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Article *in* Ecosphere · July 2021

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Noninvasive genetic sampling for flying foxes: a valuable method for monitoring demographic parameters

Mohamed Thani Ibouroi [1,2,†] Véronique Arnal,[1] Ali Cheha,[2] Said Ali Ousseni Dhurham,[2] Claudine Montgelard,[1] and Aurélien Besnard[1]

[1]*Centre for Functional and Evolutionary Ecology (CEFE UMR 5175), EPHE, PSL Research University, CNRS, University of Montpellier, SupAgro, IRD, INRA, Montpellier F-34293 France*
[2]*Sustainable Development Task Force (GIDD), Moroni, Hamramba, Comores*

Citation: Ibouroi, M. T., V. Arnal, A. Cheha, S. A. O. Dhurham, C. Montgelard, and A. Besnard. 2021. Noninvasive genetic sampling for flying foxes: a valuable method for monitoring demographic parameters. Ecosphere 12(7):e03327. 10.1002/ecs2.3327

Abstract. Establishing effective wildlife conservation measures requires accurate demographic information such as population size and survival probability: parameters that can be extremely difficult to obtain. This is especially the case for threatened species, which are often rare and sometimes occupy inaccessible areas. While noninvasive genetic sampling (NIGS) techniques are promising tools for providing demographic data, these methods may be unreliable in certain situations. For instance, fecal samples of frugivorous species in tropical areas degrade rapidly, affecting the usability of the genetic material. In this study, we compared (1) NIGS capture–mark–recapture (NIGS-CMR) with conventional CMR to determine their potential in estimating demographic parameters of fruit bats, and (2) the precision of these demographic parameters and the associated costs given varying sampling designs through simulations. Using Livingstone's fruit bats (*Pteropus livingstonii*) fecal samples, microsatellite markers were tested and genotyping success and error rates were assessed. The average genotyping success rate was 77%, and the total genotyping error rate for all loci was low (allelic dropout rate = 0.089, false alleles rate = 0.018). Our results suggested that five loci were required to identify individuals. Simulations showed that monitoring the species over a 9-yr period with a recapture rate of 0.20 or over a 6-yr period with a recapture rate of 0.30 seems appropriate to obtain valuable demographic parameters. Overall, in comparison to conventional CMR, NIGS-CMR offers a better method for estimating demographic parameters and subsequently for conducting long-term population monitoring in flying foxes due to the fact that (1) sample collection is easy and the level of genotyping errors in the laboratory is low and (2) it is cheaper, less time-consuming, and less disturbing to individual animals. We strongly advocate an approach that couples a pilot study with simulations as done in this study in order to choose the most efficient monitoring method for a given species or context.

Key words: capture–mark–recapture; fecal samples; frugivorous bats; population dynamics; species monitoring; tropical forests; wildlife conservation.

Received 14 February 2020; revised 16 July 2020; accepted 24 July 2020. Corresponding Editor: Brooke Maslo.

† **E-mail** : halibathani@yahoo.fr

Introduction

Establishing effective wildlife conservation measures requires demographic information such as population size, survival probability, and recruitment rate (Williams et al. 2002). These parameters, however, can be extremely difficult to obtain, especially for threatened species, which are often rare and sometimes occupy inaccessible areas. To estimate the population size of threatened species, many biologists use direct counts (O'Shea et al. 2003). However, direct counts are biased due to imperfect detection (Thomas et al. 1989, Kunz 2003), meaning this method provides only indices of a population size, potentially leading to erroneous conclusions regarding population trends when detection probability is not constant over time (Kunz et al. 2009, Archaux et al. 2012, Gervasi et al. 2014). The issue of imperfect detection has been the focus of methodological developments over the last 50 yr (Buckland et al. 2001, Williams et al. 2002). For instance, distance sampling methods allow modeling the detection probability of individuals given their distance to the observer and correcting for undetected individuals (Buckland et al. 2004). Yet, this method is inadequate when the population size is low or the species is rare, as is usually the case for threatened species, since the number of observations is too low to correctly estimate the detection parameters (Buckland et al. 2004). It is also inadequate for highly mobile species, which is the case of bat species (Marucco et al. 2009). More importantly, counts —corrected for detection issues or not—provide information only on population size and not on other demographic parameters such as fecundity, survival, or recruitment rates that are often crucial to develop effective conservation strategies (Hayman et al. 2012).

Over the last 50 yr, capture–mark–recapture (CMR) approaches have been developed to address the difficulties associated with estimating demographic parameters in animal populations (Nichols 1992, Pradel et al. 1997). However, despite their robustness and methodological flexibility, conventional CMR methods can be problematic to apply for secretive or wide-ranging species occurring at low density (Miller et al. 2005). As CMR requires the physical handling of individuals, it is a time-consuming approach for rare species (Hájková et al. 2009). Moreover, physical capture often disturbs and can sometimes even injure or kill animals, posing ethical issues, especially as regards threatened species (Marucco et al. 2009).

Genetic data obtained from the noninvasive sampling of diverse materials, such as hair or fecal matter, can be used to identify individuals, minimizing the risk and stress for animals (Taberlet and Luikart 1999). Noninvasive genetic sampling (NIGS) thus represents an alternative option to traditional methods to acquire the data necessary to estimate demographic parameters—data such as dispersal patterns (Valière et al. 2003), survival rates and population trends—using a CMR approach (Nichols 1992, Marucco et al. 2009). Over the two last decades, NIGS has become increasingly popular for wildlife monitoring (see, e.g., Morin and Woodruff, 1996, Taberlet and Luikart 1999, Bellemain and Taberlet 2004, Horvath et al. 2005, Boston et al. 2012) and has been successfully used to obtain information relevant to conservation issues for many species, including birds (Horvath et al. 2005), brown bears (*Ursus arctos*; De Barba et al. 2010), or large primates (e.g., *Pan troglodytes*; Arandjelovic and Vigilant 2018).

