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Letter to the editor Open Access

Mitogenomes of historical type specimens unravel the taxonomy of sportive lemurs (*Lepilemur* spp.) in Northwest Madagascar

Accurate information on name-bearing types, including corresponding type localities, is essential for proper taxonomy. However, such geographic information is often missing or unreliable. The localities of type specimens collected 100–200 years ago can be difficult to trace due to changes in local names or simple inaccuracies. Such a case can be found for the gray-backed sportive lemur (*Lepilemur dorsalis*), with its type locality imprecisely fixed as Northwest Madagascar. In recent years, eight species have been newly described for the Inter-River-Systems (IRSs) of this region, however the designation of *L. dorsalis* remains controversial due to a lack of a precise type locality. Here, we sequenced the complete mitochondrial genomes (mitogenomes) of type specimens of *L. dorsalis* and *L. grandidieri*, which is currently recognized as a synonym of *L. dorsalis* and compared their sequences with those of samples of known provenance from different IRSs. Results showed that the two type specimens of *L. dorsalis* and *L. grandidieri* had identical mitogenome sequences and clustered closely with samples collected in IRS V, indicating that the type locality could be fixed to IRS V. Consequently, *L. dorsalis* occurs in IRS V, and *L. grandidieri* and *L. mittermeieri* are junior synonyms of *L. dorsalis*. This finding demonstrates the value of type specimens for clarifying phylogeographic and taxonomic questions and clarifies the taxonomy of sportive lemurs in Northwest Madagascar.

Over the last two decades, the application of molecular methods has led to the detection and delineation of new species. This has resulted in an increase in species number in various taxonomic groups, including primates such as New World monkeys, tarsiers, lorises, galagos, and lemurs (Mittermeier et al., 2013; Rowe & Myers, 2016). Among the lemurs of Madagascar, most new species have been described for medium-to-small-bodied, nocturnal genera with only subtle phenotypic differences in pelage coloration (e.g., *Microcebus*, *Cheirogaleus*, *Avahi*, and *Lepilemur*). In contrast, for diurnal genera with more obvious phenotypic differences (e.g., *Lemur*, *Eulemur*, *Varecia, Indri*, and *Propithecus*), species number or at least the number of taxa (some subspecies have been elevated to species level) has remained relatively constant over the last few decades (Mittermeier et al., 2010, 2013; Napier & Napier, 1967; Petter et al., 1977; Rowe & Myers, 2016; Tattersall, 1982).

Sportive lemurs (*Lepilemur*) were historically classified into a single species with five subspecies (Napier & Napier, 1967; Tattersall, 1982), then later into seven distinct species (Groves, 2001; Petter et al., 1977; Rowe, 1996). At present, however, 26 species are recognized (Mittermeier et al., 2010, 2013; Rowe & Myers, 2016), two of which have been historically delineated in Northwest Madagascar: i.e., *Lepilemur edwardsi* (Forsyth Major, 1894) and *L. dorsalis* Gray, 1871. The two syntypes for *L. dorsalis*, adult male (NHMUK ZD.1868.9.7.5) and adult female (NHMUK ZD.1868.9.7.4), are housed in the Natural History Museum, London, UK (NHMUK). The female syntype was later designated as the holotype of *Lepidolemur grandidieri* Forsyth Major, 1894, but this taxon is currently regarded as a junior synonym of *L. dorsalis* (Groves, 2001, 2005; Jenkins, 1987). Unfortunately, the type localities of both specimens are imprecisely given as Northwest Madagascar (Forbes, 1894; Jenkins, 1987), so their exact provenance and whether they came from the same site remains unknown.

Molecular analyses of sportive lemurs have provided evidence for at least eight mitochondrial lineages in Northwest Madagascar (Figure 1A; Table 1), corresponding to the eight IRSs in the region proposed by Craul et al. (2007). These lineages refer to distinct species. However, as the exact type localities of *L. dorsalis* and *L. grandidieri* are unavailable, it remains unclear which of the recently described species is synonymous with *L. dorsalis* and whether *L. grandidieri* is

