RADseq data reveal a lack of admixture in a mouse lemur contact zone contrary to previous microsatellite results

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Microsatellites have been a workhorse of evolutionary genetic studies for decades and are still commonly in use for estimating signatures of genetic diversity at the population and species level across a multitude of taxa. Yet, the very high mutation rate of these loci is a double-edged sword, conferring great sensitivity at shallow levels of analysis (e.g. paternity analysis) but yielding considerable uncertainty for deeper evolutionary comparisons. For the present study, we used reduced representation genome-wide data (restriction site-associated DNA sequencing (RADseq)) to test for patterns of interspecific hybridization previously characterized using microsatellite data in a contact zone between two closely related mouse lemur species in Madagascar (Microcebus murinus and Microcebus griseorufus). We revisit this system by examining populations in, near, and far from the contact zone, including many of the same individuals that had previously been identified as hybrids with microsatellite data. Surprisingly, we find no evidence for admixed nuclear ancestry. Instead, re-analyses of microsatellite data and simulations suggest that previously inferred hybrids were false positives and that the program NEWHYBRIDS can be particularly sensitive to erroneously inferring hybrid ancestry. Combined with results from coalescent-based analyses and evidence for local syntopic co-occurrence, we conclude that the two mouse lemur species are in fact completely reproductively isolated, thus providing a new understanding of the evolutionary rate whereby reproductive isolation can be achieved in a primate.

1. Introduction

Microsatellites are tandem repeats of repetitive DNA that typically range in length from one to six nucleotides and occur at thousands of locations within the genomes of most organisms [1,2]. Individual microsatellite loci contain from as few as five to as many as 40 or more repeats, with copy number changes caused by slip-strand mispairing during DNA replication. Mutation rates for microsatellites are orders of magnitude higher than for other types of variants, including single nucleotide polymorphisms (SNPs), with the overall rate being...
a balance between the generation of replication errors and the correction of errors by proofreading and mismatch repair, all of which can vary by species [3]. Given their high rate of change, microsatellite loci have high allelic richness, often in excess of 10 alleles within humans and other primates [4]. This rich allelic diversity, combined with relatively low genotyping costs, have made microsatellites a popular genetic marker for applications ranging from paternity analysis to historical demography. In particular, they have proved useful for identifying conservation units in endangered species (e.g. [5]) as well as for revealing the presence of homoploid hybrid speciation (e.g. [6]).

Yet, their extreme sensitivity can also be cause for concern. The high rate of recurrent mutations (i.e. homoplasy) makes them poor indicators of long-term population history [2,4]. For example, the combination of homoplasy and potentially inappropriate models of mutational dynamics can yield highly inflated estimates of gene flow between populations and species [7,8]. Thus, inferences above all but the shallowest evolutionary levels should be treated with caution.

In this study, we revisit hypotheses of hybridization between two named species of mouse lemur, Microcebus murinus (sensu lato) and Microcebus griseorufus, reported from previous studies using microsatellite data [9–11]. These previous studies focused on two contact zones in the southeast of Madagascar wherein hybrids were reported to occur.

To date, seven different pairs of mouse lemur species have been shown to co-occur locally at various localities throughout Madagascar. One widespread species, *M. murinus*, is involved in five of these cases. In all but one of these seven cases of sympatry, no hybridization has been detected thus suggesting that co-occurring species are reproductively isolated. Sources of reproductive isolation among sympatric mouse lemurs are poorly known, but factors that may contribute to prezygotic isolation via differential mate choice may include divergence in acoustic [12,13] and olfactory signalling [14,15]. Additionally, opportunities for reproductive interaction may be reduced by ecological divergence manifesting, for example, in differential timing of the highly seasonal and temporally constrained reproductive season seen in mouse lemurs [16–18].

It is thus intriguing that hybridization has only been detected between *M. murinus* and *M. griseorufus*, using microsatellite loci [9,10], which is also unique among the seven cases of sympatry in consisting of a pair of sister lineages. Using the programs Structure [19] and GeneClass [20], Gligor et al. [9, p. 529] concluded that ‘most individuals within the transition zone’ had mixed ancestry (no individual-level assignments were made). Hapke et al. [10] studied a contact zone 40 km further north, and used the same set of microsatellite loci for a total of 159 mouse lemurs, with Structure and NewHybrids [21] identifying a total of 18 admixed individuals. Of these, 15 individuals showed signs of nuclear admixture (i.e. among microsatellites) whereas three had a mismatch between microsatellite and mitochondrial ancestry.