Despite the fact that NIGS is a well-established method, few studies have used this method to focus on fruit bat species in tropical forests (though see Baldwin et al. 2010). This paucity is likely due to difficulties related to the low quantity and poor quality of DNA contained in noninvasive samples from these species. In particular, fecal samples from frugivorous animals are often degraded or contain many PCR inhibitors (Baldwin et al. 2010). As fecal samples from frugivorous bats may contain non-digested fruit, this could attract insects, the presence of which could speed up DNA degradation (Palomares et al. 2002). Moreover, fecal matter from frugivorous animals is often soft or even liquid, and the high level of moisture in the sample could accelerate DNA degradation (Morin et al. 2001, Palomares et al. 2002). Taken together, these factors can lead to genotyping errors such as a high rate of allelic dropout and false alleles (Morin et al. 2001), eventually biasing demographic parameter estimates (Boston et al. 2012).

Flying foxes represent a particularly interesting case to monitor with NIGS. Most of these

tropical bat species are restricted to remote islands in the Indian and Pacific Oceans and currently face extremely high extinction risks (Ibouroi et al. 2018*a*). By virtue of their long-distance movements from roosting to feeding sites, flying foxes pollinate vegetation and disperse seeds over large distances, thus ensuring connectivity between fragmented forests as well as improving agricultural systems (Kunz et al. 2011). It is thus crucial to establish a protocol for these species for the regular monitoring of population size and dynamics in order to assess effective conservation strategies for critically endangered species.

As flying foxes live in colonies during the daytime at known roosting sites (Ibouroi et al. 2018*a*) and the collection of feces at these sites is technically easy (Ibouroi et al. 2018*b*), NIGS is potentially a promising tool for long-term monitoring. The problem of NIGS is the fact that its applicability needs some fieldwork to collect samples as other methods such as captures, but in addition implies several costs associated with genetic analysis that are further increased by the need to replicate the analysis in cases of degraded DNA. Adopting such a method may be challenging especially in developing countries. Conventional CMR might thus be a more realistic alternative in developing countries for assessing demographic parameters, though the capturing and marking of individuals. Yet, conventional CMR requires deploying intensive field efforts including physical capture of individuals that might be stressful for animals (Hämäläinen et al. 2013, Thorup et al. 2014). It is thus necessary to compare the applicability of both methods and to assess which can be the most efficient tool for assessing demographic parameters.

The Livingstone's fruit bat (*Pteropus livingstonii*) has a small population in a restricted habitat in the mountain forests of the Comoros islands of Anjouan and Mohéli (Ibouroi et al. 2018*a*). It is considered one of the most, if not the most, threatened bats in the world, classified as Critically Endangered on the International Union for the Conservation of Nature Red List (Sewall et al. 2016). As a species that requires long-term monitoring to determine and assess conservation measures, it represents an ideal model for identifying the best monitoring protocol for tropical fruit bat species.

In this study, we compared the potential of NIGS-CMR and conventional CMR in estimating demographic parameters of *Pteropus livingstonii*, a fruit bat species endemic to the Comoros archipelago. Specifically, we (1) developed a method of individual identification using NIGS, (2) compared the accuracy of demographic parameter estimates given varying sampling designs, and (3) evaluated the costs of these designs through simulations for both NIGS-CMR and conventional CMR approaches.

Materials and Methods

NIGS-CMR fecal sample collection and analysis

To assess the efficacy of a NIGS-CMR approach, field sessions were conducted on the islands of Anjouan and Mohéli during four periods: November 2014–May 2015, December 2015–April 2016, July–October 2016, and August–September 2019. During each session, fecal samples were collected at 12 *P. livingstonii* roosts by placing a plastic trap below each roosting tree from 6:00 p.m. to 8:00 a.m. Two small sticks were used as forceps to collect fresh droppings. We collected two forms of droppings: solid and soft forms that depend on the type of foods ate by the individual. This resulted in the collection of up to 30 samples of fecal materials per roost. When two or more droppings were in close proximity in the trap (~0.5 m) and had the same color and form, we assumed that they came from the same individual and only one sample was taken. Each fecal sample was then placed into a 5-mL tube containing 96% ethanol or silica gel. The samples were kept at room temperature (between 20° and 30°C) in the Comoros before being transported to France and frozen (−20°C) until processing for DNA extraction.

The DNA from the fecal samples was extracted at the degraded DNA platform at the Mediterranean Center for the Environment and Biodiversity Laboratory of Excellence (LabEx CeMEB, University of Montpellier, France) using a QIAGEN kit (DNeasy mericon Food Kit 69514) following the manufacturer's protocol. Seven microsatellite loci were amplified through polymerase chain reaction (PCR) following Ibouroi et al. (2018*b*). PCR products were run in a 16 capillary sequencer (3130 xl Genetic Analyzer, Applied Biosystems) at the genotyping–

sequencing platform at the LabEx CeMEB with the standard size GS400 (-250)LIZ500. Alleles of each sample were scored using GeneMapper v4.5 (Applied Biosystems).

In noninvasive genetic sampling, genotyping errors occur due to increased rates of (1) null alleles, (2) allelic dropout (ADO: when heterozygous genotype from at least three independent, repeated genotyping procedures showed homozygous genotypes), or (3) false alleles (FA: if a homozygote from a triplicate genotyped set showed heterozygote genotype). We applied the multiple-tubes procedure according to Taberlet et al. (1997) to assess the reliability, ADO and FA error rates of the genotyped fecal samples by independently amplifying each DNA extract in three replicates. In some cases, results were negative for at least one of the three runs. In this situation, we carried out additional runs for the sample to obtain three or more positive PCRs. Genotype profiles from the three or more replicates were compared, and the consensus genotypes were reconstructed using GIMLET v 1.3.4 (Valière 2002). The program GIMLET accepts a genotype as heterozygote if two alleles were seen at least in two replicates and as homozygote if only one allele is seen for at least three replicates. ADO and FA were quantified according to Broquet and Petit (2004). Error is considered even if one run only of the three runs or more differs. Moreover, the presence of null alleles was checked using the Micro-Checker v.2.2.3 program (Van Oosterhout et al. 2004).

