1 **Title**

2 A novel environmental DNA (eDNA) sampling method for aye-ayes from their feeding

3 traces

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## Abstract

Non-invasive sampling is an important development in population genetic monitoring of wild animals. Particularly, the collection of environmental DNA (eDNA) which can be collected without needing to encounter the target animal, facilitates the genetic analysis of cryptic and threatened species. One method that has been applied to these types of sample is target capture and enrichment which overcomes the issue of high proportions of exogenous (non-host) DNA from these lower quality samples. We tested whether target capture of mitochondrial DNA from sampled feeding traces of wild aye-ayes would yield mitochondrial DNA sequences for population genetic monitoring. We sampled gnawed wood from feeding traces where aye-ayes excavate wood-boring insect larvae from trees. We designed RNA probes complementary to the aye-aye's mitochondrial genome and used these to isolate aye-aye DNA from other non-target DNA in these samples. We successfully retrieved six near-complete mitochondrial genomes from two sites within the aye-aye's geographic range that had not been sampled previously. This method can likely be applied to alternative foraged remains to sample species other than aye-ayes. Our method demonstrates the application to next-generation molecular techniques to species of conservation concern.

**Key words:** Aye-aye, Endangered, Environmental DNA, Lemur, Madagascar, Mitogenome, Non-invasive sampling, Target capture

## Introduction

Genetic sampling of wild populations can help us to address questions of demography, individual relatedness, population structure, and other important aspects of biodiversity that cannot be answered by behavioural monitoring alone (Allendorf, Hohenlohe, &

Luikart, 2010). However, the practicality of sampling wild animals can be limited by ethical implications due to the implicit risks of sedating animals to collect blood or tissue samples, especially for arboreal species, and for rare or elusive species (including nocturnal animals), by infrequent encounter rates. (Kohn & Wayne, 1997; Goossens, Chikhi, Utami, de Ruiter, & Bruford, 2000). Even when it is possible and safe to collect invasive samples from wild individuals, the number of samples that can be obtained with this approach may be fewer than analytically desirable (Sikes & Gannon, 2011). Under these conditions, non-invasive sampling is a valuable tool in advancing our understanding of wild populations.

Over the last two decades, advances in molecular biology have facilitated the use of non-invasive sampling for genetic analyses of wild populations (Morin, Wallis, Moore, Chakraborty, & Woodruff, 1993; Joost et al., 2007). Furthermore, traces of DNA left in the environment from biological material (eDNA) can be collected without even encountering target individuals. Source materials for non-invasive or eDNA sampling that have been used in wildlife monitoring and forensic studies include feathers, faeces, egg shells, hair, saliva, urine, and snail trails (Valiere & Taberlet, 2000; Inoue, Inoue-Murayama, Takenaka, & Nishida, 2007; Beja-Pereira, Oliveira, Alves, Schwartz, & Luikart, 2009; Sastre, Francino, Sanchez, & Ramirez, 2009). Non-invasive and eDNA sampling are particularly valuable in research surrounding unhabituated populations, cryptic species, and those at risk of extinction (Goossens et al., 2000; Cushman, McKelvey, Hayden, & Schwartz, 2006; KendallL et al., 2009; Clevenger & Sawaya, 2010; Schubert et al., 2011; Morin, Kelly, & Waits, 2016; Orkin, Yang, Yang, Yu, & Jiang, 2016).

One species that could especially benefit from the advances in non-invasive genetic sampling is the aye-aye (*Daubentonia madagascariensis*), which is a rare and elusive Malagasy primate of major conservation concern (Sterling & McCreless, 2007; Schwitzer et al., 2013). The low population density, large home range size, cryptic nature, and nocturnal activity of aye-ayes make them particularly difficult to locate, and precise distributions and population densities are unclear (Sterling, 1994b). Challenges of monitoring aye-ayes and obtaining reliable population dynamics data are reflected in the volatility of aye-aye's conservation status designations over the last 70 years. In the 1950's, the aye-aye was thought to be extinct (Sterling, 1994b). After aye-ayes were rediscovered in 1957, they were classified as Endangered; in 2008, their status was changed to Near Threatened before being reassessed as Endangered in 2012 (Andriaholinirina et al., 2015). In addition to being listed as Endangered, aye-ayes are currently considered one of the world's top 25 most endangered primates (Randimbiharinirina et al., 2017 in Schwitzer et al. 2016-2018). Few encounters, along with the aye-aye's solitary social organization and long maternal investment suggest low population densities. Low nuclear genomic diversity in aye-ayes reflects these assumptions; genomic analyses estimates of heterozygosity of 0.051%, and genetic diversity across synonymous sites of $\pi$= 0.073, are the lowest of any primate species studied to date (Perry, Melsted, et al., 2012; Perry, Reeves, et al., 2012). Therefore, despite the wide distribution of aye-ayes, there are likely few individuals, increasing the risk of local and global extinction (Schwitzer et al., 2013; Gross, 2017).

The IUCN's lemur survival strategy recognises the need for biological monitoring of aye-ayes to better assess population status and conserve genetic diversity within this lineage (Schwitzer et al., 2013). In addition to their elusive behaviour making aye-ayes difficult

to find and monitor, genetic sampling of individuals once they have been located is challenging; invasive collection of blood and tissue can only be achieved during immobilization, which is risky and must be conducted by trained and experienced personnel (Cunningham, Unwin, & Setchell, 2015). Therefore, to assess genetic diversity and identify priority populations for conservation a new, reliable means of non-invasive sampling in aye-ayes is required.

