The mitochondrial DNA diversity of captive ruffed lemurs (*Varecia* spp.): implications for conservation

Rodrigo Vega, Jane Hopper, Andrew C. Kitchener, Jérôme Catinaud Delphine Roullet, Eric Robsomanitrandrasana, Jack D. Hollister Christian Roos and Tony King

Abstract Ruffed lemurs (*Varecia variegata* and *Varecia rubra*) are categorized as Critically Endangered on the IUCN Red List, and genetic studies are needed for assessing the conservation value of captive populations. Using 280 mitochondrial DNA (mtDNA) D-loop sequences, we studied the genetic diversity and structure of captive ruffed lemurs in Madagascar, Europe and North America. We found 10 new haplotypes: one from the European captive *V. rubra* population, three from captive *V. variegata subcincta* (one from Europe and two from Madagascar) and six from other captive *V. variegata* in Madagascar. We found low mtDNA genetic diversity in the European and North American captive populations of *V. variegata*. Several founder individuals shared the same mtDNA haplotype and therefore should not be assumed to be unrelated founders when making breeding recommendations. The captive population in Madagascar has high genetic diversity, including haplotypes not yet identified in wild populations. We determined the probable geographical provenance of founders of captive populations by comparison with previous studies; all reported haplotypes from captive ruffed lemurs were identical to or clustered with haplotypes from wild populations located north of the Mangoro River in Madagascar. Effective conservation strategies for wild populations, with potentially unidentified genetic diversity, should still be considered the priority for conserving ruffed lemurs. However, our results illustrate that the captive population in Madagascar has conservation value as a source of potential release stock for reintroduction or reinforcement projects and that cross-regional transfers within the global captive population could increase the genetic diversity and therefore the conservation value of each regional population.

Keywords Biodiversity, conservation, genetic diversity, Lemuridae, Madagascar, primates

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Introduction

It is often claimed that captive populations are important for biodiversity conservation, especially given continuing threats to wild populations and their habitats (Conde et al., 2011; Barongi et al., 2015). However, this claim is regularly challenged (Conway, 2003; Leader-Williams et al., 2007; Balmford et al., 2011) because of issues prevalent within zoos or in captive breeding programmes (Lacy, 2013). Most captive populations have limited viability as a result of low population sizes and inbreeding (Lees & Wilcken, 2009; Conway, 2011; Conde et al., 2013), low genetic diversity (Muñoz-Fuentes et al., 2008; Shen et al., 2009; Atkinson et al., 2018) and limited or skewed breeding success (Roullet, 2012; Kaumanns et al., 2013; Penfold et al., 2014; Edwards et al., 2015). There are also uncertainties regarding the taxonomy or geographical provenance of captive animals and issues related to subspecific or interspecific hybridization (Hvilsom et al., 2013). Additional constraints include diseases (Thompson et al., 2000), behavioural or genetic adaptation to captivity (McPhee, 2004; Frankham, 2008) and the dominance of non-threatened species over threatened species (Conde et al., 2013). Therefore, rigorous and transparent criteria that are open to scrutiny are required to justify the maintenance of captive populations for conservation (Balmford et al., 1996; IUCN/SSC, 2014), and existing captive populations need to be assessed for their conservation value (Hvilsom et al., 2013; Gilbert et al., 2017; Johann et al., 2018).

Genetic studies are being used increasingly to help assess captive populations (Ogden et al., 2020). Taxonomic uncertainties can be evaluated (Hvilsom et al., 2013; Senn et al., 2014), genetic diversity can be compared between captive populations or with wild populations (El Alqamy et al., 2012; Svengren et al., 2017), and levels of relatedness and inbreeding can be quantified (Svengren et al., 2017; Atkinson et al., 2018). Moreover, by using genetic information, the probable geographical provenance of founder animals can be ascertained. This can be particularly useful to help with decision-making in relation to the suitability of captive populations or of individual captive animals for prospective reintroduction or other release projects (Ogden et al., 2018), especially of threatened species that have been extirpated locally at some sites or that persist in isolated populations at risk of losing genetic diversity (Farré et al., 2022).