NIGS-CMR individual identification

We assessed the number of unique individuals using the GENALEX program (Peakall and Smouse 2012). This allowed us to compare multilocus genotype profiles and to determine all pairs of samples sharing the same genotype and the list of pairs of matching samples (Woods et al. 1999). If one or more samples shared the same genotype, the corresponding samples were regarded as originating from the same individual and only one was kept for subsequent analysis (Ruell et al. 2009). In a second step, we used GENALEX to determine the minimum number of microsatellites necessary for individual identification. This minimum number depends on the variation of allele frequency and the diversity at each locus. We thus applied the program GenAlex to calculate the observed number of alleles or NA (actual number of alleles in the population), the effective number of alleles or Ne (number of alleles with equal frequencies that would be necessary to give the same expected heterozygosity as in the actual population). Allelic richness (Ar) in each locus was calculated by using a rarefaction method as implemented in the program HP-Rare 1.0 (Kalinowski 2005). According to Schwartz and Monfort (2008), six microsatellite loci were sufficient to distinguish individual lynx with an identity probability of 1.55×10^{-6} (Schwartz and Monfort 2008). We thus assessed (1) the probability that one pair of two randomly selected fecal samples show identical genotypes (probability of identity, or PID hereafter) and (2) the probability that two siblings have the same genotype (the probability of identity for siblings, or PIDsibs). The PID and PIDsibs are assessed for each locus but also for all loci (Table 1), and some permutations are carried out in GENALEX in order to examine which loci are most informative for individual identification as the PID and PIDsibs are highly dependent on the diversity at each locus. Because of the small population size of *P. livingstonii* (1300 individuals), a PID of 0.001 seems a reasonable threshold for individual identification (see Waits et al. 2001).

Data simulation

In order to assess the accuracy of the demographic parameter estimations achieved with different field and genetic analysis methods, we simulated different sampling scenarios. We used values from relevant studies for the demographic parameters. The two phylogenetically closest flying fox species for which there are estimates of demographic parameters are the gray-headed flying fox (*Pteropus poliocephalus,* mean body mass of 677 g) and the spectacled flying fox (*Pteropus conspicillatus*, 600 g; Juste et al. 2000, McNab and Armstrong 2001). Fox et al. (2008) reported a first-year survival rate of 0.50 and a reproduction rate of 0.89 from age 3 for *P. conspicillatus* (Fox et al. 2008). For *P. poliocephalus*, both the adult survival rate and the reproduction rate have been estimated at between 0.80 and 0.90 (McIlwee and Martin 2002). While demographic parameters are not available for *P. livingstonii* (a flying fox species with a similar mean

Table 1. Genotyping success per locus, probability of identity (PID), and probability of identity for siblings (PIDsibs) for the seven microsatellite loci.

Locus	GTS	GTSR (%)	ADO	FA	NA	Ne	Ar	PID	PIDsibs
A1	104/143	73	0.006	0.000	3	2.287	2,535	0.262	0.530
C6	109/143	76	0.006	0.000	8	1.920	2,635	0.304	0.567
PH9	106/143	74	0.025	0.000	11	3.548	3,669	0.147	0.451
A2	110/143	77	0.013	0.000	9	4.232	3,969	0.113	0.421
CSP7	107/143	75	0.008	0.006	7	2.698	2,802	0.502	0.704
A3	105/143	73	0.029	0.000	11	1.928	2,620	0.35	0.617
B29	110/143	77	0.050	0.003	4	1.264	1,682	0.741	0.863
Value for 7 loci	110/143	77	0.089	0.012	7.62	2.63	2,840	0.000	0.027

Note: Abbreviations are ADO, allelic dropout; Ar, allelic richness; FA, false alleles rate; GTS, frequency of genotyping success; GTSR, percentage of genotyping success; NA, observed number of alleles; Ne, effective number of alleles.

body mass, 600 g, according to Smith and Leslie 2006), as demographic traits are highly correlated to body mass in animals (Ringsby et al. 2015), we posited that this species' demographic parameters may be relatively close to those of the species cited above. Based on these parameters, we used the stage-based Lefkovitch matrix on three age classes of pre-breeding females (1-year-old individuals, 2-yr-old individuals, and adult individuals) in which the survival probability was fixed at 0.90 for adults, at 0.80 for 2-yr-olds, and at 0.60 for 1-yr-olds (Crouse et al. 1987). The survival rate from birth to age one was fixed at 0.40, and fecundity was fixed at 0.60 for adult females. These values were selected to correspond to those of a long-lived species (as is the case for *P. livingstonii*) and to obtain a population growth rate of 1. In the simulations, the number of individuals in each age class in the first year was obtained from the stable stage structure provided by the stage-based Lefkovitch matrix considering a population size of 1300 individuals (Ibouroi et al. 2018*a*). From year to year, we simulated the individual trajectories (survival or death) and births to get an exhaustive census of all individuals in the population over the entire simulated period. Survival and fecundity were generated including demographic stochasticity (a random trial using a Bernoulli distribution for survival, and a random trial using a Poisson distribution for fecundity).

In a second step, we simulated a CMR sampling scheme by randomly selecting individuals in the population given the capture probability (a random trial using a Bernoulli distribution). We simulated a gradient of capture probability (from 0.10 to 0.60 by intervals of 0.10) and a gradient of monitoring duration (from 3 to 21 yr by intervals of 3 yr). The individual histories obtained were then analyzed with a seniority and survival Pradel model (Pradel 1996) using MARK (White and Burnham 1999). This allowed us to obtain an estimate of survival probability, recruitment probability (1-seniority probability) and recapture probability. Recapture probability was then used to estimate population size using the Horvitz-Thompson estimator (Overton and Stehman 1995). For each scenario of capture probability and monitoring duration, 1000 simulations were conducted. The estimates (and assessment of their precision) were derived from the quantiles of these 1000 simulations (median, 2.5 and 97.5 quantiles). These analyses were performed using the environmental R 3.3.0 package (R Development Core Team 2016) with the library RMARK (Laake 2013).

CMR sampling and cost comparison of methods

For the conventional CMR sampling, *P. livingstonii* individuals were captured using a black nylon mist net (25-mm mesh, 12 m long, 3 m high, four pockets, see Appendix S1 for more details of the capture periods). The physical capture of individuals required four strong youths (local guides) able to climb large trees in order to stretch mist nets along the natural flyways of the fruit bats. According to our observations, the physical capture rate was 1.5 individuals per day (see Appendix S1).