Here, we use restriction-site associated DNA sequencing (RADseq) data to revisit the contact zone area studied by Hapke et al. [10] and follow-up work in Lüdemann [11] detected hybridization between *M. murinus* (hereafter referred to as *murinus*) and *M. griseorufus* (hereafter referred to as *griseorufus*) using nine microsatellites and a fragment of the HV1 mitochondrial locus from individuals in the Andohahela area in southeastern Madagascar. We made use of a selection of 94 of their samples and augmented this dataset with 33 samples from distant, allopatric sites, and three Microcebus rufus samples that were used as an outgroup (electronic supplementary material, table S1, table S2).

At two of the sites examined by Hapke et al. [10], they detected unadmixed individuals of both parental species as well as individuals with admixed ancestry (individuals inferred to be admixed by Hapke et al. [10] and Lüdemann [11] are hereafter referred to as ‘putative hybrids’). From these two contact zone sites, Mangatsiaka and Tsimelahy, which we refer to as ‘sympatic’ sites, we selected 78 samples (electronic supplementary material, table S1), including 15 individuals for which Hapke et al. [10] or Lüdemann [11] had detected nuclear admixture, and an additional three with a mitonuclear ancestry mismatch. We additionally selected samples from nearby sites at which Hapke et al. [10] had exclusively (or nearly so) detected unadmixed individuals of only one of the two species: eight *griseorufus* from Hazofotsy and eight *murinus* from Ambatoabo (electronic supplementary material, table S1). We refer to these contact zone sites as ‘parapatric’ sites. ‘Allopatric’ samples, taken well away from the contact zone, were represented by 14 *griseorufus*, eight *murinus* and 11 *Microcebus ganzhorni*, a species that was recently split from *murinus* [23], from Mandena in far southeastern Madagascar (electronic supplementary material, table S2, figure 1). Below, we show that *M. ganzhorni* diverged very recently from the Andohahela area *murinus* populations, while a much deeper split occurs between western and other southeastern Madagascar populations, all of which continue to be classified as *murinus*. Therefore, we here include *M. ganzhorni* under the nomer ‘*M. murinus s.l.*’.

We used the following geographically defined population groupings for analyses where individuals are assigned to predefined groups (figure 1): western *griseorufus* (abbreviated ‘gri-W’), central/contact zone area *griseorufus* (abbreviated ‘gri-C’), western *murinus* (abbreviated ‘mur-W’), central/contact zone area *murinus* (abbreviated ‘mur-C’) and eastern *murinus* s.l. (abbreviated ‘mur-E’; this population corresponds to *M. ganzhorni sensu* Hotaling et al. [23]).

### 2. Methods

#### (a) Sampling

Hapke et al. [10] and follow-up work in Lüdemann [11] detected hybridization between *M. murinus* (hereafter referred to as *murinus*) and *M. griseorufus* (hereafter referred to as *griseorufus*) using nine microsatellites and a fragment of the HV1 mitochondrial locus from individuals in the Andohahela area in southeastern Madagascar. We made use of a selection of 94 of their samples and augmented this dataset with 33 samples from distant, allopatric sites, and three Microcebus rufus samples that were used as an outgroup (electronic supplementary material, table S1, table S2).

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#### (b) Sequencing and genotyping

We prepared RADseq libraries following the protocol of Ali et al. [24]. Libraries were sequenced using paired-end 150 bp
sequencing on an Illumina HiSeq 4000 at Duke University’s Center for Genomic and Computational Biology sequencing facility.

After read flipping, demultiplexing, trimming and mapping to the *M. murinus* reference genome (‘Mmurinus 3.0’, [25]), we performed genotype calling with GATK v. 4.0.7.0 [26], and we filtered SNPs and individuals largely according to the ‘FS6’ filter of O’Leary et al. [27] (see the electronic supplementary material for details).