Reliable genotyping from non-invasively collected material holds much promise in sampling threatened species. Environmental DNA samples that are degraded due to exposure to both biotic and abiotic factors or contain high proportions of exogenous DNA can now provide reliable genetic markers (Beja-Pereira et al., 2009; Carpenter et al., 2013; Snyder-mackler et al., 2016). Techniques such as high-throughput sequencing technologies reduce sequencing errors and improve genotyping accuracy by providing greater depth of coverage across loci. One particularly promising approach is target capture, which provides a means of isolating endogenous DNA from the high proportions of exogenous DNA in non-invasive samples (Bi, Linderoth, Vanderpool, Good, & Nielsen, 2013; Hawkins et al., 2015; Kirillova et al., 2015; Kistler et al., 2015; Mohandesan et al., 2017). Specifically, RNA probes which are complementary to particular regions or markers in the genome of the target organism are specially designed and synthesized (Gnirke et al., 2009). These probes hybridize to the endogenous DNA in the sample. After hybridization, the biotin coating of the probes allows streptavidin-coated magnetic beads to bind; the bound probes and hybridized endogenous DNA can then be isolated from the exogenous DNA by using a magnet (Gnirke et al., 2009; Giolai et al., 2016). These developments make monitoring and sampling of wild populations where individuals are difficult to locate increasingly feasible.

125

126 For primates, the application of target capture from low quality samples has largely been
127 applied to ancient DNA studies, but Perry et al., (2010) and Snyder-Mackler et al., (2016)
128 have presented effective approaches for sampling endogenous DNA from faeces in extant
129 primate species. Recently, Chiou & Bergey , (2018) demonstrated a different method that
130 isolates endogenous DNA from primate faeces by targeting the higher CpG-methylation
131 density of vertebrate taxa relative to the exogenous bacterial and plant DNA in faeces.
132 Although faecal collection is a popular source of non-invasive sampling of primates (Oka
133 & Takenaka, 2001; Quéméré, Crouau-Roy, Rabarivola, Louis, & Chikhi, 2010; Chancellor
134 et al., 2011), it is unlikely to be a feasible method of sampling for aye-ayes. Factors such
135 as aye-aye's nocturnal behaviour, the height that they travel in the canopy, and their large
136 nightly travel distances mean that defecation is difficult to observe and locating and
137 collecting faecal material is problematic (Randimbiharinirina et al., 2017); accordingly,
138 at two sites where aye-ayes have been monitored by Madagascar Biodiversity
139 Partnership since 2010, aye-aye faecal samples have only been collected through routine
140 immobilizations.. Therefore, to gain information on the genetics of aye-aye populations
141 to meet the IUCN aims, in this paper, we explore the possibility of sampling eDNA from
142 aye-ayes (Schwitzer et al., 2013).

143

144 One potential source of eDNA in aye-ayes is from their distinct feeding traces left on trees.
145 These traces are associated with their adaptations for extracting the larvae of wood-
146 boring insects from tree trunks and branches, after identifying suitable foraging locations
147 via a process of sniffing, lightly tapping, and listening (Sterling, 1994a; Erickson, 1995).
148 Aye-ayes gnaw into selected areas of trees with their elongated and continuously growing
149 incisors and extract larvae using their thin flexible third digit (Sterling, 1994a). During

150 this foraging process, the buccal cavity of the aye-aye comes into contact with the wood
151 (Sterling, 1994a; Erickson, 1995; Sterling & McCreless, 2007; arkive.org, 2017). Thus,
152 trace amounts of aye-aye biological material in the form of epithelial cells of the buccal
153 mucosa or from saliva may be deposited and accessible as an eDNA source.
154
155 We investigated the application of target capture and enrichment to obtain aye-aye DNA
156 from aye-aye feeding traces. We aimed to determine whether this method is a feasible
157 alternative to invasive sampling. If eDNA samples provide a means of remotely sampling
158 wild aye-aye populations, we predict that (1) target enrichment is an effective method of
159 obtaining aye-aye DNA from the exogenous DNA in feeding traces, and (2) we will be able
160 to obtain full mitochondrial genomes (hereafter 'mitogenomes') for population genomic
161 analysis.
162
163 **Methods**
164 **Sampling sites and techniques**
165 Initial training for researchers and local technicians on accurate identification of aye-aye
166 feeding traces and the sample collection method was conducted through the Kianjavato
167 Ahmanson Field Station (KAFS) located in the Kianjavato commune of the Vatovavy-
168 Fitovinany region in southeast Madagascar during June 2014. KAFS is a long-term field
169 site of the Madagascar Biodiversity Partnership (MBP). Individual aye-ayes present in the
170 region have been fitted with ATS (Applied Telemetry Systems®) VHS radio-collars for
171 behavioural monitoring, which afforded the opportunity to view known feeding sites in
172 an area of bamboo to become familiar with density and frequency of feeding traces, their
173 characteristics and to practice the collection technique (see below).
174

To then test the sampling method on unmonitored populations, we selected two study sites near to the limits of the aye-aye's geographic distribution, where aye-ayes have been sighted but not sampled: Manombo Special Reserve in the Atsimo-Atsinanana region in southeastern Madagascar, operated by Madagascar National Parks and at that time supported by Durrell Wildlife Conservation Trust, and the Tsingy de Beanka, Ambinda in the Melaky region in west-central Madagascar and supported by Biodiversity Conservation Madagascar (Figure 1). Between July and December 2014, MA surveyed the forest at each site with the assistance of three local technicians. Prior to sampling, local technicians cleared undergrowth from existing trail systems in the forest to use as transects. During daylight hours, these transects were walked at a slow pace of approximately 1 km/hr to look for feeding traces and searched areas on either side of the transect at 200 m intervals. Traces were observed in both live and dead plants where characteristic holes gnawed by aye-aye were identified. These were confirmed by the distinct shape of feeding traces and typically presence of teeth marks (Figure 2). The research permit (N$^{o}$ 162/14/MEF/SG/DGF/DCB.SAP/SCB) for sample collection was obtained from the Ministere de l'environment, des eaux et forets et du tourisme, in Madagascar.

