Fig. 3: *Lepilemur leucopus* from Betsimilaho (left) and Vohidava (right). Photos: Maël Jaonasy

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Genetic confirmation of the Anjiamangirana sportive lemur in the Anjajavy Forest

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Abstract

Most of Madagascar's lemurs are nocturnal, and most nocturnal lemurs are cryptic, making congeners difficult to differentiate due to their morphological similarity. Sportive lemurs (genus *Lepilemur)* are a great example and have been the subject of ongoing taxonomic debate for decades. Twenty-six sportive lemur species are currently recognized, based on early cytogenetic and more recent genetic stud-

ies. As a consequence of taxonomic rearrangements, species distributions have changed significantly over the years. During fieldwork at Anjajavy, a dry deciduous forest along the coast of the Inter River System (IRS) III, we opportunistically collected a tissue sample from a female *Lepilemur*. Although census work previously identified *L. grewcockorum* in Anjajavy and other locations in the IRS III, the only genetic confirmation for this species comes from the inland forests of Anjiamangirana and Ambongabe. We sequenced a marker gene (Cytochrome B) and compared results to a gene database assembled from GenBank. Our results genetically confirm the individual from Anjajavy as *L. grewcockorum*. Additional genetic analyses, coupled with known census sites, might render this species more widely distributed than originally thought. We encourage further survey, genetic, and behavioral work within the remaining forest patches of the IRS III to clarify the true range, population estimates, and ecological characteristics of *L. grewcockorum*. This study demonstrates the value of using genetics to identify species that are morphologically similar and to determine the boundaries of their geographic ranges.

Introduction

Madagascar is home to a rich array of lemur species, nearly all of which are threatened with extinction (IUCN, 2021). Whereas the diurnal lemurs are typically listed as flagship species for conservation efforts, somewhat ironically, the majority of lemur diversity is in the cryptic and nocturnal lineages (Mittermeier *et al*., 2010). In recent years, the nocturnal lemur lineages have undergone significant taxonomic revision (the aye-aye, *Daubentonia madagascariensis*, is a notable exception), as genetic approaches allow us to 'see' the differences between morphologically similar species (e.g., Andriantompohavana *et al*., 2007; Frasier *et al*., 2016; Schüßler *et al*., 2020). The sportive lemurs are a classic example of an understudied nocturnal lineage that has been the subject of much taxonomic debate (Lei *et al*., 2017). Sportive lemurs are elusive and challenging to research. They are widely distributed throughout Madagascar, but "are relatively uniform in appearance, morphology, behavior, and ecology" (Thalmann and Ganzhorn, 2003, p. 1336), rendering species assignments challenging.

Sportive lemurs were first classified within the *Lepilemur* genus by Geoffroy Saint-Hilaire (1851) (Dunkel *et al*., 2012) which was placed within the family Lepilemuridae by Gray (1870) (Mittermeier *et al*., 2010). The name 'sportive lemur' was given by Forbes (1894) regarding the agility of this species, as they are excellent clingers and leapers (Dunkel *et al*., 2012). Hill (1953) classified the genus instead within the Lemuridae family based on morphological and karyological evidence, but Petter *et al*. (1977) favored maintaining them separately in the Lepilemuridae family (Thalmann and Ganzhorn, 2003). Tattersall and Schwarz (1985) placed the genus as sister to the extinct *Megaladapis* genus, within the Megaladapidae family, based on dental characteristics (Thalman and Ganzhorn, 2003). By 2005, however, accruing genetics studies re-established *Lepilemur* and *Megaladapis* as independent lineages (Yoder *et al*., 1999; Karanth *et al*., 2005). Recent genomic data supports these early genetic findings and established Lepilemuridae and Cheirogaleidae as sister lineages (Marciniak *et al*., 2021).