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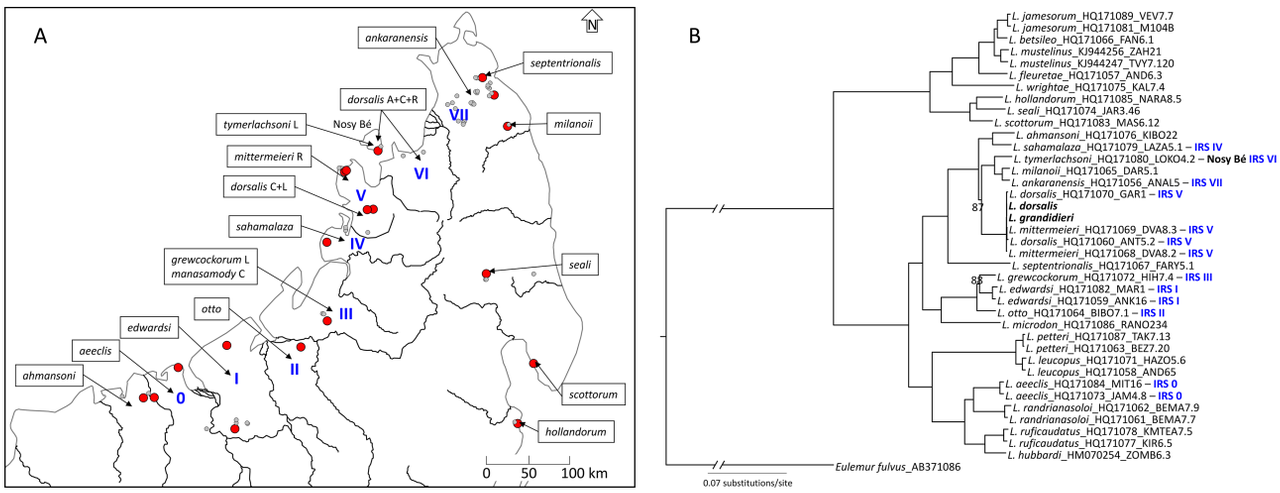

Figure 1 Sketch of northern Madagascar depicting the eight Inter-River-Systems (IRS 0–VII) (A), and maximum-likelihood tree of *Lepilemur* spp. based on complete mitogenome sequences (B)

In A, questionable species designations are indicated as: A=species in Andriaholinirina et al. (2006); C=species in Craul et al. (2007); L=species in Louis et al. (2006); R=species in Rabarivola et al. (2006). Red dots=sample location for this study; gray dots=additional sample locations of previous studies that support respective mitochondrial lineages; IRS VI includes Nosy Bé. In B, node support <100% ML BS is given at respective nodes; PP for all nodes is 1.0. Species names are followed by GenBank accession numbers and geographic identifiers (see Lei et al., 2017). Respective IRSs are in blue. Type specimens of *L. dorsalis* and *L. grandidieri* are highlighted in bold. Designation of *L. dorsalis* is according to Louis et al. (2006).

Table 1 Inter-River-System (IRS) model according to Craul et al. (2007) and corresponding *Lepilemur* species as assigned in previous studies (Andriaholinirina et al., 2006; Craul et al., 2007; Lei et al., 2017; Louis et al., 2006; Rabarivola et al., 2006; Zinner et al., 2007)

IRS	River	Andriaholinirina et al. (2006)	Louis et al. (2006)	Rabarivola et al. (2006)	Craul et al. (2007)	Zinner et al. (2007)	Lei et al. (2017)	This study
0	Mahavavy –Besiboka	*aeeclis*	*aeeclis*	*aeeclis*	*aeeclis*	*aeeclis*	*aeeclis*	*aeeclis*
I	Besiboka – Mahajamba	*edwardsi*	*edwardsi*	*edwardsi*	*edwardsi*	*edwardsi*	*edwardsi*	*edwardsi*
II	Mahajamba – Sofia	No samples	No samples	No samples	*otto*	*otto*	*otto*	*otto*
III	Sofia – Maevarano	No samples	*grewcockorum*	No samples	*manasamody*	*grewcockorum*	*grewcockorum*	*grewcockorum*
IV	Maevarano – Andranomalaza	*sahamalaza*	*sahamalaza*	*sahamalaza*	*sahamalaza*	*sahamalaza*	*sahamalaza*	*sahamalaza*
V	Andranomalaza – Sambirano	No sample	*dorsalis*	*mittermeieri*	*dorsalis*	*mittermeieri*	*dorsalis*	*dorsalis*
VI	Sambirano – Mahavy	*dorsalis*	No samples	*dorsalis*	*dorsalis*	*tymerlachsoni*	No samples	*tymerlachsoni*
VI*	Nosy Bé	*dorsalis*	*tymerlachsoni*	*dorsalis*	*dorsalis*	*tymerlachsoni*	*tymerlachsoni*	*tymerlachsoni*
VII	North of Mahavy	*ankaranensis*	*ankaranensis*	*ankaranensis*	*ankaranensis*	*ankaranensis*	*ankaranensis*	*ankaranensis*