To collect samples, we selected areas around the edge of the trace where the buccal cavity and teeth of the animal were likely to have come into contact with the tree (Figure 2). MA took 3-6 samples of tree material per trace; each sample was approximately 2 $g^{-1}$ of wood shavings. MA collected samples using a scalpel to cut wood from the site directly into a 1.5 mL Eppendorf® tube containing 500 µL RNAlater. To reduce the chance of contamination, MA wore latex gloves and used a new, sterile scalpel blade for each sample. At Manombo, we walked 9.5 km of transects over a 6 $km^2$ area. At Beanka, we

surveyed 10.8 km of transects covering a total 9 $km^2$ area. MA sampled a total of 59 traces across the two sites (Manombo: n=27; Beanka: n=32). We stored samples at ambient temperature until they could be transferred to 4 $^oC$ for storage in Antananarivo, Madagascar. MBP exported samples from Madagascar (Export Permit Number 233N-EV07/MG16) and stored the samples at Omaha's Henry Doorly Zoo and Aquarium at 4 $^oC$ for four months prior to processing.

**DNA extraction, library preparation, capture, and sequencing**

After samples were removed from storage, we extracted DNA from all samples. We vortexed each sample for 30 seconds in PBS to wash aye-aye DNA from the surface of the wood and then removed pieces of wood using sterile forceps. We added 500 µL of PBS solution to the sample and centrifuged at 10,000 g to pellet the cellular DNA and then carefully removed the supernatant so as not to disturb the precipitate. We performed all DNA extractions on the precipitate using EZNA® blood DNA mini kit following the buccal swab protocol with the following modifications: we lysed samples overnight, and we eluted DNA in 50 µL elution buffer, incubated for 20 minutes and then re-eluted with the same eluate. Preliminary tests to confirm the presence of aye-aye DNA were conducted using a microsatellite marker for loci AYE33 of approximately 279 bp, with the following primer pair: forward 5'-3' GTCTGCTACTCCTTAGGTGCTG and reverse 5'-TGGCTCAGGGCAATACAAT-3'. We amplified the extracted DNA in a 15 µL reaction containing 0.25 µL of each 10 uM primer, 7.5 µL buffer containing 1.5 mM $MgCl_2$ (1X) and 0.2 mM of each dNTP (1X), 0.12 µL KAPA3G plant DNA polymerase, 4.9 µL $H_2O$, and 2 µL DNA sample. Cycling parameters began with an initial enzyme activation temperature of 95 $^oC$ for 3 min, followed by 32 cycles of: denaturation at 98$^oC$ for 20 s, primer annealing at 58 $^oC$ for 15 s and elongation at 72 $^oC$ for 30 s, then one cycle of final elongation at 72

225 °C for 1 min. We visually confirmed the presence of the expected 279 bp PCR product by
226 gel electrophoresis with 5 µL of PCR product and using 2 µL of a 1 kb ladder.
227
228 Extracted DNA was transported to Pennsylvania State University, University Park, PA for
229 library preparation and DNA capture. We quantified the DNA concentration and fragment
230 size of ten samples using a bioanalyzer (Agilent Technologies, Santa Clara, CA) at the Huck
231 Institute of the Life Sciences (HILS), University Park, PA, genomics core facility. We
232 selected a subset of 22 samples that were estimated to have been between 14 h-6 months
233 old at the time of collection based on observations during surveys and discolouration of
234 wood. We prepared DNA sequencing libraries from each of these samples using the
235 Illumina TruSeq nano® kit. The TruSeq nano® library preparation protocol recommends
236 200 $ng^{-1}$ of input DNA. Due to the typically low quantities of DNA extracted from each
237 individual wood sample (<300 pg/uL), we combined DNA extracted from multiple
238 samples taken from the same trace into one sample per feeding trace to increase total
239 amounts of library preparation input DNA per sample to approximately 5-42 $ng^{-1}$ DNA
240 (based on bioanalyzer readings for a subset of samples). To account for the low input DNA
241 quantities, we adapted the protocol to use half quantities of all reagents and volumes. We
242 sheared each DNA sample to a mean size of 500 bp with the Covaris-M220® using the
243 following settings: 34 seconds, peak incident of 50, duty factor of 20% and 200 cycles per
244 burst. For library preparation wash steps, 180 µL ethanol was used and an additional
245 initial wash step was included to remove residual salts from eluting extracted DNA. We
246 used the Bioanalyzer (Agilent Technologies, Santa Clara, CA) to quantify the final library
247 and confirm a mean library size of 750 bp fragments, as expected for 550 bp libraries
248 once the sequencing adapters and unique barcodes are attached to the 550bp DNA
249 fragments.

As a preliminary test to check for the presence of aye-aye DNA in these libraries, we shotgun sequenced ten DNA libraries. Libraries were included as part of a larger multiplexed sequencing pool (Table A1) and sequenced using the Illumina NextSeq® 500 2x150 pair end chemistry at UCLA Clinical Microarray Core Facility. Samples were demultiplexed at the sequencing facility.

RNA probes for capture of the aye-aye mitogenome were designed through MyBaits® at Arbor Biosciences™. Baits were designed based on the aye-aye reference mitochondrial DNA genome sequence (GenBank® accession NC_010299.1), bait length was 80 bp at 4x tiling and these are available as a predesigned MyBaits® Mito panel. We conducted captures as per MyBaits® manual v.3 and a single round of captures was conducted on 19 of the eDNA (foraging remains) samples. Post capture products were quantified using Bioanalyzer (Agilent Technologies, Santa Clara, CA) at the genomics core facility at the HILS and pooled for sequencing (Table A2). These capture products were sequenced as one pool of 17 samples and two of the samples (BTS38 and BTS108) were included as part of a different sequencing pool. The final mitochondrial capture pool of 17 Mito Bait captured DNA libraries was sequenced on one lane of the NextSeq 500 mid-output 2x150 bp pair end chemistry UCLA Clinical Microarray Core Facility. Samples were demultiplexed and barcodes removed at the sequencing facility.