While gaining clarity into the higher-level relationships between sportive lemurs and other lemurs, recent years have also seen a rapid increase in the number of species within the genus. Historically, only two species were included in the *Lepilemur* genus: *L. mustelinus* in the east and *L. ruficaudatus* in the west and south (Thalmann and Ganzhorn, 2003). Petter *et al*. (1977) elevated 5 additional subspecies to species status, based on karyological evidence, though Tattersall (1982) favored synonymizing them all as subspecies within *L. mustelinus* (Thalmann and Ganzhorn, 2003). By 2000, genetic studies and karyological evidence led the field to largely recognize 7 full species (Thalmann and Ganzhorn, 2003). Since the early 2000s, accruing molecular, morphometric, and karyological studies support at least 26 species distributed around Madagascar (Andriaholinirina *et al*., 2006; Craul *et al*., 2007; Lei *et al*., 2017; Louis *et al*., 2006; Rabarivola *et al*., 2006; Rumpler *et al*., 2008). Many of these species were first described, and remain known today, only from single type localities and few samples or individuals. As more species within this genus continue to be described, questions remain regarding each species' geographic distributions and ecological characteristics.

Here, we add to our growing knowledge about the *Lepilemur* genus by sequencing a marker gene (cytochrome B) from an individual sportive lemur that was opportunistically sampled in the Anjajavy forest. Anjajavy, a dry deciduous forest in northwest Madagascar, sits along the coast between the Sofia and Maevarano rivers in the Inter River System (IRS) III. Based on the new lemur assessments released by the IUCN Red List of Threatened Species (2020), and the potential for rivers to establish lemur biogeographical patterns (Wilmet *et al*., 2014), we predict the sportive lemur from Anjajavy to be *L. grewcockorum.*

Lepilemur grewcockorum, also known as the Anjiamangirana sportive lemur, was first identified by Louis *et al*. (2006) as *L. grewcocki* in the Classified Forest of Anjiamangirana (15°09'14.9"S, 47°43'41.0"E) in the former range of *L. edwardsi,* based on mitochondrial DNA. Near the same locality, Craul *et al*. (2007) described specimens as *L. manasamody,* from Ambongabe (15°19'38.3"S, 46°40'44.4"E) and Anjiamangirana 1 (15°09'24.6"S, 47°44'06.2"E). Zinner *et al.* (2007) indicated that *L. manasamody* is probably a junior synonym of *L. grewcocki*, as sampling sites were separated by less than two kilometers, with no obvious geographic barrier. The synonymizing of *L. grewcockorum* and *L. manasamody* was confirmed by a molecular genetic analysis by Lei *et al*. (2017). During this period of taxonomic ambiguity for the Ambongabe samples, Hoffmann (2009) noted that *L. grewcocki* was an incorrect original spelling and the species name was amended to *L. grewcockorum*.

The Anjiamangirana sportive lemur is found in northwestern Madagascar (Louis *et al*., 2020). The known distribution is limited to the inland sites of Ambongabe and Anjiamangirana, as confirmed by genetic analysis (see Fig. 1). Both sites are situated in the IRS III which is delimited by the Sofia River in the south and Maevarano river in the north (Olivieri *et al*., 2005; Craul *et al*., 2007). During census surveys, Randrianambinina *et al*. (2010) reported *L. grewcockorum* at three additional sites, including Anjajavy (S15°01'39.6" E47°16'38.4"), Ambarijeby (S14°53'20.9" E47°43'17.8") and Bekofafa (S14°53'20.9" E47°43'17.8"), though none have been confirmed genetically. According to these surveys, the encounter rates of *L. grewcockorum* are rare (Randrianambinina, 2010). The species is currently listed as Critically Endangered, due to its tiny extent of occurrence (EOO) covering only 143 km^2 (IUCN, 2020), which does not include the census sites that lack genetic confirmation.

