of neutral coalescence, however,
460 implies that the magnitude of genetic drift is a good predictor of species limits. At least when
461 considering reproductive isolation (i.e., biological species), this can be problematic, given that
462 the role of drift in speciation is generally thought to be small (Rice and Hostert 1993; Czekanski-
463 Moir and Rundell 2019; but see Uyeda et al. 2009). Therefore, additional measures of divergence
464 should be taken into account, including those that do not depend on population size, such as rates
465 of gene flow and divergence time itself (Yang and Rannala 2010; Leaché et al. 2019).

466 *Sympatric Occurrence and the Tempo of Speciation in Mouse Lemurs*

467 Sympatric *Microcebus* species were found at two study sites, with a representative of
468 each of the two focal clades in Anjiahely (*M. macarthurii* and *M. mittermeieri*) as well as in
469 Ambavala (*M. sp. #3* and *M. lehilahytsara*; Fig. 1). These cases of sympatric occurrence, with
470 no evidence for recent admixture, imply that the two clades are reproductively isolated. Though
471 our methods cannot address the mechanisms underlying reproductive isolation, possible barriers
472 include male advertisement calls, which tend to differ strongly among species (Braune et al.
473 2008), and timing of reproduction, which has previously been found to differ among sympatric
474 mouse lemur species (Schmelting et al. 2000; Evasoa et al. 2018) including the focal species

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475 (Schüßler et al., in revision). Only six other cases of sympatry among mouse lemur species are
476 known, five of which include *M. murinus* as one of the co-occurring species (Radespiel 2016;
477 Sgarlata et al. 2019).

478 Given that the sympatrically occurring species were estimated to have had a common
479 ancestor as recently as ~700-800 kya (i.e., the divergence time between Clade I and Clade II, see
480 Fig. 5), this suggests rapid evolution of reproductive isolation and a short time to sympatry
481 among mouse lemurs. By comparison, Pigot & Tobias (2015) estimated that after 5 Ma of
482 divergence, only 21–23% of primate species have attained sympatry. In fact, the one sympatric
483 pair within their dataset of 74 sister species pairs younger than 2.5 million years consisted of
484 *Galago gallarum* and *G. senegalensis* (Pigot and Tobias 2015), which are also Strepsirrhini.
485 Moreover, Curnoe et al. (2006) compiled data for naturally hybridizing primate species, and
486 found the median estimated divergence time to be 2.9 Ma. More broadly, primate speciation rates
487 do not appear to be lower than those for other mammals or even vertebrates (Curnoe et al. 2006,
488 Upham et al. 2019). It should be noted, however, that the temporal estimates reported in our
489 study are based on MSC analyses using mutation rates estimated from pedigree studies, whereas
490 dates for other primate clades were largely calculated from fossil-calibrated relaxed clock
491 methods.

492 *Complexities of Divergence Time Estimates*

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493 There are two noteworthy discrepancies in divergence time estimates highlighted by this
494 study. First, the age estimate between the *M. mittermeieri* and *M. lehilahytsara* lineages
495 increased from approximately 100 kya (Fig. 5b) to more than 500 kya (Fig. 5c) when the
496 MSC model allowed for gene flow. The substantial effect of incorporating or disregarding gene
497 flow on divergence time estimation has been previously noted (Leaché et al. 2014; Tseng et al.
498 2014) and we here reiterate its significance. Second, the coalescent-based estimates of
499 divergence times presented here differ drastically from estimates based on fossil-calibrated
500 relaxed-clock methods. In the present study, we estimated the mean age of the most recent
501 common ancestor (MRCA) of mouse lemurs to be under 1.5 Ma, with the highest upper bound of
502 95% HPDs across models at 2.40 Ma. This age estimate is in stark contrast to previous fossil-
503 calibrated estimates of 8 - 10 Ma (Yang and Yoder 2003; dos Reis et al. 2018).

504 Several factors likely contribute to this large difference. First, the MSC estimate uses a *de*
505 *novo* mutation rate sampled from a distribution based on available pedigree-based mutation rates
506 in primates, including mouse lemurs (Campbell et al., 2019). This rate is nearly two-fold higher
507 than the estimated substitution rate for *M. murinus* (dos Reis et al., 2018). Second, converting
508 coalescent units to absolute time also requires a generation time estimate. We attempted to
509 account for uncertainty in generation time by similarly drawing from a distribution based on
510 empirical parent age estimates (Zohdy et al., 2014; Radespiel et al., 2019) in mouse lemurs.
511 Thus, either overestimation of the mutation rate and/or underestimation of the generation time

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512 would lead to divergence time estimates that are too recent. However, theoretical considerations
513 suggest that instead, mouse lemur divergence time estimates from fossil-calibrated clock models
514 are too old.