Lemurs are one of the most threatened groups of primates (Schwitzer et al., 2014). Ruffed lemurs (including the black-and-white ruffed lemur *Varecia variegata* and the red ruffed lemur *Varecia rubra*) occur only in the eastern rainforests of Madagascar (Fig. 1) and are particularly sensitive to habitat loss and disturbance (Vasey, 2003). Both species are categorized as Critically Endangered on the IUCN Red List (Borgerson et al., 2020; Louis et al., 2020). As with most lemur species, ruffed lemurs are threatened by habitat loss because of deforestation and climate change (Morelli et al., 2020), and hunting for food and live-trapping for the illegal pet trade are additional threats (Golden, 2009; Reuter & Schafer, 2017; Borgerson et al., 2022). Habitat protection remains the priority for ensuring the survival of ruffed lemurs (King et al., 2013a,b; Schwitzer et al., 2013a), with 10 sites supporting *V. variegata* and two sites supporting *V. rubra* populations, all listed as priority lemur conservation sites in the IUCN lemur conservation strategy (Schwitzer et al., 2013b). Nevertheless, large numbers of ruffed lemurs are held in captivity, with > 800 *V. variegata* and > 600 *V. rubra* reported globally (Schwitzer et al., 2013a; Louis et al., 2020). Smaller numbers of both species are also held in Madagascar in recognized facilities (including 35 *V. variegata* according to a 2014 census) and illegally (Schwitzer et al., 2013a; Reuter et al., 2016; Louis et al., 2020). Therefore, the two species have been identified as having high potential for integrating in situ and ex situ conservation planning (Schwitzer et al., 2013a), with the North American captive population of *V. variegata* having already been used as a source of captive-born lemurs for reinforcing one small, isolated wild population (Britt et al., 2004). An assessment of the conservation value of the captive populations of these two species would aid conservation decision-making.

Unresolved subspecific taxonomy complicates the current assessment of the conservation value of the captive population of *V. variegata* (King et al., 2013a; Baden et al., 2014). Three subspecies are recognized (Louis et al., 2020): *V. variegata subcincta*, *V. variegata editorum* and *V. variegata variegata*. *Varecia v. subcincta* occurs in the north of the species range, *V. v. editorum* in the south and *V. v. variegata* in the area between *V. v. editorum* and *V. v. subcincta* (Louis et al., 2020). Yet the most comprehensive genetic study to date of wild *V. variegata* populations found a genetic distinction between *V. variegata* populations located to the north and to the south of the Mangoro River (Baden et al., 2014). Although this is a major biogeographical barrier for many taxa in Madagascar (Ganzhorn et al., 2006; Wilmé et al., 2006), it is not traditionally considered to represent the distributional limit between the two southern subspecies of *V. variegata*. Current texts consider *V. v. editorum* to occur on both sides of the Mangoro River, with the distribution limit and possible overlap with *V. v. variegata* located in the general region of Zahamena National Park (Louis et al., 2020), which is > 200 km north of the Mangoro River. Additionally, genetic evidence suggests that *V. v. subcincta*

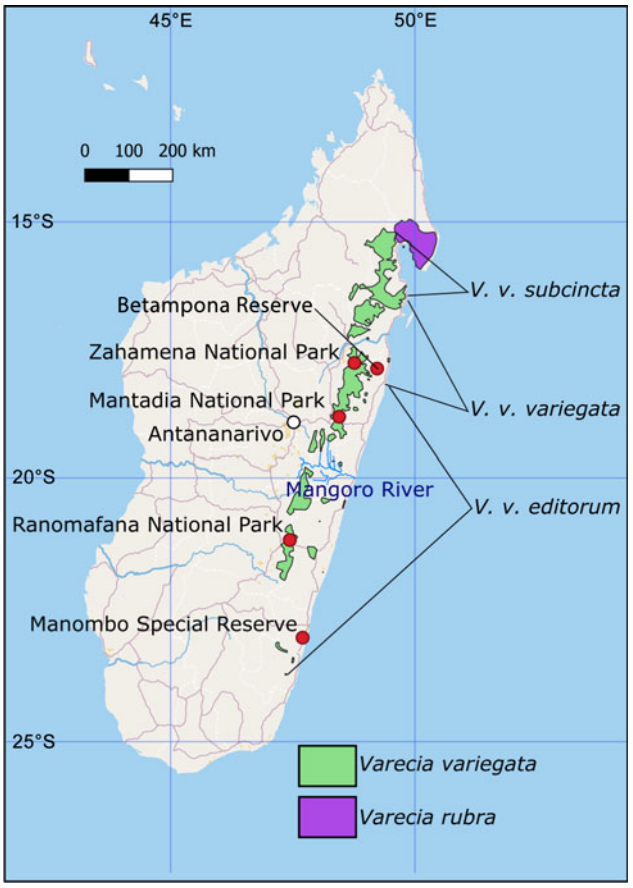

FIG. 1 Geographical distribution of ruffed lemurs. The red ruffed lemur *Varecia rubra* occurs in the north-east, whereas the black-and-white ruffed lemur *Varecia variegata* has a wider and scattered distribution from north to south. Although three subspecies of *V. variegata* are recognized (*V. variegata variegata*, *V. variegata subcincta* and *V. variegata editorum*), the taxonomic status and distribution of the subspecies are not fully resolved.