Methods

Sample collection

The subject was a female sportive lemur opportunistically sampled from the Anjajavy forest. The individual was caught on July 15th, 2018, by hand from a tree hole, while searching

Fig. 1: Map of Madagascar showing the IUCN ranges in the northwest of *L. grewcockorum* in the IRS III and the neighboring *L. otto* in the IRS II, *L. edwardsi* in the IRS I, and L. sahamalaza in the IRS IV against the Maevarano, Sofia, Mahajamba rivers. Sampling locale at Anjajavy is depicted as a star.

for a radio-collared dwarf lemur that was hibernating in the adjacent tree. While in hand, the individual was placed in a cloth bag and brought back to the campsite to be given a physical exam by project veterinarians. At camp, the sportive lemur was briefly anesthetized with Ketamine (10 mg/kg body mass) for morphometric data collection, and a small tissue biopsy was obtained from the left ear for genetic analysis. The sample was immediately submerged in 90% ethanol and stored at room temperature until extraction and subsequent analysis. The individual was given water after recovery and released at her initial capture location at sunset the same day. Although this individual was a by-catch, and not the target of our research project, we followed approved research practices for nocturnal species, following the guidelines established by the International Primatological Society in "International Guidelines for the Acquisition, Care and Breeding of Nonhuman Primates". In addition to sampling, this was an opportunity to conduct a comprehensive biomedical exam by two early-career wildlife veterinarians (ER & HAR) overseen by an expert lemur veterinarian (RS).

DNA extraction and amplification

DNA was extracted from the tissue sample *in situ* at Anjajavy within 2 weeks of capture using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany). DNA concentration was quantified on a Qubit fluorometer (Thermo Fisher Scientific, Waltham, MA, USA).

We used primers CYT-LEP-L (5'- AATGATATGAAAAACCATCGTTGTA -3') and CYT-LEP-H (5'- GGCTTACAAGGCCGGGGTAA -3') following Andriaholinirina et al. (2006) in the U.S. to amplify the mitochondrial cytochrome B (*cytb*) gene. The 25 μL PCR reaction included 12.5 μL Qiagen HotStartTaq Master Mix, 2.0 μL Ambion Ultrapure non-acetylated Bovine Serum Albumin (20 mg/mL), 1.0 μL each of 10 μM forward and reverse primers and 4.0 μL of template DNA. Following an activation step at 95°C for 15 min, PCR cycling conditions (40 cycles) were: 94°C for 60 sec, 50°C for 60 sec, 72°C for 90 sec. The final extension was at 72°C for 10 min. PCR product was visualized via agarose gel electrophoresis, enzymatically purified and sequenced at the Duke DNA Analysis Facility on an Applied Biosystems 3730 Genetic Analyzer using both the PCR primers and internal sequencing primers CYT-LEP-L400 5'- TGAGGACAAATATCATTCTGAGG – 3' and CYT-LEP-H545 5'- TGGAGTGCGAAGAATCGGGT– 3' following Andriaholinirina et al. (2006). The chromatogram was visually inspected using FinchTV v 1.5.0 (Geospiza).

Data analysis

We downloaded available (n=146) sportive lemur complete cytochrome B sequences in GenBank, representing all 26 currently-recognized sportive lemur species (IUCN, 2021). We removed duplicate sequences, resulting in a final dataset of 124 sequences. The newly generated data for the sportive lemur was collated to the datamined sequences and aligned using MUSCLE v3.8.31 (Edgar, 2004). The alignment was visually inspected using AliView (Larsson, 2014). The best scoring maximum likelihood tree was estimated using RAxML (Stamatakis, 2006) using the rapid bootstrap analysis algorithm (Stamatakis *et al*., 2008) with 1000 bootstrap replicates and a general time-reversible (GTR) nucleotide substitution model with a gamma distribution for rate heterogeneity. A GTR model was chosen because it has been found to perform at least as well as other models in phylogenetic reconstruction under a variety of conditions (Arenas, 2015). An eastern woolly lemur (*Avahi laniger;* DQ451106.1) was used as an outgroup. For the construction of the final tree, we removed a handful of samples with unclear provenance in GenBank and identical sequences from conspecifics.

Results

The Anjajavy sample is placed as sister to the *L. grewcockorum* sequence (Fig. 2), collected from Anjiamangirana (Lei *et al*., 2017). Bootstrap support for this placement was high (98). We provide morphometrics from the focal subject in Tab. 1, along with published values and descriptions for individuals from other sites.