**Data analysis**

All computational analyses were conducted on servers provided by WestGrid (www.westgrid.ca) at Compute Canada (www.computecanada.ca). Raw reads were submitted to the NCBI SRA project under the accession PRJNA434884. The following

processing and alignment pipeline was used for the shotgun and captured DNA sequencing pools. We assessed sequence read quality using Fast-QC (Andrews, 2007) and filtered reads based on minimum read length of 120bp and mean quality of 20 using PRINSEQ-lite (Schmieder & Edwards, 2011). We removed any potential human contaminant reads using the bbsplit package of bbmap (Bushnell, 2016); sequencing reads were aligned to both the aye-aye and human genome and any reads which matched better to the human genome than the aye-aye genome were removed from the dataset. Paired reads were aligned to the aye-aye reference genome GCA_000241425.1 (Perry, Reeves, et al., 2012), using the bwa-mem alignment algorithm (Li & Durbin, 2009; Li, 2013). We used the MarkDuplicates tool in PicardToolsv.1106 (Broad Institute, 2017) to remove sequencing and PCR duplicates, and reads flagged as clipped were filtered from the dataset. We generated consensus sequences for the eDNA samples with near-complete mitogenomes using samtoolsv.1.3, bcftoolsv.1.3 and vcfUtilsv.1.3 (Li et al., 2009; Danecek et al., 2011; Li, 2011). To confirm the base calls in these consensus sequences, we compared the samtoolsv.1.3 generated sequences to those generated using IGV (Robinson et al., 2012) to ensure the same nucleotide bases were found by each software program.

We compared the mitogenome sequences from our eDNA samples with the GenBank® aye-aye mitogenome data to confirm that they were novel (Perry et al., 2013; Kistler et al., 2015). We called variants using GATK (McKenna et al., 2010), and used haplotype caller to generate individual gVCFs and then the joint variant caller. To filter for biallelic SNPs only, we used VCFtoolsv.1.12 and set maximum and minimum alleles to two.

We used PGDSpiderv2.3 to convert SNP variant calls from vcf to ped format (Lischer & Excoffier, 2012). We used the --cluster and --matrix commands in Plinkv.1.7 (Purcell et al., 2007) to calculate the pairwise proportion (identity by state, IBS) of shared alleles at SNP loci among all complete mitogenome sequences. For the Plink analysis, we used the complete mitochondrial genomes excluding the hyper-variable region given the potential for homoplasy in this region and the possibility for multiple mutations per site (Ballard & Whitlock, 2004; Gonder, Mortensen, Reed, De Sousa, & Tishkoff, 2007).

## Results

### Pre-capture processing

After library preparation, shotgun sequencing of libraries from eDNA samples did not yield sufficient mitochondrial DNA for mitogenome analysis. Of the 10 libraries that were shotgun sequenced, seven yielded fewer than 10 reads aligning to the aye-aye mitochondrial genome; one sample, with the highest number of mitochondrial reads, was MSR58 with 189 unique reads (0.000274%) (Table 1).

### Target capture DNA sequencing

Target captures of the mitogenome increased the proportion of on-target mitochondrial DNA reads by several orders of magnitude compared to reads yielded from shotgun sequencing without capture (Table 1). For samples for which it was possible to calculate the fold enrichment (i.e., the samples that were both shotgun sequenced and sequenced following DNA capture), the percent of unique on-target reads increased by over 100 times. For captured samples, the percent of on-target reads ranged from 0.009% to 27.5% of the total reads, whereas the percent unique on-target reads ranged from 0.001% to 0.05% (Table 2).

We recovered near-complete mitogenomes for six of the 19 captured samples (31.6%). The depth and breadth of coverage varied between samples (Table 1). For these six mitogenomes, the mean depth of coverage was 16x (range 8-25x). For the 13 samples with less than 90% coverage, the percent of the genome covered ranged from 10-71% (mean 32.58%) with a mean depth of 1.7 (+/-0.3-2.8). Five of these 13 samples had a breadth of coverage between 45-70% and were all identical at SNP loci to the samples with greater breadth of coverage. We only included samples with coverage greater than 90% in downstream analysis.

**Mitogenome analysis**

Based on multiple sequence alignment of these seven near-complete mitogenomes, we identified one unique mitogenome sequence from Manombo Special Reserve and two unique mitogenomes from Tsingy de Beanka. Alignment with previously published GenBank® sequences that the mitogenome sequences obtained from the eDNA samples were unique to all previously published aye-aye mitogenomes. For the GenBank sequences sample locations from northern Madagascar, we follow the population names in Perry et al., (2013); North, East, and West (Figure 1). When we compared SNP loci among samples, all mitogenomes clustered into distinct populations according to their collection location (Figure 3). Pairwise comparison of genotypes at polymorphic loci indicate that there are four distinct populations (Figure 3). The Tsingy de Beanka population showed moderate differentiation from the other populations (Figure 3). There was also a split between the East population and Manombo in the southeast, while the North population shared the fewest alleles with all other populations.