*: Nosy Bé is here regarded as a part of IRS VI (see Andriaholinirina et al., 2006 and Craul et al., 2007).

indeed synonymous with *L. dorsalis*. Zinner et al. (2007) showed that the teams that described new *Lepilemur* species in 2006 and 2007 (Andriaholinirina et al., 2006, 2017; Craul et al., 2007; Louis et al., 2006; Rabarivola et al., 2006) had differing opinions on the type locality of *L. dorsalis* (Table 1), and thus the validity of *L. tymerlachsoni*, *L. mittermeieri*, and possibly *L. sahamalaza* depends on which opinion is correct.

One way to clarify these questions is to compare the mitochondrial DNA (mtDNA) sequences of the type specimens of *L. dorsalis* and *L. grandidieri* with those of geo-referenced sportive lemur samples from Northwest Madagascar, assuming that the type specimens will cluster with one of the lineages in one of the IRSs, as mtDNA haplotypes of *Lepilemur* show geographic structure (Andriaholinirina et al., 2006; Craul et al., 2007; Louis et al., 2006; Rabarivola et al., 2006; Zinner et al., 2007).

To trace the geographic origin of the type specimens of *L. dorsalis* and *L. grandidieri* and to determine which recently described sportive lemur species is/are synonymous with *L. dorsalis* and/or *L. grandidieri*, we sequenced the complete mitochondrial genomes (mitogenomes) of both types and compared them with the mitogenomes of geo-referenced sportive lemurs representing all 26 currently recognized species.

We collected dry skin samples (ca. 4×4 mm) from the two syntypes of *Lepilemur dorsalis* (NHMUK ZD.1868.9.7.4 (later designated as the holotype of *Lepidolemur grandidieri*) and NHMUK ZD.1868.9.7.5). Both specimens were collected before 1868, but the exact collection date is unknown. Sampling was performed with single-use tweezers and scalpels to avoid human and particularly cross-sample

contamination. Samples were stored dry and in the dark in 1.5 mL tubes until further processing.

To avoid cross-sample contamination, NHMUK ZD.1868.9.7.5 was processed at the University of Potsdam (Potsdam, Germany), while NHMUK ZD.1868.9.7.4 was handled at the German Primate Center (Göttingen, Germany). DNA extraction and library preparation were performed in the respective ancient DNA laboratories, in which all standards for such laboratories were implemented (e.g., UV light decontamination before and after use, positive air pressure, separate sterile working areas, protective clothing, negative controls during DNA extraction and sequencing library preparation). For DNA extraction, we applied a column-based method specifically designed to recover degraded DNA fragments (Dabney et al., 2013; Rohland et al., 2004). After extraction, DNA concentrations were measured with a Qubit 4.0 fluorometer (ThermoFisher Scientific, USA), and DNA quality and degradation were checked on a Bioanalyzer 2100 (Agilent Technologies, USA) or a TapeStation 4200 (Agilent Technologies, USA). Genomic DNA (50 ng) was then used to construct shotgun sequencing libraries with the NEBNext Ultra II DNA Library Prep Kit (New England Biolabs, USA). All standard protocols of the manufacturer were followed, except DNA fragmentation before library preparation was omitted due to the degraded status of the DNA. After end repair, adapter ligation, and ligation cleanup without size selection, libraries were indexed with multiplex oligos and then cleaned with the kit's purification beads. Library concentration and size distribution were measured with a Qubit fluorometer and Bioanalyzer or TapeStation, respectively, and molarity was quantified via quantitative polymerase chain reaction (qPCR) using the NEBNext Library Quant Kit (New England Biolabs, USA). Sequencing was conducted on an Illumina NextSeq (75 bp paired-end read) at the University of Potsdam, or an Illumina HiSeq 4000 (50 bp single-end read) at the NGS-Integrative Genomics Core Unit (NIG) of the University Medical Center Göttingen (Germany). Raw sequencing reads were demultiplexed with Illumina software. Subsequent bioinformatic analyses were performed with the Geneious 11.1.3 package (https://www.geneious.com/). We first trimmed and quality-filtered the reads with BBDuk 37.64 of the BBTools package (https://jgi.doe.gov/data-and-tools/bbtools/) and then removed duplicate reads with Dedupe 37.64 (BBTools package), both with standard settings. For mitogenome assembly, reads were mapped onto the mitogenomes of *L. dorsalis* (GenBank accession No.: HQ171070) and *L. mittermeieri* (GenBank accession No.: HQ171069) using the Geneious assembler with standard settings. Both newly produced mitogenomes were manually checked and then annotated with Geneious. Sequences were submitted to GenBank and are available under accession Nos. MW023869 and MW023870.