515 When incomplete lineage sorting is common, clock models that assume a single topology
516 underlies all loci can overestimate species divergences compared to MSC estimates that allow
517 gene trees to vary (Stange et al., 2018; Feng et al., 2020). This is likely to apply to mouse lemurs
518 given that high levels of incomplete lineage sorting have been previously documented (Heckman
519 et al., 2007; Weisrock et al., 2010; Hotaling et al., 2016). Moreover, due to the absence of a post-
520 K-Pg terrestrial fossil record for Madagascar, clock-model estimates of divergence times in
521 mouse lemurs have relied on fossil calibrations from the distantly related African sister lineage of
522 lemurs, the Lorisiformes (Seiffert et al., 2003), as well as from anthropoid primates and other
523 mammals. This scenario - estimation of divergence times for younger, internal nodes with
524 calibrations placed on much older nodes - should lead to overestimation of divergence times
525 (Angelis and dos Reis 2015). Therefore, it is likely that divergence times between mouse lemur
526 species have been overestimated by previous studies with fossil-calibrated clock models (e.g.
527 Yang and Yoder 2003; dos Reis et al., 2018), and we suggest that the mutation rate-calibrated
528 MSC divergence times presented here are more accurate.

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529 Our estimates of divergence times imply that the entire mouse lemur radiation originated in
530 the Pleistocene, in turn suggesting that Pleistocene climatic oscillations represent a likely factor
531 leading to geographic isolation and subsequent genetic divergence. Periods of drought during
532 glacial maxima are hypothesized to have caused dramatic contraction of forest habitats (Burney
533 et al. 1997; Gasse and Van Campo 2001; Wilmé et al. 2006; Kiage and Liu 2016) and to isolation
534 of previously connected populations. Notably, the patterns of differentiation observed in this
535 study are consistent with the predictions of Wilmé et al. (2006) wherein Madagascar's river
536 drainage systems created high-elevation retreat-dispersal corridors during periods of climatic
537 oscillation. That is, whereas the lineages in Clade I (highly differentiated and low N_e) appear to
538 occur only in lowland forests, those in the Clade II (poorly differentiated and high N_e) occur at
539 both higher and lower elevations (Schüßler et al., in revision). Moreover, the Mananara river
540 runs between the fairly distinct northern and southern populations of *M. sp. #3*, further
541 emphasizing the potential of large rivers to act as phylogeographic barriers in lemurs (Martin
542 1972; Pastorini et al. 2003; Goodman and Ganzhorn 2004; Olivieri et al. 2007).

543 *Population Size Dynamics*

544 A long-term decline in population size was inferred for the lineage leading to *M. sp. #3*.
545 While changes in inferred N_e may be confounded by changes in population structure – especially
546 for single-population sequential Markovian coalescent (PSMC/MSMC) models that do not
547 explicitly consider population subdivision (Mazet et al. 2016; Chikhi et al. 2018) – we recovered

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548 similar results with both MSMC and G-PhoCS analyses (Fig. 6A). This congruence is especially
549 persuasive given the underlying differences between the G-PhoCS and MSMC models and their
550 input data. Moreover, Markovian coalescent approaches are robust to genome assembly quality
551 (Patton et al. 2019), yielding further confidence in the results. The inferred decline and
552 population subdivision of *M. sp. #3* was initiated long before anthropogenic land use, supporting
553 the emerging consensus that human colonization in Madagascar alone does not explain the
554 occurrence of open habitats and isolated forest fragments (Quéméré et al. 2012; Vorontsova et al.
555 2016; Yoder et al. 2016; Salmona et al. 2017, 2020; Hackel et al. 2018). Conversely, results for
556 the *M. mittermeieri* lineage do not indicate a declining N_e through time (Fig. 6B). This latter
557 result may well be a simple corollary of the evidence described above, that this lineage is part of
558 a single species complex represented by Clade II and thus occurs at both higher and lower
559 elevations in northeastern Madagascar.

560 *Conclusions*

561 We have shown that substantial mouse lemur diversity exists within a 130-km-wide stretch
562 in northeastern Madagascar, including two instances of sympatric occurrence between
563 representatives of two closely related clades. Within one of these clades, our comprehensive
564 approach that uses a variety of genomic analyses indicates that the undescribed lineage *M. sp. #3*
565 represents a distinct species, while the two named species in the other clade, *M. mittermeieri* and
566 *M. lehilahytsara*, are better considered a single, widespread species with significant population

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567 structure. Given that the original description of *M. lehilahytsara* precedes that of *M.*
568 *mittermeieri*, primate taxonomists should synonymize the two as *M. lehilahytsara*.

569 The divergence times calculated here using pedigree-based mutation rate estimates with
570 the MSC are much younger than those of previous studies that used external fossil-based
571 calibrations with relaxed-clock methods. The younger dates suggest rapid evolution of
572 reproductive isolation in mouse lemurs as well as a Pleistocene origin of the radiation, likely
573 following population isolation due to climatic oscillations. This departure from previous
574 hypotheses of mouse lemur antiquity emphasizes the need for future studies focused on resolving
575 discrepancies in divergence time estimates, both in mouse lemurs and in other recently evolved
576 organismal groups for which such comparisons have yet to be made.

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