## Discussion

We tested whether sampling of aye-aye feeding traces is a feasible method of non-invasive sampling of this species. Our first prediction, that target capture would provide a means of sampling aye-aye DNA from these feeding traces, was supported by the increased amount of endogenous DNA obtained from target capture compared to shotgun sequencing. The number of on-target reads from the shotgun sequencing was not at sufficient coverage across the mitogenome to identify polymorphic sites and genotype individuals. The high level of enrichment from captured libraries compared to shotgun sequencing indicates the efficacy of a target capture approach (Gnirke et al., 2009). Our second prediction, that mitogenomes could be obtained from these samples and used for population genomic analysis, was also supported. We were able to obtain near complete mitogenomes for 31.6% of the processed samples. Previous studies that have sampled eDNA from foraged material have typically only retrieved partial D-loop fragments rather than whole mitogenomes (Nichols, Konigsson, Danell, & Spong, 2012; Caniglia, Fabbri, Mastrogiuseppe, & Randi, 2013; Wheat, Allen, Miller, Wilmers, & Levi, 2016). While partial fragments can be useful in metabarcoding studies for species detection, whole mitogenomes provide greater phylogenetic resolution and reduce the likelihood of including nuclear copies of mitochondrial DNA(numts) (Johnsen, Kearns, Omland, & Anmarkrud, 2017).

### Future directions for methodological improvements for sampling aye-aye eDNA

The method developed here is a valuable tool to extend sampling of unmonitored aye-aye populations. Yet, improved efficacy of the method to increase amount of aye-aye DNA obtained and therefore reduce the cost per sample could make it a more attractive application for conservation monitoring (Ekblom & Galindo, 2010). Low proportions of

374 on-target, unique sequence reads recovered are common when working with
375 environmental samples (Ávila-Arcos et al., 2015; Nielsen et al., 2017) and adequate
376 sampling effort is required to account for this issue. A mode of three different samples
377 was taken per feeding trace, and it would be feasible to double this number. Therefore,
378 we recommend more intensive sampling of each feeding trace to allow for multiple
379 library preparations per trace, whilst maintaining sufficient amounts of input DNA for
380 TruSeq nano® library preparation. Multiple library preparations for each trace prior to
381 capture may increase the number of unique DNA fragments within the samples and
382 reduce PCR bias. The proportion of unique reads aligning to the target region was
383 relatively low (0.4-12 %). To better assess the number of PCR cycles to run post-capture
384 to achieve sufficient concentrations for sequencing, qPCR quantification may be valuable
385 to ensure reduced PCR duplication, therefore improved sequencing efficacy and
386 ultimately increased cost effectiveness (Enk, Rouillard, & Poinar, 2013).
387

388 **Application towards addressing the IUCN's objectives for aye-ayes**

389 We demonstrated a novel method that can be applied across the species range for
390 population genomic monitoring. This method helps to meet objective seven of the IUCN's
391 lemur survival plan set out by Schwitzer et al., (2013:page32) which is "Fill knowledge
392 gaps in population ecology and biodiversity of lemurs, and increase training of Malagasy
393 scientists". Our method provides a means of genomic biodiversity monitoring using
394 cutting edge molecular techniques. Application of this method allowed us to successfully
395 recover six aye-aye mitogenomes from sites in the south-east and west of Madagascar
396 where aye-ayes have not been sampled previously. The wide distribution of aye-ayes
397 across Madagascar means sampling across environments is key in effective population
398 monitoring at local and national scales (Schwitzer et al., 2013). The different habitat types

399 that aye-ayes are distributed in range from rainforests in southeast Madagascar, a habitat
400 with relatively humidity and rainfall, to the dry deciduous forest in the west of
401 Madagascar, with less dense canopy cover and likely greater exposure to direct UV
402 radiation (Rakoto-Joseph, Garde, David, Adelard, & Randriamanantany, 2009;
403 Andriamisedra, Aylward, Johnson, Louis, & Raharivololona, 2015). Our recovery of
404 mitogenomes from distinct environments indicates these abiotic factors do not
405 necessarily preclude the ability to sample eDNA from these areas and this method can
406 likely be applied to sample in habitat across the species' geographic distribution (Sterling
407 & McCreless, 2007). The mobility of this sampling technique along with little training
408 required for sample collection provides a means of surveying large areas of aye-aye
409 habitat in a relatively short time frame. The ease of sampling and ability to survey large
410 areas provide the opportunity for sampling multiple individuals across a population and
411 accessing mitogenome diversity within forest fragments.
412
413 Our preliminary analysis revealed high differentiation between the North population and
414 all other sampled locations, consistent with the whole genome analysis of Perry et al.,
415 (2013) which showed the North population to be the most genetically distinct of three
416 regional populations. Given that our relatively sparse sampling strategy revealed two
417 genetically distinct populations, we encourage the application of this method and the use
418 of the predesigned aye-aye Mito panel from MyBaits® to sample additional areas of aye-
419 aye habitat.
420
421 **Applications to other species**
422 The aye-aye is arguably one of the most elusive lemur species in Madagascar (Schwitzer
423 et al., 2013), and other endangered species that are difficult to locate could also benefit