may not be a valid subspecies (Baden et al., 2014; Louis et al., 2020). Further work is underway to resolve these taxonomic issues (Louis et al., 2020). However, previous genetic research (Baden et al., 2014) provides a baseline for ascertaining the geographical provenance of captive ruffed lemurs. Work is also needed to determine the genetic diversity of captive ruffed lemurs in Europe and Madagascar and their relationships with ruffed lemurs in North America and in the wild.

Here we assess the mitochondrial genetic diversity of captive ruffed lemurs in Madagascar, Europe and North America, focusing primarily on *V. variegata*, using analyses of new samples from lemurs in Madagascar and Europe and data published previously on lemurs in North America. We compare the results from captive lemurs with published data from wild lemurs, with a particular emphasis on ascertaining the geographical provenance of the founders of the global captive population. Our findings will help inform decision-making regarding the potential conservation value and roles of captive populations and the integration of ex situ and in situ conservation activities for ruffed lemurs.

Methods

Molecular biology techniques

We obtained 51 new samples for this study, including nine muscle samples from *V. variegata* in the CryoArks Biobank at National Museums Scotland (derived from animals in UK zoos during 1989–2012) and 42 hair (with follicle), whole-blood and Whatman FTA card (Merk, Darmstadt, Germany) blood samples from captive ruffed lemurs collected by zoo and/or veterinary professionals in Madagascar and Europe, including samples from *V. rubra*, *V. v. subcincta* and *V. variegata* of undetermined subspecies (but phenotypically not of *V. v. subcincta*; Supplementary Table 1). In addition, we retrieved 229 mitochondrial DNA (mtDNA) D-loop sequences (accession numbers KJ700486–KJ700626, AF173507–AF173547, AF475865–AF475904, AF493668–AF493671 and AY584494) from GenBank (we later removed AF173519, AF173521, AF173522 and AF173530 from the analysis because of their short sequence length).

We obtained DNA from hair, whole-blood and muscle tissue samples using the GeneJET Genomic DNA Purification Kit (ThermoFisher Scientific, Waltham, USA). We obtained DNA from dried blood samples from Whatman FTA cards following a custom method for DNA extraction (Supplementary Material 1). We performed PCR to amplify the mtDNA D-loop region of ruffed lemurs using heavy strand dLp5 (5′-CCATCGWGATGTCTT ATTTAAGRGGAA-3′; Baker et al., 1993) and light strand dLp1.5 (5′-GCACCCAAAGCTGARRTTCTA-3′; Wyner et al., 1999) primers following standard protocols, resulting in 536 base-pair fragments (Supplementary Material 1).

Data analysis

We analysed 276 DNA sequences of ruffed lemurs (Supplementary Table 1). We aligned all sequences using *BioEdit 7.2.6* (Hall, 1999). We divided the sample into nine groups (Table 1) for downstream analysis according to the known species or subspecies classification, the geographical origin of wild *V. v. editorum* samples (north or south of the Mangoro River), the status of the individuals (wild or captive) and whether captive individuals were part of the captive population in Madagascar, the European Association of Zoos and Aquaria Ex-situ Programme (EEP; formerly known as European Endangered Species Programme), the North American Association of Zoos and Aquariums Species Survival Plan Programmes (SSP) or a non-EEP European population (samples from Fenn Bell Conservation Project; FBC).

We imported DNA sequences into *DnaSP 6.12.03* (Rozas et al., 2017). We obtained several DNA polymorphism indices in *DnaSP*, including number of segregating sites (S), number of haplotypes (H), haplotype diversity (Hd), nucleotide diversity (π) and average number of nucleotide differences (k). We evaluated changes in population size using Ramos-Onsins and Rozas' R_2 and Fu's Fs values calculated with *DnaSP*, with significance tested using the coalescent process for a neutral infinite-sites model and assuming a large constant population size (1,000 replications).