For the set of individuals from the contact zone area, we additionally produced two datasets using more lenient filtering procedures to be able to examine admixture using more individuals and SNPs: (i) a dataset produced by omitting the last round of removal of SNPs and individuals based on missing data; and (ii) a dataset produced using the FS6 filter without the individual-filtering steps that retained two additional putative hybrids and two individuals with mitonuclear discordance.

Based on GATK-called genotypes, we also produced full-sequence FASTA files for each RAD locus (see the electronic supplementary material for details).

(c) Detection of hybrids using clustering approaches

For the detection of admixed individuals, we used complementary model-free and model-based approaches. First, we used principal component analysis (PCA) as implemented in the SNPRelate R package v. 1.17.2 [28], using the snpgdsPCA() function. Second, we used the program ADMIXTURE v. 1.3.0 [29] to detect clusters and assign individual-level ancestry proportions from each cluster. Third, we used the program NEWHYBRIDS
among populations.

W, (F1 x unadmixed individuals. Second, we used TREEMIX v. 1.13 [33] to estimate tree and thus shows phylogenetic relationships while also allowing population migration, was continued for F2 and backcross individuals. An average of five admixed alleles per locus was drawn from an unadmixed individual of each species. The procedure, using data from the appropriate population, was continued for F2 and backcross individuals. In total, 60 simulated individuals were created: 20 each of six predefined categories: griseorufus, murinus, F1 hybrid (griseorufus x murinus), F2 hybrid (F1 x F1), griseorufus backcross (F1 x griseorufus) and murinus backcross (F1 x murinus). Five hundred thousand iterations were used as burn-in, with another 1500000 iterations after that, using Jaffereys-like priors. A run was considered successful if it passed a test for convergence implemented in the hybrid-detective R package [30].

(d) Reanalysis of microsatellite data
We reanalysed the Hapke et al. [10] and Lüdemann [11] microsatellite data using only the samples included in this study. Like in Hapke et al. [10], we used the Bayesian classification methods STRUCTURE v. 2.3.4 [19]; see the electronic supplementary material for details) and NEWHYBRIDS v. 1.1 to detect hybrids. For STRUCTURE, 20 runs using K = 2 were used to calculate the average membership coefficients by creating an optimal alignment using the full-search algorithm implemented in CLUMPP v. 1.1.2 [31]. To keep the results directly comparable with Hapke et al. [10], we used the same threshold for the detection of hybrids: a sample was considered a hybrid when the posterior probability for assignment to the species of their mitochondrial haplotype was ≤0.9 for STRUCTURE or ≤0.5 in NEWHYBRIDS, and part of a specific hybrid category when the corresponding probability was greater than 0.5.

(e) Comparison of microsatellites and single nucleotide polymorphisms using simulations
Using simulations, we compared the performance of microsatellites and SNPs for detecting hybrids. The hybrid-detective R package [30] was used to generate multi-generational hybrids from both the microsatellite and SNP data. First, unadmixed murinus and griseorufus individuals were created by randomly drawing two alleles per locus from the allopatric reference populations, without replacement. For subsequent F1 samples, one allele per locus was drawn from an unadmixed individual of each species. This procedure, drawing from the appropriate population, was continued for F2 and backcross individuals. In total, 60 simulated individuals were created: 20 each of unadmixed griseorufus and murinus, and five each of F1, F2, F1 x unadmixed griseorufus, and F1 x unadmixed griseorufus. Ancestry assignment was compared between microsatellites and SNPs by running STRUCTURE and NEWHYBRIDS, as described above, on the simulated genotypes.

(f) Phylogenetic inference
To enable subsequent tests of gene flow and demographic modelling, we determined relationships among all murinus s.l. and griseorufus individuals sampled by our study, using three M. rufus individuals as an outgroup. First, we used the NeighborNet method implemented in SPLITSTREE v. 4.14.4 [32]. This method visually displays phylogenetic conflict in an unrooted tree and thus shows phylogenetic relationships while also allowing for the detection of potentially admixed populations and individuals. Second, we used TreeMix v. 1.13 [33] to estimate relationships among predefined populations (gri-W, gri-C, mur-W, mur-C and mur-E) both with and without admixture events among populations.