424 from eDNA sampling. The Critically Endangered *Prolemur simus* leave traces on bamboo
425 plants which are similar to those of sympatric bamboo lemurs that feed on the same plant
426 species (Tan, 1999; Ravaloharimanitra et al., 2011). Application of the method presented
427 here could confirm the presence of these threatened species which are typically sparsely
428 distributed and difficult to locate in the wild (Wright et al., 2008; Ravaloharimanitra et
429 al., 2011; Frasier et al., 2015). Similarly, Critically Endangered *Eulemur cinereiceps* could
430 be sampled via traces on gnawed fronds of *Cecropia peltata* trees (Ralainasolo,
431 Ratsimbazafy, & Stevens, 2006). Although the method developed here is novel, sampling
432 of saliva from foraged material has been used previously as a means of non-invasive
433 sampling; gorillas and golden monkeys discard plant material (Smiley et al., 2010),
434 ungulates leave saliva traces on foraged twigs (Nichols et al., 2012), and large carnivores
435 leave saliva on prey (Blejwas, Williams, Shin, Dale, & Jaeger, 2006; Sundqvist, Ellegren, &
436 Vila, 2008; Glen et al., 2010; Caniglia et al., 2013; Wheat et al., 2016). This method of target
437 capture could be applied to these sources to sample mitogenome eDNA from a range of
438 species.
439
440 **NGS molecular techniques and conservation**
441 This method provides an example of the utility of next-generation molecular techniques
442 for non-invasive samples towards sampling wild populations in a species that requires
443 conservation attention. The application of target capture and enrichment has been used
444 previously in non-human primates to sample faecal and ancient DNA (Perry et al., 2010;
445 Kistler et al., 2015; Snyder-mackler et al., 2016). We demonstrated the application of
446 similar molecular techniques to sample from an Endangered lemur species, revealing
447 broad-scale population structure across the aye-aye's geographic range. One area of
448 discussion that arises from reviews of the application of next-generation molecular

449 techniques to conservation is often the necessity of these novel techniques over more
450 conventional approaches, given the increased cost and amount of data generated
451 (Allendorf et al., 2010; Mcmahon, Teeling, & Höglund, 2014; Shafer et al., 2015). Here we
452 present both a unique challenge and solution to sampling a low-density, elusive, and
453 Endangered species. These methodological developments are valuable tools that have
454 enabled us to sample and monitor a cryptic species that otherwise has limited genomic
455 sampling potential. As the field of conservation genomics expands methods such as the
456 one presented here can be applied to achieve direct conservation action.
457
458
459 **Author contributions**
460 EL, GP, MA and SJ conceived idea and designed methodology; MA collected samples; MA
461 and AS conducted laboratory work. All authors contributed critically to drafts and gave
462 final approval for publication.
463
464
465 **Data accessibility**

466 Raw sequence reads are available from GenBank SRA accession SRP133213

## Figures and tables

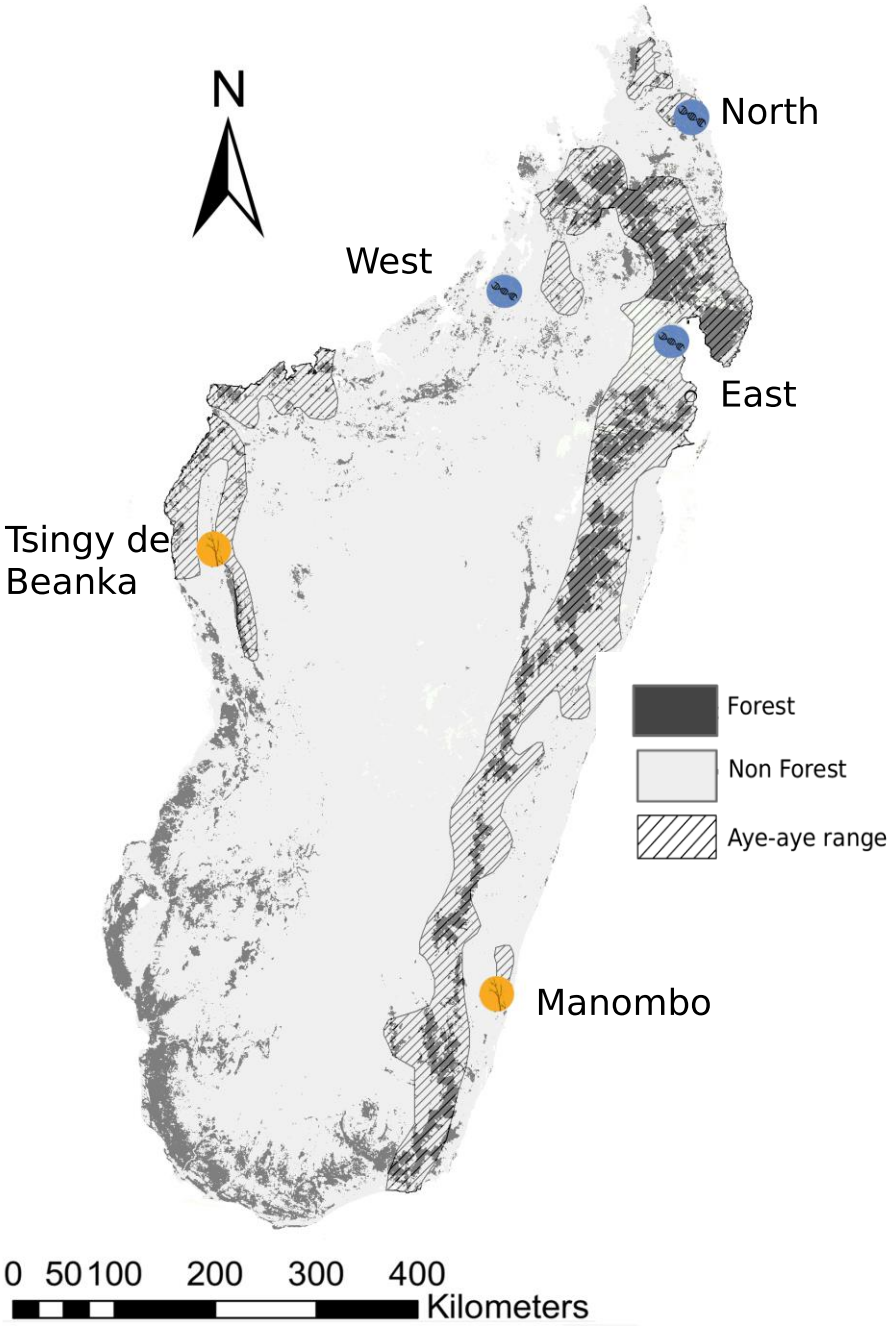


**Figure 1. A map of the island of Madagascar showing sample locations targeted in this study. The orange circles highlight the two locations surveyed for aye-aye feeding traces and eDNA samples were collected. The blue circles indicate the locations that were sampled in Perry et al. 2013, from which the mitogenome sequences from Kistler et al. 2015 were sampled.**