To estimate the genetic structure amongst the groups, we obtained pairwise genetic differentiation (F_{ST}) values and the numbers of net nucleotide substitutions per site between groups (Da) using *DnaSP*. We carried out an analysis of molecular variance in *Arlequin 3.5.2.2* (Excoffier et al., 2005). We generated a phylogenetic network from the haplotypes using *PopART* (Leigh & Bryant, 2015), with the *Median Joining* algorithm selected.

Results

We obtained 51 new D-loop sequences belonging to the genus *Varecia* (GenBank accession numbers MZ615228–MZ615278; Supplementary Table 1). These included 23 from captive lemurs in Madagascar, of which three were *V. v. subcincta* and 20 were *V. variegata* (unidentified subspecies), four from the EEP *V. rubra* (from ZooParc de Beauval), 20 from the EEP *V. variegata*, of which three were *V. v. subcincta* (from Port Lympne Reserve) and 17 were *V. variegata* of undetermined subspecies but not *V. v. subcincta* phenotypically (six from Howletts Wild Animal Park, two from Marwell Zoo and nine from specimens at National Museums Scotland), and four from

TABLE 1 DNA polymorphism and population expansion test of ruffed lemurs (*Varecia* spp.). Bold indicates significant test of neutrality ($P < 0.05$).

Species[1]	n[2]	S[3]	H[4]	Hd[5]	π[6]	k[7]	R_2[8]	Fs[9]
V. rubra (WC)	6	6	2	0.533	0.007	3.200		
V. variegata subcincta (WC)	33	17	9	0.843	0.013	5.705		
V. variegata editorum-S (W)	115	3	4	0.406	0.001	0.415	0.041	**−4.766**
V. variegata editorum-N (W)	32	11	4	0.768	0.012	5.212	0.237	10.537
V. variegata variegata (W)	34	27	9	0.850	0.025	11.091	0.202	6.723
V. variegata-Madagascar (C)	20	20	10	0.911	0.026	11.705	0.201	1.299
V. variegata-EEP (C)	17	17	2	0.485	0.001	0.485	0.243	1.233
V. variegata-SSP (C)	15	26	7	0.781	0.017	7.676	0.139	2.275
V. variegata-FBC (C)	4	1	2	0.500	0.001	0.500		
Total[10]	276	44	36	0.877	0.023	10.278		

[1]W, wild; C, captive; S/N, south/north of the Mangoro River; EEP, European Endangered Species Programme; SSP, Species Survival Plan Programmes; FBC, Fenn Bell Conservation Project.
[2]n, number of sequences used. [3]S, segregating sites. [4]H, number of haplotypes. [5]Hd, haplotype (gene) diversity. [6]π, Jukes–Cantor nucleotide diversity. [7]k, average number of nucleotide differences. [8]R_2, Ramos-Onsins and Rozas' value. [9]Fs, Fu's Fs value. [10]Values calculated based on the full dataset of DNA sequences.

captive *V. variegata* of undetermined subspecies held in Europe but not included within the EEP (from FBC).

Genetic diversity

The DNA sequence alignment for the whole dataset (276 D-loop sequences) was 613 base pairs long and contained 455 sites (excluding gaps and missing data) from which 411 were invariable, 41 were informative and three were singletons. We found the highest Hd and π values in the sample from captive *V. variegata* in Madagascar, and the lowest values in the sample from wild populations of *V. v. editorum* south of the Mangoro River (Table 1). Genetic diversity in the sample from the EEP *V. variegata* was much lower than in the SSP sample, and the EEP sample had the second lowest diversity value across all groups (Table 1).

Consistent with the low genetic diversity values, we found small pairwise differences between sequences in the samples from wild populations of *V. v. editorum* south of the Mangoro River and from the EEP *V. variegata*, whereas other groups showed some larger pairwise differences, with frequencies of 5–16% (Supplementary Fig. 1). None of the R_2 and Fs values were significantly different from neutral expectations, with there being no indications of sudden population expansions or contractions except for the sample from wild populations of *V. v. editorum* south of the Mangoro River, which showed a significantly low and negative Fs value (Table 1).