(g) Formal admixture statistics
The D-statistic and related formal statistics for admixture use phylogenetic invariants to infer post-divergence gene flow between non-sister populations. We used the qpDstat and F4RatioTest programs of ADMIXTOOLS v. 4.1 [34] to compute four-taxon D-statistics and f4-ratio tests, respectively, to test for gene flow among the predefined mouse lemur populations. For all tests, M. rufus was used as the outgroup. Significance of D-values was determined using the default Z-value reported by qpDstat, which uses weighted block jackknifing.

(h) Demographic modelling
We ran the coalescent-based approaches implemented in G-PHOCS v. 1.3 [35] and BPP v. 4.2 [36], using Markov chain Monte Carlo to jointly infer population sizes, divergence times and migration rates for the three murinus populations (mur-W, mur-C and mur-SE) and the two griseorufus populations (gri-W and gri-SE). While G-PHOCS implements an isolation-with-migration model with continuous gene flow during potentially long periods, the multispecies-coalescent-with-introgression model in BPP models discrete introgression events. As input for G-PHOCS and BPP, we created full-sequence FASTA files with loci for three individuals per population based on the GATK genotypes (see the electronic supplementary material for details).

We converted the migration rate parameter m to the population migration rate (2Nm), which is the number of haploid genomes (i.e. twice the number of migrants) in the source population that arrive each generation by migration from the target population. Divergence times, population sizes and the proportion of migrants per generation (m × μ) were converted using empirical estimates of the mutation rate (1.52 × 10−8, [37]) and generation time. For the generation time, we used a log-normal distribution with a mean of ln(3.5) and a standard deviation of ln(1.6) based on two available estimates for Microcebus (4.5 years from [38] and 2.5 years from [39]).

3. Results
(a) Genotyping
GATK genotyping followed by the standard (FS6) filtering procedure for all individuals resulted in a VCF file with 83 individuals and 60 460 SNPs. The equivalent VCF file with only samples from sympatric and parapatric sites in the contact zone area (Andahohela area, figure 1) contained 69 individuals, 12 of which were putative hybrids, and 7180 SNPs. The two less stringent filtering procedures (see Methods) for the contact zone set resulted in the retention of 78 individuals (13 putative hybrids) and 48 556 SNPs, and 79 individuals (18 putative hybrids) and 1360 SNPs, respectively. Sixteen individuals, among which two putative hybrids, did not survive the filtering steps for any of the final VCF files. The full-sequence FASTA file produced for G-PHOCS analyses contained 12 952 loci with an average length of 475 bp. For a comparison of quality control and filtering statistics among populations, see the electronic supplementary material.

(b) No evidence for ongoing hybridization in the contact zone
admixture identified K = 2 as the optimal number of clusters among individuals from the contact zone area (figure 2a - top). All individuals, including the 12 putative hybrids that passed filtering, were entirely assigned to one of the two clusters (figure 2a - bottom), with no signs of admixture. Results were also plotted for K = 3, for which a third cluster corresponded to differentiation between sympatric (Mangatsiaka,
Tsimelahy) and parapatric (Hazofotsy) sites in *griseorufus* (electronic supplementary material, figure S11). PCA with individuals from the contact zone revealed a wide separation between two groups along the first principal component axis (PC1), which explained around tenfold more of the variation compared to PC2. The separation along PC1 corresponded to differentiation between *griseorufus* and *murinus*, and importantly, all putative hybrids fell within one of those two groups, with none occupying an intermediate position (figure 2b). Similar to the *Admixture* results at K = 3, PC2 mostly corresponded to differentiation between sympatric and parapatric sites in *griseorufus* (see also the electronic supplementary material, figure S12 for a within-species PCA).

*NewHybrids* was run with and without assigning individuals from the parapatric populations to reference parental species, and in both cases, all individuals were assigned to one of the two parental species and none were assigned to one of the hybrid categories. Assignment to species matched perfectly with *Admixture* assignments and PCA results.