To determine which of the currently recognized species is synonymous with *L. dorsalis* and/or *L. grandidieri*, we reconstructed phylogenetic trees. We expanded our dataset with an additional 36 *Lepilemur* spp. mitogenome sequences from GenBank (accession Nos. HQ171056–HQ171087, HQ171089, KJ944247, KJ944256, HM070254; Lei et al., 2017), representing all currently recognized *Lepilemur* spp., with 1–2 representatives per species. We used *Eulemur fulvus* (GenBank accession No.: AB371086) as an outgroup. A total of 39 sequences were aligned using Muscle 3.8.31 (Edgar, 2010) in AliView 1.18 (Larsson, 2014), then manually checked. The final alignment was 17 566 bp in length and contained 5 453 parsimony-informative and 1 832 parsimony-uninformative variable sites.

Uncorrected pairwise differences between sequences were calculated with PAUP 4.0a169 (Swofford, 2002). Phylogenetic trees were reconstructed using maximum-likelihood (ML) in IQ-TREE 1.6.1 (Nguyen et al., 2015) and Bayesian inference (BI) in MrBayes 3.2.6 (Ronquist et al., 2012). For both tree reconstructions, we treated the mitogenome as a single partition and applied the optimal substitution model (GTR+I+G) determined by ModelFinder (Chernomor et al., 2016; Kalyaanamoorthy et al., 2017) in IQ-TREE under Bayesian Information Criterion (BIC). We reconstructed the BI tree via two independent Markov Chain Monte Carlo (MCMC) runs, each with 10 million generations, tree and parameter sampling every 100 generations, and burn-in of 25%. To check the convergence of all parameters and adequacy of burn-in, we calculated the uncorrected potential scale reduction factor (PSRF) (Gelman & Rubin, 1992) in MrBayes. The BI posterior probabilities (PPs) and consensus phylogram with mean branch lengths from the posterior density of the trees were calculated in MrBayes. Node support for the ML tree was obtained from 10 000 ultrafast bootstrap (BS) replications (Minh et al., 2013). Phylogenetic trees were visualized and edited with FigTree 1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/).

For the type specimen of *L. dorsalis*, we obtained 6 906 138 sequencing reads after trimming, quality-filtering, and duplicate removal, 6 190 (0.09%) of which were mapped to the reference genomes, resulting in 100% mitogenome coverage and an average sequencing depth of 17. For the type specimen of *L. grandidieri*, 5 037 921 sequencing reads remained after filtering, 15 281 (0.30%) of which were mapped to the reference genomes, resulting in 100% mitogenome coverage and an average sequencing depth of 45.

The two newly generated mitogenomes were identical and 17 101 bp in length. They contained the 22 transfer RNA (tRNA) genes, two ribosomal RNA (rRNA) genes, 13 protein-coding genes, and control region in the structure and order commonly found in mammals. All protein-coding genes were correctly transcribed without any premature stop codons and tRNAs exhibited typical secondary structure, suggesting that the mitogenomes did not contain any nuclear mitochondrial DNA sequences (numts).