Discussion

Our results support the assignment of the sportive lemur from Anjajavy as *L. grewcockorum*. This represents a confirmed range expansion for the species, which is currently listed in the IUCN Red List for Threatened Species in only a tiny fragment far inland of our locality. Importantly, census data placed *L. grewcockorum* as variably distributed at intermediate locations between Anjajavy and Anjiamangirana (Randrianambinina, 2010), suggesting that this species is present throughout the IRS III. It is becoming clearer that sportive lemur species, like mouse lemurs, are allopatric in the northwest and confined to specific IRS (Olivieri *et al*., 2007; Roos *et al*., 2021; Wilmet *et al*., 2014). We encourage the IUCN to update the range maps for this species to include Anjajavy and the census sites of Ambarijeby and Bekofafa. We also encourage further survey, genetic, and behavioral work within the remaining forest patches of the IRS III to clarify the true range, population estimates, and ecological characteristics of *L. grewcockorum*.

The case of *L. grewcockorum* highlights the importance of using genetics to confirm the boundaries of species' ranges. Within those boundaries, morphological characteristics can be used as general descriptors to guide census, behavioral, and survey work. But morphological and visual features, like coat color, can be subjective and variable across popula-

Tab. 1: Morphometrics from published sources and this study: BM: body mass in kg, BL: body length in cm, TL: tail length in cm, HW: head width in cm. NA: data not available

Species	BM (Mean + SD)	BL (Mean + SD)	TL (Mean + SD)	HW (Mean + SD)	Notes
L. grewcockorum (n=3)*	0.78 (0.20)	24.8 (2.1)	28.5 (1.8)	NA	Predominantly gray color pattern. Area around the mandible and dorsal surface of the snout is whitish-pink in coloration. A dark stripe is present on the dorsal midline surface of the head…Unlike *L. edwardsi*, which has a consistently white-tipped tail, the tail of *L. grewcockorum* is entirely gray.
L. manasamody (now *L. grewcockorum*, n=8)**	0.939 (96.97)	NA	28.1 (15.24)	37.61 (2.29)	Dorsal pelage is predominantly grey-brown. The ventral pelage is generally grey to creamy. Face and forehead are essentially grey. From the middle of the upper skull, a dark diffuse line runs down the spine. Tail is grey-brown to deep brown, sometimes with a white tail.
*Louis *et al.*, 2006; **Craul *et al.*, 2007					
Anjajavy (This study)	1.09	29	30	NA	Predominantly gray color pattern with a white-grey ventral side and a pronounced white tail tip.

tions and individuals (see Tab. 1). Species described from a small number of lemurs within single populations might miss some morphological variations. This is the case with the white tail-tip, which was thought to be descriptive of *L. edwardsi* and absent in *L. grewcockorum* (Louis *et al.*, 2006) but also turns out to be variably present among *L. grewcockorum* individuals (Craul *et al.*, 2007; this study).

The case of *L. grewcockorum* at Anjajavy, coupled with the recent confirmation of sympatric *M. danfossi* (Blanco *et al.* 2020), also highlights the potential for research-informed conservation at Anjajavy. Anjajavy boasts a new protected area under Category V (Harmonious Landscape) that comprises >10,000ha of mangrove, tsingy, dry deciduous forest, and recovering agricultural land. Although the site is perhaps best known for its high-end ecotourism in the smaller private reserve, a growing research program across the entire protected area aims to characterize and monitor the endangered species endemic to this heterogenous landscape.