Datasets produced by less stringent filtering procedures included an additional four putative hybrids that did not pass all filtering steps but could still be assessed using a more limited number of SNPs (electronic supplementary material, figure S13). *Admixture* and *NewHybrids* analyses of these datasets similarly showed no evidence for admixed individuals with the exception of mitonuclear discordance: for two of the individuals for which Lüdemann [11] had detected *griseorufus* ancestry in nuclear DNA but *murinus* mitochondrial DNA haplotypes mitonuclear discordance, we could confirm that the nuclear DNA has pure *griseorufus* ancestry (electronic supplementary material, figure S13). The third sample for which Lüdemann [11] detected mitonuclear discordance did not pass filtering at all. No other cases of mitonuclear discordance were found (figure 2a; electronic supplementary material, table S1.)
In a reanalysis of the Hapke et al. [10] microsatellite data for only the individuals that were included in this study, 11 individuals identified as hybrids in Hapke et al. [10] were no longer identified as such by either NEWHYBRIDS or STRUCTURE. Only a single sample was now identified as a hybrid by NEWHYBRIDS, but STRUCTURE did not support this inference (figure 3a; electronic supplementary material).
figure S14). As noted above, admixture was not detected for any individuals in the RADseq data, including those that had been identified as hybrids in the original microsatellite analyses.

In analyses of simulated microsatellite data, NEWHYBRIDS inferred that 4 out of 40 unadmixed individuals were hybrids, whereas STRUCTURE found no false positives. False negatives occurred with both NEWHYBRIDS (2 out of 20) and STRUCTURE (6 out of 20) for microsatellite data. On the other hand, NEWHYBRIDS and STRUCTURE analyses of simulated RADseq data were 100% accurate in inferring ancestry (figure 3b; electronic supplementary material, figure S15).

(d) Phylogenetic approaches clarify relationships within murinus

A SplitsTree NeighborNet phylogenetic network (electronic supplementary material, figure S16A) of the SNP data showed a very clear separation between griseorufus and murinus with little phylogenetic conflict, and strong intraspecific structure in murinus. All putative hybrids fell squarely within one of the two clades, with individual assignments in perfect agreement with clustering approaches. Similarly, a NeighborNet network using only contact zone individuals showed little to no phylogenetic conflict (electronic supplementary material, figure S17).

TreeMix (electronic supplementary material, figure S16B) was run with murinus and griseorufus individuals assigned to the five populations and M. rufus as the outgroup, and confirmed the relationships within murinus suggested by SplitsTree: mur-W was the most divergent and mur-C and mur-E were sisters. No significant migration edges were found between murinus and griseorufus, with instead several significant edges between M. rufus and griseorufus, and M. rufus and murinus (electronic supplementary material, figure S18). When M. rufus was excluded, significant migration edges between griseorufus and murinus did emerge, but did not include any between contact zone area populations (gri-C and mur-C) (electronic supplementary material, figure S19).

(e) No current—but some ancestral—interspecific gene flow

D-statistics showed an over-representation of shared derived sites between both griseorufus populations (gri-W and gri-C) and the two southeastern murinus populations (mur-C and mur-E; relative to their sister mur-W, western murinus) (electronic supplementary material, figure S20A). Values of D were highly similar regardless of which of the griseorufus or southeastern murinus populations were used, which suggests historical admixture between the ancestral griseorufus and southeastern murinus lineages, as well as a lack of ongoing gene flow in the contact zone. A lack of ongoing gene flow was further supported by values of D very close to (and not significantly different from) zero for comparisons testing for excess derived allele sharing between contact zone populations of both species relative to their sister populations (electronic supplementary material, figure S20A). f2-ratio tests similarly indicated ancestral admixture between griseorufus and the ancestor of contact zone (mur-C) and eastern murinus (mur-E) populations, specifically estimating that after divergence from western murinus, this ancestral southeastern murinus population experienced about 4.0–4.4% admixture with griseorufus (electronic supplementary material, figure S20B).

Demographic modelling using G-PHOCs and BPP supported the presence of non-zero but low levels of historical gene flow between ancestral murinus and griseorufus populations, but a lack of gene flow between extant contact zone area populations of griseorufus and murinus (figure 4a,b).

4. Discussion

We re-examined a contact zone between two species of mouse lemur in southeastern Madagascar, where significant hybridization had previously been reported based primarily on evidence from microsatellite data [10]. With RADseq data, we found no evidence for the presence of admixed individuals, and using simulations and re-analyses of microsatellite data, we showed that previously detected hybrids were probably false positives. By including allopatric populations and performing multispecies coalescent analyses, we furthermore found a general lack of ongoing gene flow, and very low levels of ancestral gene flow, between these two species.