To compare NIGS-CMR and conventional CMR in order to evaluate which method is most relevant for the long-term monitoring of tropical

fruit bat species, we assessed the cost of each approach. For conventional CMR, we assessed the cost of fieldwork, that is, collecting one sample in the field (cost of mist nets and other biological products, foods, travel, salary for local guides etc.), the annual cost to achieve 130 captured individuals, the total cost of monitoring 20% or 30% of the total population over a three-year period (260 or 390 captured individuals over a three-year period), the total cost of monitoring the population during a six-year period with a recapture rate of 0.3, and the total cost of monitoring the population during a nine-year period with a recapture rate of 0.2. For NIGS-CMR, we assessed also the cost of fieldwork, that is, collecting one sample in the field (fecal sample collection, cost of plastic traps and other biological products, foods, travel, salary for local guides, etc.), the cost of processing one sample in the laboratory including cost for molecular products, the cost of successful but also failed samples, the cost for replicated samples, salaries for the laboratory analyses, the annual cost for sampling 190 fecal material in order to successfully identify 130 Livingstone's flying foxes with three successful replicates, the total cost of monitoring 20% or 30% of the total population over a three-year period, the total cost of monitoring the population during a six-year period with a recapture rate of 0.3 as for conventional CMR, and the total cost of monitoring the population during a nine-year period with a recapture rate of 0.2 also as for conventional CMR.

Ethics statement

All animal procedures were carried out in conformance with the Comoros research protocol and animal handling guidelines, and both handling permission (N°13-08/DGEF/DG) and endangered species permits (N° 002/KM/15/DNEF) were granted by the Direction of Environment and Forest of Comoros.

Results

Individual identification by genotyping

Genotype consensus across replicates was established with seven loci (Appendix S2). In total, 143 fecal samples (of the 244 extracted) were amplified with microsatellites. Of these, 109 were successfully genotyped (94 fecal samples successfully genotyped at all loci, 11 samples at 6 loci, and 4 samples genotyped at 5 loci), corresponding to a success rate of 77%. All samples genotyped at less than 5 loci were considered as unsuccessful. From these fecal samples, we identified 98 (68%) different genotypes across the two islands of Anjouan (76 genotypes) and Mohéli (22 genotypes), corresponding to about 7.5% of the total population (98 over about 1300 individuals). The genotyping success rate was high for all loci, varying from 73% (locus A1 and A3) to 77% (locus A2 and B29, Table 1). According to our results, no null alleles were detected. The total allelic dropout (ADO) and false alleles (FA) were low, corresponding, respectively, to 0.089 and 0.012 (Table 1). The number of alleles per locus varied from 3 (A1) to 11(A3). Allele richness varied across locus from 1.68 (B29) to 3.96 (A2, Table 1). Permutations between loci carried out with Genalex indicated that five loci only were required to achieve a PID of <0.001 for P. livingstonii individuals and a PIDsibs of <0.05. PID and PIDsibs were, respectively, 0.0001 and 0.038 for five loci (A1, C6, PH9, A2, and CSP7) or 0.0001 and 0.027 for the seven loci (Table 1).

Demographic parameter estimates using simulated CMR datasets

As expected, the precision of demographic parameters was positively correlated with the number of years of study and with the recapture probability (Figs. 1–3). The results clearly showed that a three-year study, whatever the field effort conducted each year, does not allow parameter estimates with a sufficient level of precision to be useful. Likewise, a field effort achieving 0.10 recapture probability does not provide sufficient data whatever the duration of the study. Simulations showed that the minimum effort required would be a nine-year study with a recapture rate of 0.20, or a six-year study with a recapture rate of 0.30, in order to obtain estimates with a level of precision potentially high enough for a population viability analysis (Figs. 1–3). For instance, the population size estimated with a six-year study and a recapture rate of 0.10 was 1350 (95% CI 500–2000). For a recapture rate of 0.30 with the same duration, it was 1350 (95% CI 1250–1550; Fig. 1). For a nine-year study and a recapture rate of 0.20, the population size was estimated at 1350 (95% CI 1200–1500;