Genetic structure

We found significant pairwise F_{ST} values amongst all groups (Table 2). The values ranged from 20.0% between the samples from captive *V. variegata* in Madagascar and the samples from wild populations of *V. v. variegata*, to 97.7% between the samples from the EEP *V. variegata* and the samples from wild populations of *V. v. editorum* south of the Mangoro River. These values are even higher than those from pairwise comparisons of different species (e.g. *V. rubra* and *V. variegata*). We found the highest pairwise divergence values (Da) to be between *V. rubra* and all *V. variegata* groups followed by pairwise comparisons between *V. v. subcincta* and *V. v. variegata* and *V. v. editorum*, and we found the lowest Da values in pairwise comparisons between *V. v. variegata* and *V. v. editorum* (Table 3). The analysis of molecular variance showed that 29.1% of the variation was between wild vs captive animals, and 41.3% of the variation was amongst groups and 29.5% was within groups.

The phylogenetic network reflected the taxonomic classification of ruffed lemurs (Fig. 2). The *V. rubra* and *V. v. subcincta* haplotypes appeared separately from each other and from other ruffed lemur groups by several mutational steps, and *V. v. editorum* haplotypes appeared closer together than to any other *V. variegata* haplotypes. The remaining haplotypes showed a mix of small and large numbers of mutational steps obtained from samples from wild populations of *V. v. variegata* and from the four groups of captive *V. variegata* (in Madagascar, the EEP, the SSP and FBC). The most common haplotypes were Hap_5 and Hap_20 (with 84 and 29 sequences, respectively), all belonging to the sample from wild populations of *V. v. editorum* south of the Mangoro River, followed by Hap_4 (27 sequences) belonging to a mix of groups. The EEP and FBC samples were represented by only two haplotypes (Hap_2 and Hap_4), whereas the SSP sample was represented by seven haplotypes. The captive animals in Madagascar had the highest haplotype diversity, with 10 different haplotypes. Hap_12 was shared between the sample from wild populations of *V. v. editorum* north of the Mangoro River and the

TABLE 2 Pairwise differentiation values (F_{ST}) amongst groups of ruffed lemurs (*Varecia* spp.). *Varecia variegata*-Fenn Bell Conservation Project (C) is not included because of small sample size.

Species[1]	1	2	3	4	5	6	7	8
1 *V. rubra* (WC)								
2 *V. v. subcincta* (WC)	0.598							
3 *V. v. editorum*-S (W)	0.936	0.800						
4 *V. v. editorum*-N (W)	0.782	0.579	0.627					
5 *V. v. variegata* (W)	0.479	0.416	0.631	0.342				
6 *V. variegata*-Madagascar (C)	0.545	0.464	0.706	0.397	0.200			
7 *V. variegata*-EEP (C)	0.729	0.838	0.977	0.917	0.670	0.784		
8 *V. variegata*-SSP (C)	0.262	0.555	0.840	0.590	0.289	0.413	0.270	

[1]W, wild; C, captive; S/N = south/north of the Mangoro River; EEP, European Endangered Species Programme; SSP, Species Survival Plan Programmes.

TABLE 3 Pairwise divergence values (Da) amongst groups of ruffed lemurs (*Varecia* spp.). *Varecia variegata*-Fenn Bell Conservation Project (C) is not included because of small sample size.

Species[1]	1	2	3	4	5	6	7	8
1 *V. rubra* (WC)								
2 *V. v. subcincta* (WC)	0.016							
3 *V. v. editorum*-S (W)	0.033	0.029						
4 *V. v. editorum*-N (W)	0.033	0.025	0.008					
5 *V. v. variegata* (W)	0.032	0.026	0.018	0.015				
6 *V. variegata*-Madagascar (C)	0.031	0.023	0.011	0.009	0.003			
7 *V. variegata*-EEP (C)	0.034	0.029	0.017	0.010	0.018	0.012		
8 *V. variegata*-SSP (C)	0.030	0.023	0.013	0.006	0.008	0.005	0.001	

[1]W, wild; C, captive; S/N = south/north of the Mangoro River; EEP, European Endangered Species Programme; SSP, Species Survival Plan Programmes.

captive samples from Madagascar and the SSP, which indicates that those captive animals from Madagascar and the SSP not identified to subspecies level could have a *V. v. editorum* maternal origin. Furthermore, several haplotypes belonging to wild *V. v. variegata* and to captive *V. variegata* from Madagascar, the EEP, the SSP and FBC were distantly related to other *V. v. variegata* haplotypes. Specifically, Hap_9, Hap_10 and Hap_11 (all from wild *V. v. variegata*) were separated by eight mutational steps and distantly related to all other haplotypes.