(a) Reconciling the lack of evidence for hybrids with microsatellite results

We found no admixed nuclear ancestry in any of the individuals from the contact zone. Our RADseq data are expected to have high power in species assignment and hybrid detection, given the combination of the relatively high number of genetic markers used [40,41] and the pronounced genetic differentiation between these two species (estimated divergence time in a no-migration scenario: approximately 600 ka ago; figure 4; average Fst in the contact zone area: 0.40; electronic supplementary material, table S5). Furthermore, in a re-analysis of microsatellite data using the same methods as the original studies [10,11], though restricted to the individuals used in this study, all but one of the previously detected hybrids were no longer classified as such (figure 3d).

Considering the clear and robust RADseq results, it is highly unlikely that true hybrids were missed in our analyses. Instead, our results suggest that the hybrids inferred in Hapke et al. [10] were false positives, and more generally, that the inference of hybridization using microsatellites can be sensitive to such false positives, particularly when using the program NEWHYBRIDS.

In our simulations with microsatellites, STRUCTURE suffered from false negatives only, whereas NEWHYBRIDS produced four false positives among 40 simulated unadmixed individuals (figure 3b). Additionally, in our reanalysis of the microsatellite data, the single individual that NEWHYBRIDS continued to assign hybrid ancestry to did not show signs of admixture using STRUCTURE (figure 3a). In Hapke et al. [10], their fig. 5), STRUCTURE did not consistently infer admixed ancestry for several of the putative hybrids. This was especially apparent when parapatric populations were included, in which case only four out of the 12 NEWHYBRIDS positives showed admixed ancestry using STRUCTURE (and three out of those four were still assigned less than 10% admixed ancestry by STRUCTURE, [10], their fig. 5). Even though NEWHYBRIDS appears considerably more prone to false positives than STRUCTURE, the latter did show admixed ancestry for seven individuals in an analysis using only
individuals from the contact zone site Mangatsiaka (versus nine with NEWHYBRIDS). At the same time, both programs had 100% accurate assignments with simulated SNP data, suggesting that the false positives found in the microsatellite analysis stem mostly from challenges with this type of molecular marker, to which NEWHYBRIDS appears to be more sensitive than STRUCTURE.

(b) Evolutionary resolution of microsatellite versus single nucleotide polymorphism data

The results of our simulation analysis suggest that microsatellite data are vulnerable to both false positive and false negative detection of admixture between species. This effect will be especially significant when parental lineages are sufficiently phylogenetically diverged such that the rate of recurrent or backward mutation will obscure the true evolutionary signal [2,4]. To our knowledge, this study is the first to directly compare microsatellite and SNP data in a population genetic analysis within mammals. As reviewed by Sunde et al. [42], such ‘head-to-head’ studies are extremely rare and are presently limited to plants and fishes. Nonetheless, relative strengths and weaknesses of the two data types are emerging. Whereas earlier assessments of microsatellite data posited that their extremely high evolutionary rate would make them ideal for revealing subtle population genetic parameters [4,7], direct comparison with SNP data is showing the opposite to be true. Indeed, these studies

Figure 4. Demographic inferences using G-PHOCS and BPP. (a–c) Summary of results for G-PHOCS models without (a) and with (b) gene flow and for BPP (c; with gene flow). Each box represents an extant (bright colours: gold for griseorufus, purple for murinus) or ancestral (faded colours) lineage, with box width indicating $N_e$ and box height indicating time. Gene flow was estimated reciprocally between three pairs of lineages, as depicted by the arrows (using the same units as panels d and e). (d) Point estimates and 95% highest posterior densities (HPDs) of BPP introgression probabilities ($\phi$). (e) Point estimates and 95% HPDs of G-PHOCS population migration rates (2Nm). (Online version in colour.)
indicate that SNP data are more sensitive across a broad range of evolutionary parameters, including phylogenetic structure, admixture, population subdivision and measures of heterozygosity [42–44]. Recent work is also clarifying the degree to which SNP data are robust to small organismal datasets, even those with as few as \( n = 2 \) [44]. These observations and assessments are further supported by both the simulation and empirical results reported in this study.