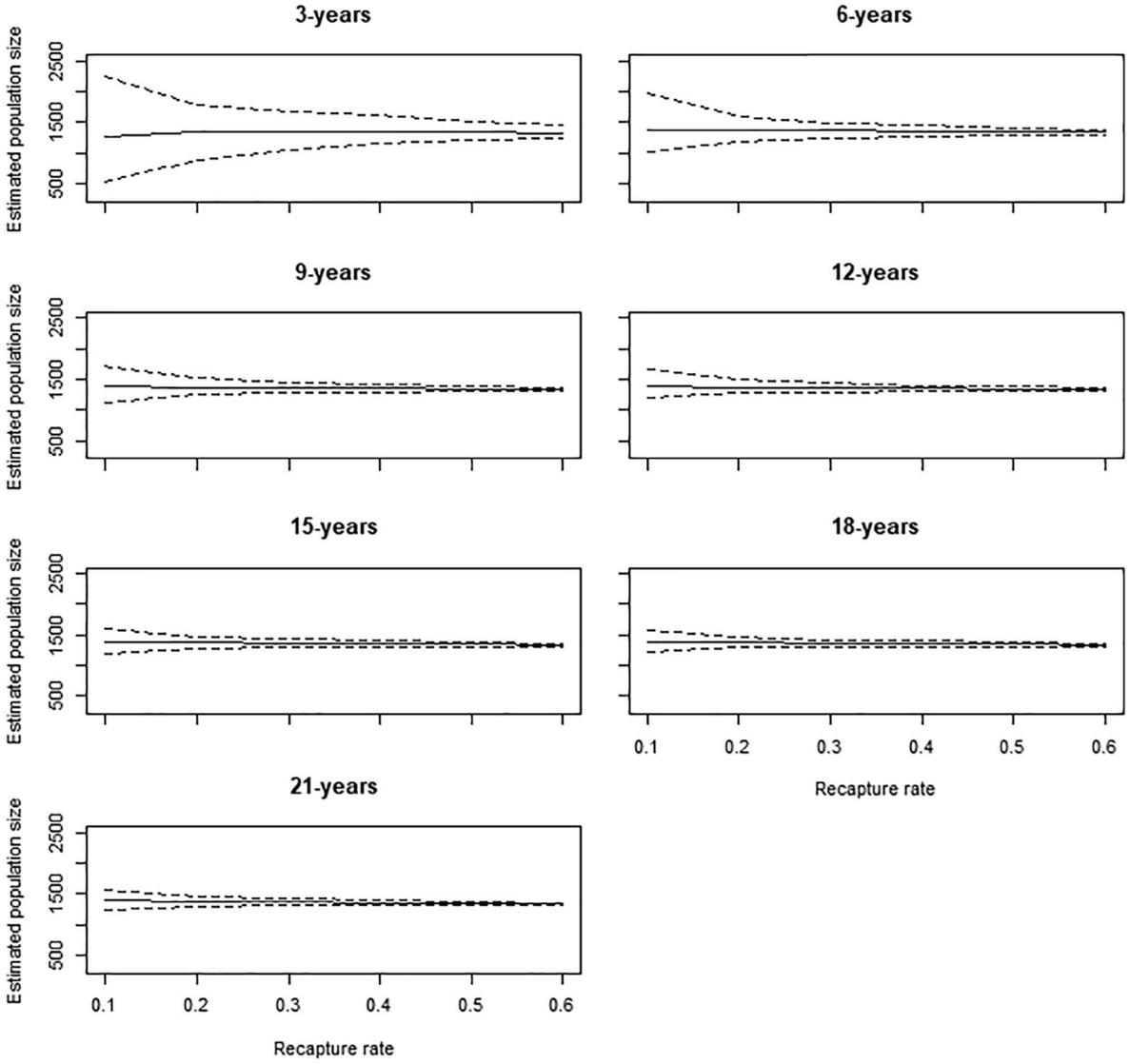

Fig. 1. Estimated population size and 95% CI given varying recapture rates and years of study of *P. livingstonii*; the true population size estimate was considered to be 1300 individuals according to Ibouroi et al. (2018*a*); simulations of population size precision were carried out using 1000 bootstraps and were derived from the quantiles (median, 2.5 and 97.5 quantiles).

Fig. 1). These estimates would allow the detection of a 1.6% decrease in the population each year at the end of the study period.

Monitoring cost of both methods

The cost of the NIGS-CMR method, when considering five microsatellite loci (five microsatellite are the number of loci required to achieve a PID of <0.001 and a PIDsibs of <0.05 for *P. livingstonii* individuals) and a field effort to collect 191 samples annually in order to reach 382 samples over three years and identify 20% of the actual population (see Table 2 for more details) or to reach 574 samples over three years and identify 30% (Table 2), is estimated to be $10,126 annually (including the cost of fieldwork and laboratory processing, Table 2). The total cost for the three-year period is estimated to be $22,926 with a capture