**Figure 2. Aye-aye feeding traces. These images indicate the typical aye-aye feeding traces showing distinct shapes and teeth marks. These traces centre on a hole where the aye-aye has used its specialized extractive foraging strategy to access wood boring insect larvae. We sampled pieces of wood around the edge of the trace where the buccal cavity of the aye-aye comes into contact with the tree.**

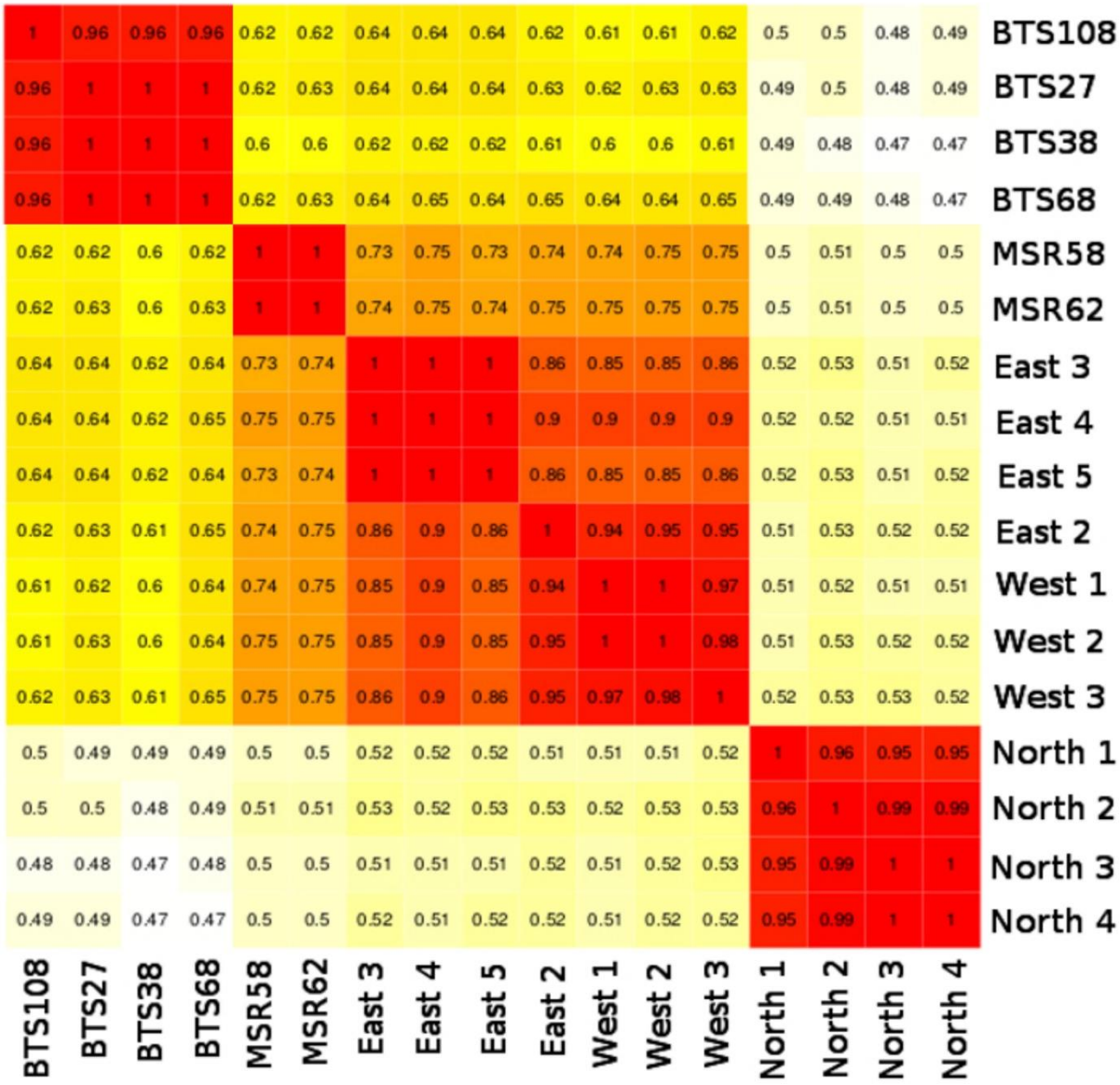


**Figure 3. Identity by state matrix on the left, which shows the proportion of pairwise shared alleles at each SNP site for mitogenomes excluding the d-loop region. We compare eDNA samples from feeding traces with previously published mitogenome sequences (Kistler et al., 2015). Warmer colours indicate greater proportions of shared alleles at these loci.**

**Table 1. The fold increase in percent of unique on-target reads aligning to the aye-aye mitochondrial genome target MitoBait captures compared to shotgun sequencing of the DNA libraries. Data show for the ten libraries that were shotgun sequenced prior to capture.**

| Sample ID | Number of on-target reads | Unique on-target reads, shotgun sequencing (%) | Unique on-target reads, from captured DNA libraries (%) | Fold enrichment |
|---|---|---|---|---|
| MSR01 | 0 | 0.000000 | 0.0027 | - |
| MSR44 | 8 | 0.000038 | 0.0538 | 1416 |
| MSR46 | 0 | 0.000000 | 0.0019 | - |
| MSR50 | 31 | 0.000063 | 0.0248 | 394 |
| MSR58 | 189 | 0.000274 | 0.0408 | 149 |
| BTS04 | 0 | 0.000000 | 0.0051 | - |
| BTS38 | 6 | 0.000103 | 0.0267 | 259 |
| BTS60 | 0 | 0.000000 | 0.0005 | - |
| BTS108 | 24 | 0.000124 | 0.0139 | 112 |
| BTS112 | 0 | 0.000000 | 0.0056 | - |