(c) Lack of ongoing gene flow and implications for speciation

The presence of at least two individuals with mitonuclear discordance (a *griseorufus*-type mitochondrial haplotype, and *murinus* nuclear DNA) may suggest some ongoing or recent gene flow between the two species. However, consistent with the lack of evidence for nuclear admixture in contact zone sites, we found no evidence for ongoing gene flow using multiple methods, including a phylogenetic network (electronic supplementary material, figure S16A), TreeMix (electronic supplementary material, figure S16B), formal admixture statistics (electronic supplementary material, figure S20) and two multispecies coalescent methods (G-PhoCS and BPP; figure 4f). Combined with syntopic occurrence in at least one of the contact zone sites (figure 2), these findings strongly suggest that *murinus* and *griseorufus* are currently reproductively isolated, which is striking giving the estimated divergence time of less than 1 Myr (see also [45]).

Little is known about the relative importance of different types of reproductive isolation in mouse lemurs. Across their ranges, *murinus* and *griseorufus* occur in distinct habitat types, with *griseorufus* mostly limited to spiny forests that appear to be too arid for *murinus* [46,47]. Separation by habitat (e.g. [48]) at larger scales could therefore minimize or even prevent sympatric co-occurrence despite nominal sympathy in the contact zone area, thus limiting interactions between the species. At one of the two sympatric sites included in this study, Tsimelalhy, species-specific sampling locations are indeed consistent with separation by habitat, but at the other, Mangatsiaka, the two species co-occur even at a very fine spatial scale (figure 4f; see also [45]). Therefore, the observed lack of gene flow is unlikely to simply be a by-product of separation by habitat, and additional sources of pre- and/or postzygotic reproductive isolation need to be invoked.

5. Conclusion

Using RADseq data, we found no evidence for admixture between two species of mouse lemurs in a contact zone in southern Madagascar. This is in sharp contrast to a previous study that found widespread hybridization among the same samples using microsatellites. Our results suggest that the hybrids inferred by the previous study were probably false positives, and we urge caution when using microsatellites to infer hybridization. Thus, our results support concerns around the usage of microsatellites—most importantly, that rates of evolution in microsatellites are simply too high for use at interspecific levels given their propensity for homoplasy beyond the intrapopulation level [7,49]. Finally, we estimate a divergence time of less than 1 Myr and a lack of historical gene flow, which in combination with local syntopic occurrence and no evidence for admixture, suggests the rapid development of reproductive isolation between these species.

Ethics. Field work has been carried out under the Accord de Collaboration between Madagascar National Parks, the University of Antananarivo and the University of Hamburg. All samples were exported and imported with appropriate CITES permits (755C-09; MG17; 045C-EA02/MG09; E-03782/17; E03342/09; E-4681/09; E-0117/07; E-2866/07; W-14946/17; W-09260/18; W-09260/18; W-03396/21; 14US19101B/9; 18US217642/9; 18US19101B/9).

Data accessibility. Sample metadata can be found in the electronic supplementary material, table S1. Additional metadata and processed data, such as VCF files and analysis input and output files can be found at the Dryad Digital Repository at https://doi.org/10.5061/dryad.1jostgc3 [50]. All code used to run the analyses and produce the figures in this manuscript can be found on GitHub at https://github.com/jelmerp/lemurs_contactzone_grimur. Raw sequence data is available through the NCBI (Bioproject PRJNA661727).

Data are provided in the electronic supplementary material [51].

Authors’ contributions. J.W.P.: conceptualization, formal analysis, methodology, writing—original draft, writing—review and editing; B.K.M.: data curation, formal analysis, resources, writing—review and editing; Z.Y.: data curation, formal analysis, writing—review and editing; S.J.R.: resources, writing, review and editing; P.H.: data curation, formal analysis, resources, writing—review and editing; S.I.R.: resources, writing—review and editing; P.H.: data curation, formal analysis, resources, writing—review and editing; N.S.: data curation, resources, supervision, writing—original draft, writing—review and editing; A.D.Y.: conceptualization, funding acquisition, investigation, project administration, resources, supervision, writing—original draft, writing—review and editing; A.D.Y.: conceptualization, funding acquisition, investigation, project administration, resources, supervision, writing—original draft, writing—review and editing.

All authors gave final approval for publication and agreed to be held accountable for the work performed therein.

Conflict of interest declaration. We declare we have no competing interests.

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