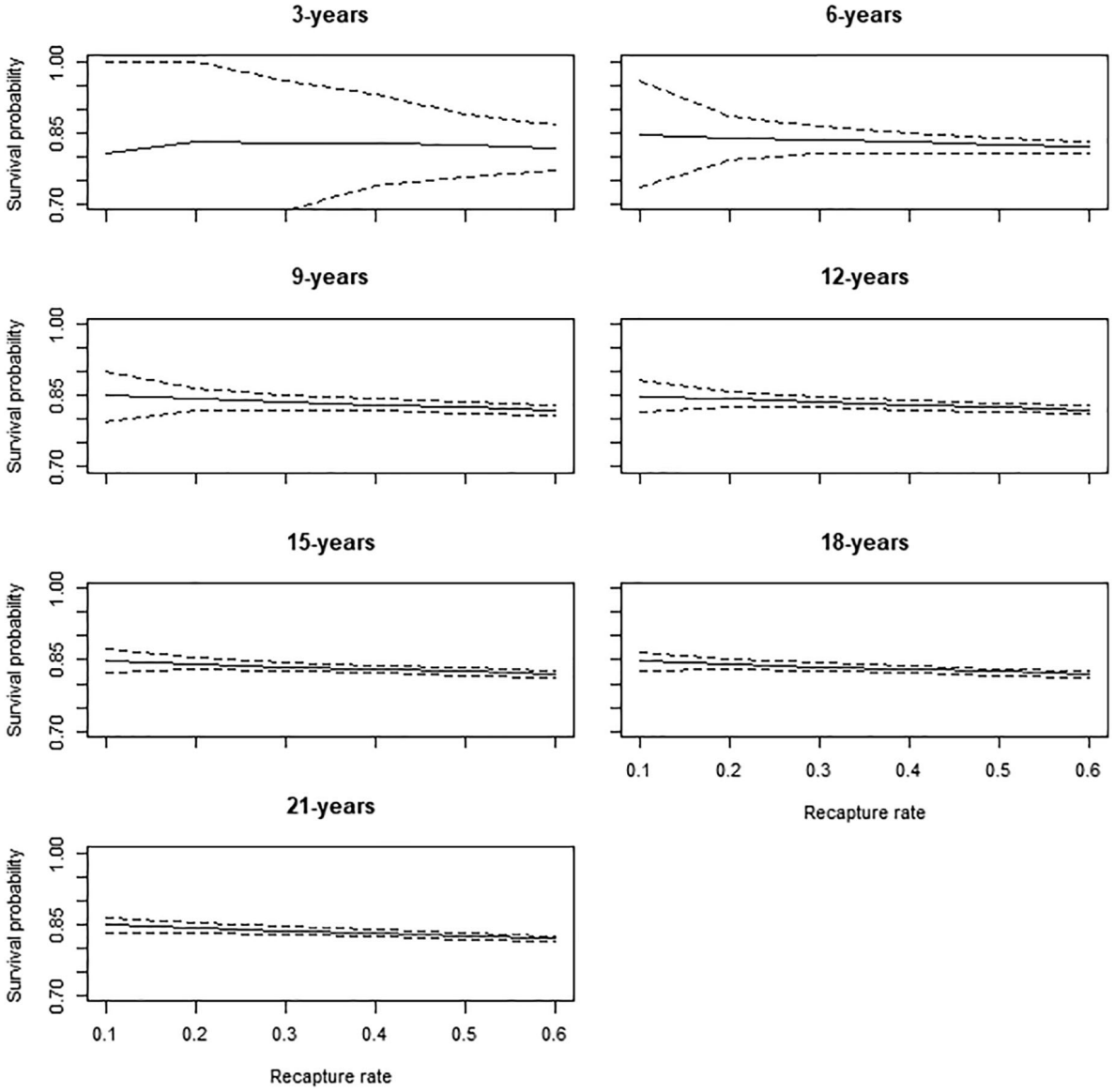

Fig. 2. Estimated survival probability and 95% CI given varying recapture rate and years of study of *P. livingstonii*; the true survival probabilities were considered at 0.90 for adults, 0.80 for 2-yr-olds, and 0.60 for 1-yr-olds (McIlwee and Martin 2002, Fox et al. 2008); simulations of survival probability precisions were carried out using 1000 bootstraps and were derived from the quantiles (median, 2.5 and 97.5 quantiles).

rate of 0.20 or $30,426 with a capture rate of 0.30 (Table 2). The cost per successfully identified sample is approximately $62. For conventional CMR, to achieve the same rate of successfully identified samples (130 captured individuals or 68% of the 191 samples collected in NIGS-CMR), the annual cost is estimated to be $21,580 (Table 3). The cost for a three-year period is estimated to be $43,160 with a capture rate of 0.20 or $64,740 with a capture rate of 0.30 (Table 3). Considering a nine-year monitoring study with a recapture rate of 0.20, the total cost would be $129,480 for conventional CMR compared to $68,778 for NIGS-CMR (almost double the cost for conventional CMR compared to NIGS-CMR). Considering a six-year study with a recapture rate of 0.30, the total monitoring cost

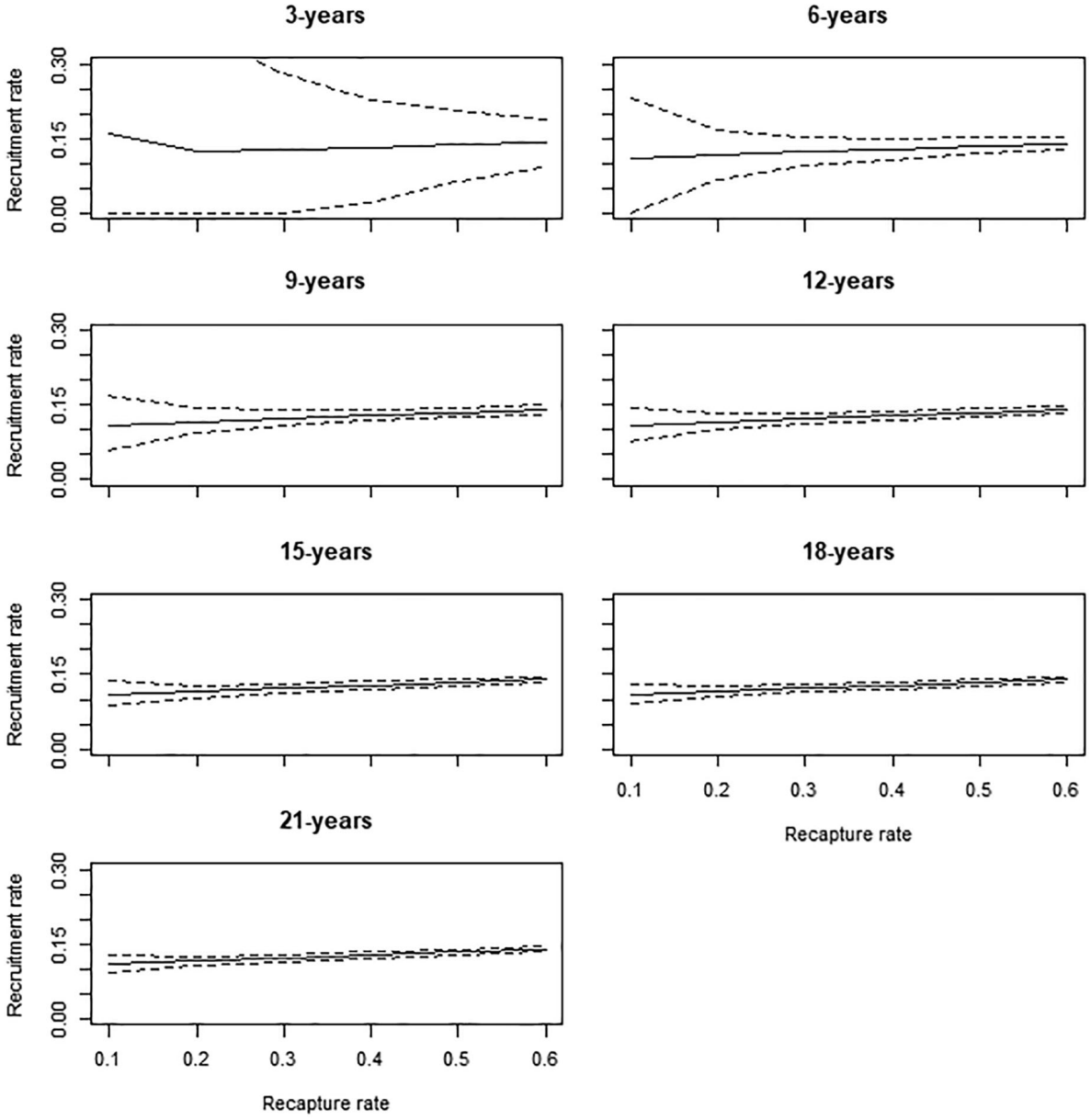

Fig. 3. Estimated recruitment probability and 95% CI given varying recapture rate and years of study of *P. livingstonii;* simulations of recruitment rate precisions were carried out using 1000 bootstraps and were derived from the quantiles (median, 2.5 and 97.5 quantiles).

would remain $129,480 for conventional CMR compared to $60,852 for NIGS-CMR (over twice the cost).