**Table 2. Sequencing summary statistics showing number of reads and depth and breadth of coverages for MitoBait captures. Grey shading indicates samples with over 90% breadth of coverage that were used for comparisons with published mitogenomes.**

| Sample site | Sample ID | Reads on-target (%) | Unique reads on-target (%) | Mean read depth (SD) | Mean % coverage |
|---|---|---|---|---|---|
| Tsingy de Beanka | BTS04 | 0.06 | 0.0015 | 1.56 (0.9) | 59.3 |
| Tsingy de Beanka | BTS27 | 0.38 | 0.0063 | 13.0 (5.3) | 99.4 |
| Tsingy de Beanka | BTS38 | 0.22 | 0.027 | 23.3 (5.9) | 98.0 |
| Tsingy de Beanka | BTS60 | 0.02 | 0.0005 | 1.8 (2.2) | 45.6 |
| Tsingy de Beanka | BTS68 | 4.3 | 0.0088 | 8.5 (6.0) | 98.9 |
| Tsingy de Beanka | BTS102 | 0.26 | 0.0013 | 1.8 (1.4) | 27.3 |
| Tsingy de Beanka | BTS105 | 0.5 | 0.0005 | 1.7 (1.2) | 22.1 |
| Tsingy de Beanka | BTS108 | 0.4 | 0.014 | 15.1 (5.6) | 99.5 |
| Tsingy de Beanka | BTS112 | 0.46 | 0.0056 | 1.3 (0.6) | 10.9 |
| Manombo Special | MSR01 | 0.05 | 0.0027 | 2.0 (1.4) | 65.9 |
| Manombo Special | MSR23 | 0.04 | 0.039 | 1.2 (0.4) | 12.3 |
| Manombo Special | MSR37 | 0.16 | 0.0002 | 1.1 (0.3) | 11.1 |
| Manombo Special | MSR39 | 27.54 | 0.0024 | 5.0 (11.83) | 45.5 |
| Manombo Special | MSR44 | 22.25 | 0.05 | 1.5 (1.2) | 21.9 |
| Manombo Special | MSR46 | 4.11 | 0.0019 | 1.3 (0.5) | 10.8 |
| Manombo Special | MSR50 | 12.19 | 0.025 | 2.5 (2.07) | 70.9 |
| Manombo Special | MSR56 | 0.024 | 0.0001 | 1.3 (0.6) | 15.0 |
| Manombo Special | MSR58 | 2.71 | 0.04 | 12.4 (7.2) | 98.5 |
| Manombo Special | MSR62 | 2.20 | 0.015 | 25.1 (8.8) | 99.8 |

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468 **References**
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## Supporting information

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**Table A1. Sequencing pool parameters for shotgun sequencing of eDNA libraries.**

| Sample | Conc. ng/ul | Vol. in pool | Total ng | % pool by amount |
|---|---|---|---|---|
| MSR01 | 0.26 | 2 | 0.528 | 6.81 |
| MSR44 | 0.17 | 5.88 | 0.999 | 6.58 |
| MSR46 | 0.46 | 2.17 | 0.999 | 6.58 |
| MSR50 | 0.39 | 2.56 | 0.998 | 6.58 |
| MSR58 | 0.96 | 1.04 | 0.994 | 6.58 |
| BTS04 | 0.53 | 1.89 | 1 | 6.6 |
| BTS38 | 0.03 | 1 | 0.03 | 0.2 |
| BTS60 | 0.70 | 1.43 | 1 | 6.59 |
| BTS108 | 0.20 | 1 | 0.2 | 1.32 |
| BTS112 | 0.34 | 2.94 | 0.999 | 6.58 |

**Table A2. Sequencing pool parameters for MitoBait captures from eDNA samples.**

| Sample | Conc. ng/ul | Vol. in pool | ng total | % pool by amount |
|---|---|---|---|---|
| BTS04 | 0.36 | 11 | 3.96 | 6.7 |
| BTS27 | 6.8 | 3 | 4.08 | 6.9 |
| BTS60 | 0.515 | 7.8 | 4.02 | 6.8 |
| BTS68 | 0.633 | 6.3 | 3.99 | 6.75 |
| BTS102 | 4.4 | 0.8 | 3.52 | 5.96 |
| BTS105 | 1.56 | 2.6 | 4.06 | 6.87 |
| BTS112 | 0.23 | 16.7 | 2.87 | 4.86 |
| MSR01 | 0.43 | 9.4 | 4 | 6.77 |
| MSR23 | 0.546 | 7.3 | 3.99 | 6.75 |
| MSR37 | 0.389 | 10.3 | 4.01 | 6.79 |
| MSR39 | 0.848 | 4.7 | 3.9 | 6.75 |
| MSR44 | 0.003 | 10 | 0.03 | 0.05 |
| MSR46 | 0.003 | 10 | 0.03 | 0.05 |
| MSR50 | 0.0 | 10 | 0 | 0.00 |
| MSR56 | 0.932 | 4.3 | 4.01 | 6.79 |
| MSR58 | 0.209 | 10 | 2.09 | 3.54 |
| MSR62 | 2.66 | 1.5 | 3.99 | 6.75 |
| BTS108 | 0.42 | 6 | 2.5 | N/A |
| BTS38 | 2.2 | 1.8 | 3.96 | N/A |