Discussion

We assessed the mitochondrial genetic diversity of captive ruffed lemurs in Madagascar, Europe and North America and compared this to published data from wild lemurs to ascertain the geographical provenance of the founders of the global captive population. The results could inform decision-making regarding the potential conservation value and roles of the captive ruffed lemur populations and the potential integration of ex situ and in situ conservation practices for ruffed lemurs. We report 10 mtDNA haplotypes that have not yet been recorded from wild or captive *Varecia* populations. We found one of the novel haplotypes in the four samples from the European captive *V. rubra* population but there was only one published *V. rubra* haplotype from wild populations for comparison, with which the captive haplotype clustered. All three haplotypes found in captive *V. v. subcincta* (one from the European captive population and two from Madagascar) have not been reported previously but clustered with the six published haplotypes from wild *V. v. subcincta* populations. We found only two other mtDNA haplotypes in the European captive *V. variegata* population, both of which were identical to previously published haplotypes from wild populations. Conversely, out of 10 mtDNA haplotypes present in the non-*V. v. subcincta* captive population in Madagascar, only four had been reported previously from wild populations and six are newly reported here.

There are 18 recognized founders of the non-*subcincta* EEP population and four founders of the *V. v. subcincta* EEP population (Johann et al., 2018; Louis et al., 2020). Seven of the 18 founders of the non-*subcincta* EEP population were female, of which we were able to sample four indirectly in this study through their descendants along uninterrupted maternal lines. As several of the founders of the EEP population are also founders of the North American captive population (SSP), another seven of the EEP founders had been sampled previously in a study of the SSP population (Wyner et al., 1999), including one female founder sampled indirectly through a maternal-line descendant and six male founders sampled directly. Therefore, we have been able to identify the mtDNA haplotypes of 11 of the 18 founders of the EEP captive