Discussion

Our findings indicate that the individual identification of flying foxes using fecal matter is feasible. The simulations suggest that identifying (whether through capture or other means) 30% of *P. livingstonii* population is the minimum to obtain data allowing an accurate estimation of population size and other demographic parameters. Thus, in terms of cost and feasibility, NIGS-CMR seems a more suitable approach for the long-term monitoring of tropical fruit bat species

Table 2. Evaluation of the simulated expenditure required for noninvasive genetic sampling to assess demographic parameters of *P. livingstonii*.

Sampling and processing activities	Cost for one individual ($)	Annual cost ($), 191 samples	Scenario 1 Cost ($) of 3 yr and 0.3 of recap prob, 574 samples	Scenario 2 Cost ($) of 3 yr and 0.2 of recap prob, 382 samples	Scenario 3 Cost ($) of 6 yr and 0.3 of recap prob	Scenario 4 Cost ($) of 9 yr and 0.2 of recap prob
Field effort	14	2674	8022	8022	16,044	24,066
DNA extraction	9	1719	5166	3438	10,332	10,314
DNA genotyping (77%) of all samples	39	5733	17,238	11,466	34,476	34,398
Total cost	62	10,126	30,426	22,926	60,852	68,778

Notes: Scenario 1, for a three-year period, 574 fecal samples collected of which 77% (441 samples) are successfully genotyped and 68% (390 individuals or 30% of the total population) are identified to the level of individuals. Scenario 2, for a three-year period, 382 fecal samples collected of which 77% (294 samples) are successfully genotyped and 68% (260 individuals or 20% of the total population) are identified to the level of individuals. Scenario 3, for a six-year period with a capture rate of 0.3 based on scenario 1. Scenario 4, for a nine-year period with a recapture rate of 0.2 based on scenario 2.

Table 3. Estimated cost for long-term monitoring of the Livingstone's flying fox using conventional CMR (physical capture), with a capture rate averaging 1.5 individuals per day.

Sampling activities	Cost ($) of one sample	Annual cost ($), 130 individuals	Scenario 1 Cost ($) of 3 yr and 0.3 recap prob, 390 inds	Scenario 2 Cost ($) of 3 yr and 0.2 recap prob, 260 inds	Scenario 3 Cost ($) of 6 yr and 0.3 recap prob	Scenario 4 Cost ($) of 9 yr and 0.2 recap prob
Food, travel and materials	33	4290	12,870	8580	25,740	25,740
Local guides (4)	133	17,290	51,870	34,580	103,740	103,740
Total cost	166	21,580	64,740	43,160	129,480	129,480

Notes: As for NIGS-CMR, the simulated scenarios were as follows. Scenario 1, capture about 30% of the total population of the species in a three-year period (390/1300 individuals). Scenario 2, capture about 20% of the total population of the species in a three-year period (260/1300 individuals). Scenario 3, scenario 1 but over a six-year period. Scenario 4, scenario 2 but over a nine-year period.

compared to conventional CMR which costs twice as much.

To our knowledge, this is the first study assessing individual identification using noninvasive genetic sampling of flying fox species. Our genotyping success rates (77%, Table 1) for the *P. livingstonii* are high in comparison to those obtained by Baldwin et al. (2010) for the gray-headed flying fox (*Pteropus poliocephalus*: 57%) a fruit bat species from the tropical forests of Queensland, Australia. However, our success rates are low/comparable to those obtained in studies of other mammal species: European insectivorous bats (*Rhinolophus hipposideros* 96%, Puechmaille and Petit 2007; *Myotis mystacinus* 72–88% and *Myotis nattereri* 72–90%, Boston et al. 2012) and large primates (*Pan troglodytes* 79%, Morin et al. 2001). Our genotyping errors (Table 3) are also low in comparison to those obtained by Baldwin et al. (who found a total genotyping error rate of 49%, a total ADO rate of 45% and a total FA rate of 3.7%) for *Pteropus poliocephalus*. Yet, these success rates extracted from different studies have to be compared with caution as the context and method used to select successful samples and genotypes can differ from studies to others. The fact that we used a specialized extraction kit and worked in a laboratory for degraded and sensitive DNA may have reduced genotyping errors and increased the accuracy of our genetic results (Puechmaille and Petit 2007, Boston et al. 2012).

Of the seven polymorphic loci we successfully amplified, five were sufficient for identifying

individuals (probability of identity <0.001 and probability of identity for siblings <0.05, Table 1). According to Woods et al. (1999), a minimum PID of 0.001 is required to distinguish between individuals, while a minimum PIDsibs of 0.05 is required to distinguish between siblings (see also Woods et al. 1999, Brinkman et al. 2009, Marucco et al. 2009). This value of PID was sufficiently low to discriminate between individuals accurately since the expected population size was not greater than a few hundred individuals (Woods et al. 1999, Brinkman et al. 2009, Marucco et al. 2009, Gray et al. 2014). The low values of PID and PIDsib obtained for five loci suggest that individuals can be identified using a low number of loci, ranging between 5 and 7 (Woods et al. 1999, Brinkman et al. 2009, Marucco et al. 2009). If the number of loci is too low, it does not allow the differentiation of individuals, potentially leading to an underestimated population size (Woods et al. 1999), conversely a high number of loci can also have a negative impact on individual identification (Fickel and Hohmann 2006). For example, our results estimated a total proportion of false alleles of 0.012 for seven microsatellite loci, corresponding to a mean FA proportion of 0.0017 per locus. Increasing the number of loci can decrease this value, thus generating false unique genotypes, which in turn can lead to an overestimated population size (Fickel and Hohmann 2006). Woods et al. (1999) reported that in brown bears, for instance, between 4 and 6 microsatellites are sufficient to accurately distinguish individuals and siblings (Woods et al. 1999). For *P. livingstonii*, we considered that five microsatellite loci are sufficient to identify individuals given that the values of PID and PIDsib are below the threshold (Table 1).