The ML and BI trees revealed identical tree topologies with overall strong node support (BS 87%–100%, PP 1.0; Figure 1B). Both type specimens clustered with two *L. mittermeieri* specimens (HQ171068, HQ171069) and two *L. dorsalis* specimens (HQ171060, HQ171070), as classified by Louis et al. (2006). Uncorrected pairwise differences between the mitogenomes of the type specimens of *L. dorsalis* and *L. grandidieri* and those of the two *L. mittermeieri* and two *L. dorsalis* specimens were 0.04%–0.06% and 0.09%–0.14%, respectively. Accordingly, the geographic origin of the type

specimens of *L. dorsalis* and *L. grandidieri* could be fixed to IRS V.

We successfully sequenced the complete mitogenomes of two *Lepilemur* museum specimens (>150 years old) that represent the types for *L. dorsalis* and *L. grandidieri* with moderate to high sequencing depth. Both mitogenome sequences were identical. Laboratory contamination can be excluded, as both type specimens were processed in different ancient DNA laboratories. Likewise, contamination during sampling was highly unlikely as single-use tweezers and scalpels were used.

Given the identical mitogenomes of the *L. dorsalis* and *L. grandidieri* types, the synonymy of both is clearly supported, as suggested by Jenkins (1987) and Groves (2001, 2005). Consequently, *L. grandidieri* is a junior synonym of *L. dorsalis*. As the description of *L. dorsalis* was based on two syntypes, one of which (NHMUK ZD.1868.9.7.4) was later designated as the holotype of *L. grandidieri*, the second syntype (NHMUK ZD.1868.9.7.5) is the only name-bearing type available for *L. dorsalis* and hence we designate this specimen as the lectotype of *L. dorsalis* (ICZN, Article 74.1 and 74.7).

Both types clustered closely with samples collected in IRS V, supporting the hypothesis that the sportive lemurs of IRS V are taxonomically *L. dorsalis*, as proposed by Louis et al. (2006), Craul et al. (2007), and Lei et al. (2017). Thus, *L. mittermeieri*, described as the sportive lemur species of IRS V by Rabarivola et al. (2006), becomes a synonym of *L. dorsalis*. Consequently, the sportive lemurs of IRS VI refer to *L. tymerlachsoni* and since Andriaholinirina et al. (2006) and Craul et al. (2007) have already shown that the mitochondrial lineages of Nosy Bé and IRS VI constitute the same clade, *L. tymerlachsoni* occurs also on Nosy Bé. The assignment of all sportive lemur species of Northwest Madagascar to corresponding IRSs is given in Table 1.

The exact provenance of the two museum specimens (types of *L. dorsalis* and *L. grandidieri*) within IRS V remains unclear. The collector, D.C. van Dam, did not provide any relevant information. Based on sites where the same collector sampled other lemurs (*Hapalemur* and *Mirza*), Kappeler et al. (2005) and Louis et al. (2020) proposed either the coast of the Passandava Bay (=Ampasindava; about S13°40', E48°15') or alternatively Mourountsang (=Anorontsangana; S13°55', E47°55'), which are both located within IRS V.

Our study highlights the great value of (historical) museum samples in settling open taxonomic and biogeographic questions by molecular methods, even more so with the advance of high-throughput sequencing technologies (Raupach et al., 2016). Our study also emphasizes the importance of investigating name-bearing types, particularly of cryptic species, to reassess their geographic origins. Considering the synonymy of *L. mittermeieri* with *L. dorsalis*, the genus *Lepilemur* now contains a total of 25 species (*L. aeeclis*, *L. ahmansoni*, *L. ankaranensis*, *L. betsileo*, *L. dorsalis*, *L. edwardsi*, *L. fleuretae*, *L. grewcockorum*, *L. hollandorum*, *L. hubbardorum*, *L. jamesorum*, *L. leucopus*, *L. microdon*, *L. milanoii*, *L. mustelinus*, *L. otto*, *L. petteri*, *L. randrianasoloi*, *L. ruficaudatus*, *L. sahamalaza*, *L. scottorum*, *L. seali*, *L. septentrionalis*, *L. tymerlachsoni*, and *L. wrightae*).

DATA AVAILABILITY

Mitochondrial genome sequences were submitted to GenBank and are available under accession Nos. MW023869 and MW023870.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

C.R., M.H., and D.Z. conceived and designed the study. R.P.M. and R.S. provided valuable samples from the Natural History Museum, London. E.E.L. provided field data. C.R. and M.H. generated the data. C.R. analyzed the data. C.R. and D.Z. wrote the paper. All authors discussed the data and read and approved the final version of the manuscript.

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