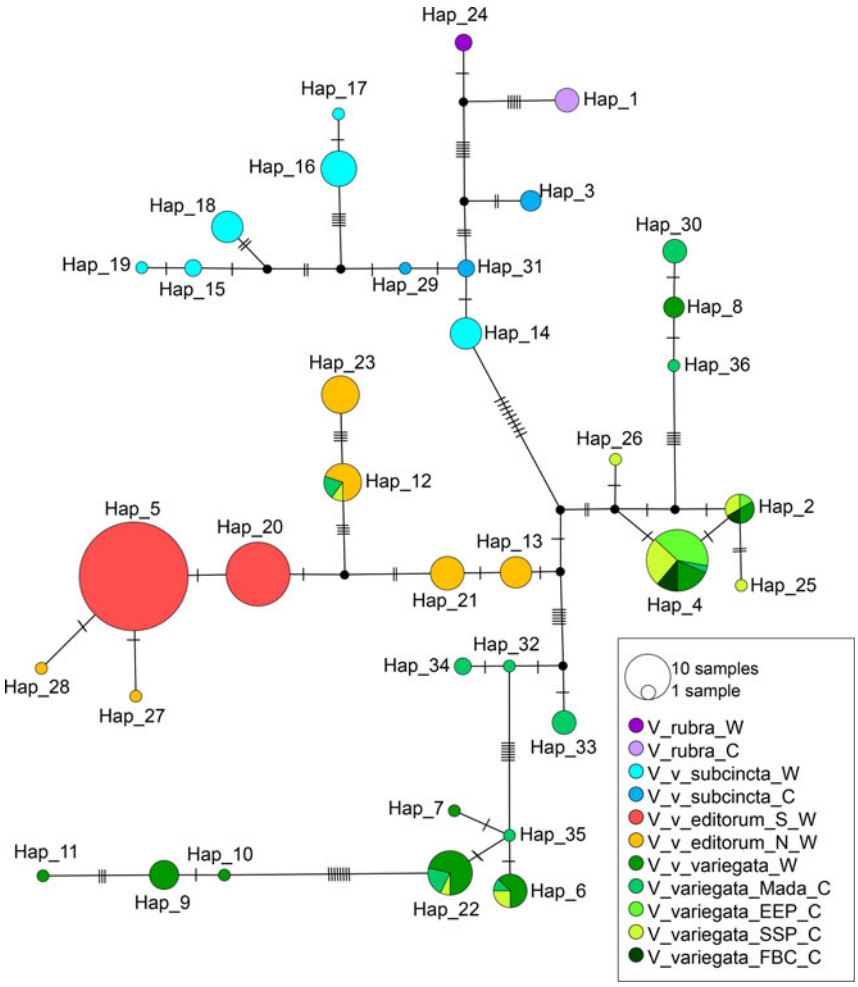

FIG. 2 Phylogenetic haplotype network of ruffed lemurs (*Varecia* spp.) based on D-loop mitochondrial DNA sequence data. The node sizes are proportional to the number of sequences belonging to the haplotypes. Mutations between haplotypes are shown using hatch marks. Inferred internal nodes are shown in black. W, wild; C, captive; S/N, south/north of the Mangoro River; Mada, Madagascar; EEP, European Endangered Species Programme; SSP, Species Survival Plan Programmes; FBC, Fenn Bell Conservation Project. (Readers of the printed journal are referred to the online article for a colour version of this figure.)

population. Studbook analysis illustrates that these 11 founders have contributed 72% of the genetic diversity of the EEP population, illustrating that despite sampling only a small proportion of the EEP population our results are representative of the majority of the living population.

We identified only four different mtDNA haplotypes within the historic non-*subcincta* EEP population (including only two within contemporary samples), despite having sampled 11 of the 18 founder animals directly or indirectly. Six of the sampled founders share Hap_4 (founders 4, 5, 11, 13, 21 and 25) and three share Hap_2 (founders 14, 15 and 35). Putative founders having the same haplotype suggests they could share common maternal ancestry, and if so, perhaps should not be considered as unrelated founders for the purposes of the analysis of theoretical summary genetic statistics for captive populations or for the purposes of calculating mean kinship and founder representation, and for making breeding recommendations. We recommend additional genetic analysis to ascertain the true levels of relatedness of the putative founders of the captive ruffed lemur populations, as has been done for other species in captivity (Svengren et al., 2017; Atkinson et al., 2018). This would allow a more accurate assessment of the genetic diversity of the original founder populations, with implications for our understanding of the conservation value of the existing captive populations and of the specific breeding recommendations to maximize their conservation potential.

Studbook analysis illustrates that the six founders sharing Hap_4 have contributed 46% of the genetic diversity of the non-*subcincta* EEP population and the three founders sharing Hap_2 have contributed 19% of this genetic diversity. Therefore, these nine founders sharing only two mtDNA haplotypes have contributed 65% of the genetic diversity of the EEP population. At over 300 individuals (Johann et al., 2018), the European captive population of non-*subcincta* *V. variegata* is relatively large but our results show that mitochondrial genetic diversity is relatively low and that the founders of the population represent only a small proportion of the genetic diversity of the species. Nevertheless, one haplotype in the non-*subcincta* EEP population is not represented in the captive population from Madagascar, and exchanges between the various regional captive populations could increase the genetic diversity of each and therefore increase their conservation value. The genetic diversity of the global *V. rubra* captive population is also considered to be low (Borgerson et al., 2020).

Geographical origins of captive ruffed lemurs

All mtDNA haplotypes reported from captive ruffed lemurs were either identical to or clustered with published haplotypes originating from wild *Varecia* populations located north of the Mangoro River. There is currently no evidence for the presence of lemurs from south of the Mangoro River being incorporated into global captive populations. Moreover, the distantly related haplotypes within *V. variegata* and specifically those seen in wild *V. v. variegata* suggest a cryptic genetic structure in ruffed lemurs, warranting further genetic characterization of individuals in the wild.

Our results confirm the low haplotype diversity found in a previous genetic study (Wyner et al., 1999) but contradict the conclusion from that study that the captive-born *V. variegata* from North American zoos, which were released into Betampona Reserve from 1997 to 2001 to reinforce a small, isolated wild population (Britt et al., 2004), probably originated from the south of the species' range and therefore were not particularly appropriate for release in this Reserve because of its location in the northern part of the species range (Wyner et al., 1999). This previous study used population aggregation analysis to test for phylogenetic clusters based on diagnostic nucleotide positions and used a restricted baseline from wild populations for comparison. Instead, our results show that the mtDNA of the North American captive population originates from wild populations north of the Mangoro River and therefore that the lemurs released were more suitable genetically for the population reinforcement project than suggested previously (Wyner et al., 1999). Should further releases of captive *V. variegata* be considered appropriate in Madagascar within the context of integrating in situ and ex situ lemur conservation (King et al., 2013a; Schwitzer et al., 2013a), our results suggest that the current global captive population would be more suitable from a genetic perspective for releases in sites located north of the Mangoro River. However, there are many other issues that would need to be considered prior to any potential releases, including behavioural assessments such as potential naivety to predators, disease risk analyses and socioeconomic considerations, as detailed in international guidelines (IUCN/SSC, 2013).