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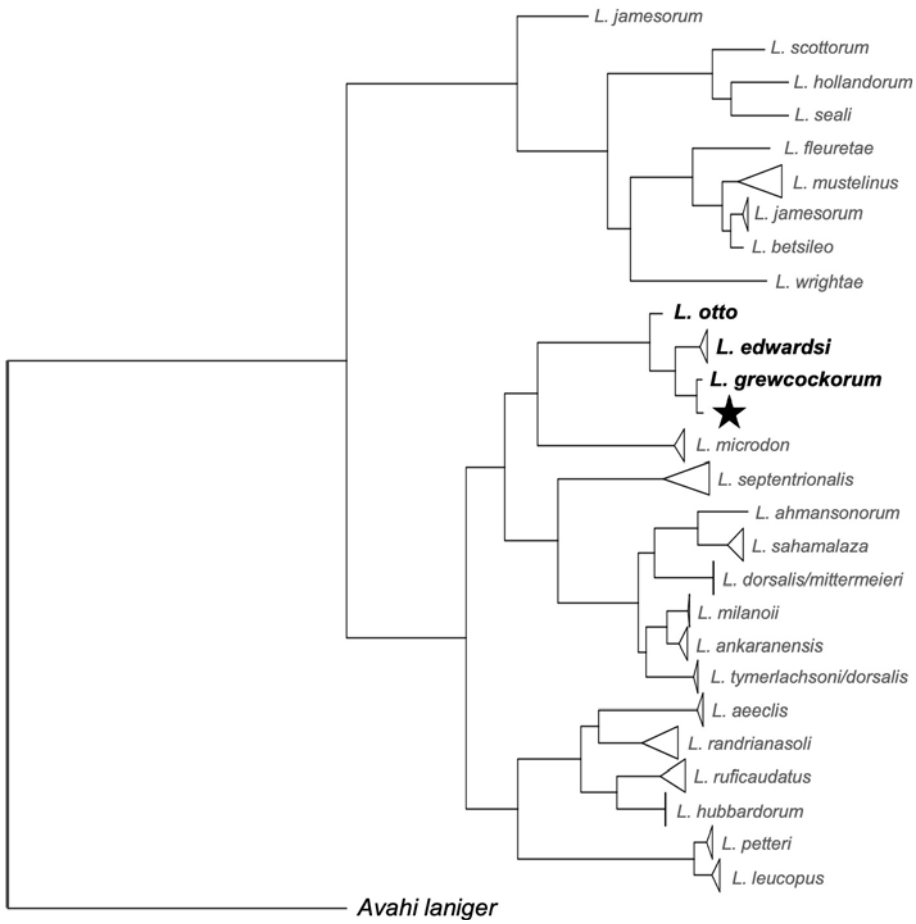

Fig. 2: Maximum likelihood phylogenetic tree of cytochrome B sequences with bootstrap support. The star denotes the sample from Anjajavy. The length of the branch connecting Avahi to the sportive lemur clade is minimized for ease of visualization.

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Description of the gastrointestinal parasites of *Propithecus diadema* (Primates: Lemuridae) in the New Protected Area of Maromizaha, Eastern Madagascar

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Abstract
The aim of this work is to identify and describe the gastrointestinal (GI) parasites of the lemur *Propithecus diadema* from the New Protected Area of Maromizaha – Andasibe, East Madagascar. 218 fecal samples were analyzed from adult females and males from two different groups. These *Propithecus diadema* host six morphotypes of GI parasites including: 1) four Nematode, of which two Oxyuridae (*Lemuricola* sp. and unidentified sp.), one Trichostrongylidae (*Pararhabdonema* sp.), and one other Nematode unidentified sp.; 2) one Cestode (*Hymenolepis* sp.); and 3) one Protozoan of the Coccidia order. This study expands upon the known GI parasites of diademed sifaka.

Introduction
Parasites affect host survival and reproduction and thus are an important selective force shaping host physiology, ecology, and behavior (Coltman *et al*., 1999; Nunn and Altizer, 2006; Wood and Johnson, 2015, cited in Springer and Kappeler, 2016). Specifically, intestinal helminths and protozoa can lead to decreased energy absorption, pathological damage, and decreased reproductive success in their hosts (Hudson *et al*., 1992, 1998; Delahay *et al*, 1995; Hillegass *et al*., 2010, cited in Springer and Kappeler, 2016). Thus, the study of parasites is proving to be necessary to aid in the conservation of animal species (Altizer *et al*., 2007)