According to the international union for conservation of nature (UICN 2012), for a population less than 10,000 individuals, a population declining of about 1% each year can be considered as vulnerable. The Livingstone's flying fox population size is estimated to be only 1300 individuals and our simulations predict a population decline rate of 1.6% each year, a number that can be considered as relatively high for this long-lived species giving its low population size, so that this level of precision should be a minimum to reach. According to our results, the precision of the demographic parameter estimates was low for a recapture rate of 0.10 during a 3-yr study (Figs. 1–3). Two sampling designs seemed most effective to obtain useful estimations of demographic parameters: (1) monitoring the species with a sampling effort reaching a recapture rate of 0.20 over a 9-yr period; and (2) monitoring the species with a sampling effort reaching a recapture rate of 0.30 over a 6-yr period.

When applying NIGS-CMR for monitoring tropical bat species, the cost of the first option (sampling during a nine-year period with a recapture rate of 0.20) at $68,778 is higher than the second (sampling during a six-year period with a recapture rate of 0.30) at $60,852. This is because it is less costly to increase the number of fecal samples collected in the same year than to add an additional year of field sessions (collecting more fecal samples when in the field does not increase the cost, whereas increasing the duration of fieldwork, which includes transportation, etc., does). Moreover, the first option has the disadvantage of requiring more time to get accurate demographic parameter estimates, while for threatened species it is important to obtain data as rapidly as possible. In conventional CMR, the two sampling designs have the same cost constraints, as increasing the field effort to capture individuals would considerably increase the field cost (number of local guides, number of mist nets, more time for the survey, etc.). For example, a high number of captures can be obtained by increasing the duration of field session (then the salary of the guides) or by increasing the number of field agents and nets during a short period so that the cost is about the same.

When comparing the two methods, NIGS-CMR appears cheaper than conventional CMR. In addition, data collection is easier in NIGS-CMR and may also involve less capture heterogeneity, as with physical capture some individuals may be more difficult to catch than others. In conventional CMR, the physical capture of *P. livingstonii* is difficult. The species roosts in tall trees on steep slopes, requiring the expertise of local guides to assist in sampling. The number of captures per day was thus very low (1.5), as flying foxes fly very high in the canopy and can avoid nets. Another advantage of NIGS-CMR is that it also avoids trap dependence, a typical source of bias when capture conditions are stressful for

individuals (Boonstra and Krebs 1976). Conventional CMR involves capturing and recapturing certain individuals, which can cause disturbance at the roosting site and may lead to roost abandonment. Moreover, physical capture with nets poses ethical questions for highly threatened species—particularly if the goal is to capture and manipulate 30% of the population every three years as suggested by our simulations for *P. livingstonii*. In this context, while the cost of NIGS-CMR is high, the difficulty of physical capture means the costs of conventional CMR would be doubles that of NIGS-CMR to reach the same capture rate.

Conclusion and Recommendations

Demographic parameters including population size are among the most important data to acquire to develop relevant conservation strategies. The results of our study confirm the potential of using NIGS-CMR to provide such information for critically endangered tropical frugivorous bats, including for species for which physical capture is an option. Yet, the protocol we used could be further optimized in order to provide higher rates of amplification for microsatellite loci. First, collecting fecal samples from frugivorous animals in wet forests during the rainy season is tricky since the feces are relatively liquid due to the frugivorous diet and because of the rainfall. In such conditions, using a plastic trap alone for feces collection, as we did, might not be the optimal protocol. Placing a cloth just above the plastic would allow fecal samples to dry quickly by filtration. Secondly, as has been highlighted by many studies (Puechmaille and Petit 2007, Boston et al. 2012), higher rainfall degrades fecal samples. We thus recommend sampling during the dry season if possible. If using the silica gel conservation method, we recommend checking samples regularly for potential moisture saturation and contamination by mildews. In some cases, silica gel should be replaced to avoid mildew and saturation. Also, according to some studies, the PCR success rate decreases with the length of time the sample is preserved before DNA extraction (Boston et al. 2012), so we recommend a minimum conservation duration to minimize DNA degradation. As regards processing in the laboratory, we recommend testing different extraction kits and protocols to select the most efficient one. Taberlet et al. (1997) found that the use of a multiple-tubes approach allows the genotyping error rate to be verified, increasing result accuracy and reducing false interpretations.

The NIGS-CMR approach is feasible for monitoring flying fox species as long as multiple strategies for minimizing errors are taken into account during field sampling and laboratory analysis. Moreover, this method is arguably more appropriate for conservation purposes than conventional CMR approaches as it is less harmful for individuals, and is also less expensive compared to conventional CMR. An additional benefit is that within the same NIGS-CMR analysis other types of information—such as genetic diversity and social structure/ organization (Solberg et al. 2006) or dispersion between roosts or between seasons—can also be provided (Mengüllüoglu et al. 2019). To make NIGS-CMR easier and less expensive in contexts with cost constraints, collaborative sampling can be undertaken with other national and international NGOs and institutions. For instance, in the Comoros, direct counts at roosts are usually conducted annually by national and international NGOs (e.g., Dahari and Bat Conservation International for *P. livingstonii*). In these field sessions, fecal material could be collected, rendering the field effort to collect fecal samples even cheaper. Beyond *P. livingstonii*, our approach combining a pilot study and simulations could be widely applied to other rare species of conservation concern—fruit bats or other mammals from tropical habitats—in order to choose the most efficient monitoring method. We strongly advocate adopting such an approach when defining monitoring protocols.

Acknowledgments

We would like to thank the Comoros Department of the Environment and Forests for granting permission to carry out our fieldwork and to export samples (authorization number 002/KM/15/DNEF). The fieldwork was funded through research support grants from the Rufford Foundation (grant nos. 26731-2 for M.T.I., 19010-1 for A.C., and 21803-1 for S.AO.D.) and by the French National Center of Scientific Research (CNRS) Center of Functional and Evolutionary Ecology

(CEFE). The data used in this study were obtained using the technical equipment at the degraded DNA and genotyping–sequencing platforms at the Mediterranean Center for the Environment and Biodiversity (CeMEB) Laboratory of Excellence in Montpellier, France. At last, we would like to thank the two anonymous reviewers of this manuscript; they provided extensive and critical corrections and comments for the improvement.

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Supporting Information

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