The most prevalent mtDNA haplotype in the European captive EEP population (Hap_4) has been reported in the wild only from Zahamena National Park (Baden et al., 2014), suggesting that much of the genetic diversity of the EEP probably originated from this part of the species' range. The second most prevalent haplotype (Hap_2) has been reported in the wild from Betampona Reserve only, but the wild samples were collected after the release of captive-bred lemurs from the North American captive SSP population, so this haplotype could be derived from the released lemurs rather than the original wild population of Betampona Reserve. This haplotype is similar to Hap_4 and so could have originated from closer to Zahamena National Park; a letter from the 1970s regarding one of the founders with Hap_2 (ISB35) claims that this individual was captured 50 miles north-east of Ambatondrazaka, which would be in or near Zahamena National Park. Betampona Reserve and Zahamena National Park are at similar latitudes in the species' range, so the distinction is unlikely to be significant from an evolutionary perspective. Of the two remaining haplotypes identified from the EEP founders, Hap_26 has not been reported from wild populations but is also similar to Hap_4 from Zahamena National Park, whereas Hap_12 is different and has been obtained from wild lemurs identified as *V. v. editorum* in the Mantadia, Andasibe and Torotorofotsy sample sites at the southern end of the Ankeniheny–Zahamena Corridor (Baden et al., 2014). The three haplotypes that probably originated from in or around Zahamena National Park can be traced back to 10 founder individuals who have contributed at least 60% of the genetic diversity of the EEP population, providing strong evidence for the probable geographical origins of a large proportion of the captive EEP population.

Using mtDNA, this study has helped to ascertain the geographical provenance of several of the founders of the global captive ruffed lemur population, provided insights into the taxonomic classification of captive individuals and determined the genetic diversity of captive ruffed lemurs. Although the use of mtDNA has limitations in comparison with other molecular markers (Nielsen et al., 2020), it is useful for species identification and for wildlife forensics (Alacs et al., 2010). The use of mtDNA to identify the probable population of origin of captive animals in Madagascar could help us to understand where ruffed lemurs or other species are being captured illegally from wild populations (Reuter et al., 2016; Reuter & Schafer, 2017). Our results illustrate that several captive ruffed lemurs in Madagascar have mtDNA haplotypes that have not yet been identified from wild populations. Therefore, we recommend prioritizing the genetic analysis of wild populations that have not yet been sampled, including utilizing historical museum specimens of known origin if available and non-invasive samples from populations under community-based conservation initiatives such as the Andriantantely lowland forest and the western Ankeniheny–Zahamena Corridor (King et al., 2013b,c; Louis et al., 2020). This would provide us with a better understanding of the genetic diversity of wild ruffed lemur populations and could provide baseline genetic diversity for identifying where lemurs are being captured illegally, especially if there is a particular sampling focus on areas where illegal capture is most likely to be occurring (e.g. forests within relatively easy reach of the major markets in Toamasina). Areas that are identified as probable sites of illegal captures or of other threats to ruffed lemurs or their habitats should also be considered for urgent conservation interventions to mitigate these threats. Community-based

conservation of forests and lemurs is a well-established model in Madagascar (King et al., 2013b,c; Rasolofoharivelo et al., 2013; Ravaloharimanitra et al., 2015; Louis et al., 2020) and could be implemented or increased for any remaining wild ruffed lemur populations through appropriate local community support.

Conclusion

Given the continuing crisis facing rainforests and ruffed lemurs in Madagascar (Jenkins et al., 2011; Seaman et al., 2018; Morelli et al., 2020), the use of all available tools to tackle these issues should be considered an urgent priority for lemur conservation. Genetic analyses, such as those presented here, can help to inform conservation decision-making. The results of this study indicate that the large global captive population of ruffed lemurs could have some value as a source of potential release stock for reintroduction or reinforcement projects, that the much smaller captive population in Madagascar has higher genetic diversity and greater potential for contributing suitable release candidates, and that effective conservation of wild populations should be considered the highest priority for the conservation of ruffed lemurs and their remaining genetic diversity.

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Conflicts of interest None.

Ethical standards This research abided by the *Oryx* guidelines on ethical standards. Ethical guidelines and considerations by Canterbury Christ Church University, The Aspinall Foundation and the Fenn Bell Conservation Project were followed for procuring hair and blood samples from captive lemurs.

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