Primate Conservation 2014 (28): 9–35

Revision of Madagascar's Dwarf lemurs (Cheirogaleidae: *Cheirogaleus*): Designation of Species, Candidate Species Status and Geographic Boundaries Based on Molecular and Morphological Data

Runhua Lei[1]*, Cynthia L. Frasier[1], Adam T. McLain[1], Justin M. Taylor[1], Carolyn A. Bailey[1], Shannon E. Engberg[1], Azure L. Ginter[1], Richard Randriamampionona[2], Colin P. Groves[3], Russell A. Mittermeier[4] and Edward E. Louis Jr.[1,2]

[1]*Grewcock Center for Conservation and Research, Omaha's Henry Doorly Zoo and Aquarium, Omaha, NE, USA*
[2]*Madagascar Biodiversity Partnership, Manakambahiny, Antananarivo, Madagascar*
[3]*School of Archaeology and Anthropology, Australian National University, Canberra, ACT Australia*
[4]*Conservation International, Arlington, VA, USA*

Abstract: The genus *Cheirogaleus*, the dwarf lemurs, is a radiation of strepsirrhine primates endemic to the island of Madagascar. The dwarf lemurs are taxonomically grouped in the family Cheirogaleidae (Infraorder: Lemuriformes) along with the genera *Microcebus, Mirza*, *Allocebus*, and *Phaner*. The taxonomic history of the genus *Cheirogaleus* has been controversial since its inception due to a paucity of evidence in support of some proposed species. In this study, we addressed this issue by expanding the geographic breadth of samples by 91 individuals and built upon existing mitochondrial (cytb and COII) and nuclear (FIBA and vWF) DNA datasets to better resolve the phylogeny of *Cheirogaleus*. The mitochondrial gene fragments D-loop and PAST as well as the CFTR-PAIRB nuclear loci were also sequenced. In agreement with previous genetic studies, numerous deep divergences were resolved in the *C. major*, *C. minor* and *C. medius* lineages. Four of these lineages were segregated as new species, seven were identified as confirmed candidate species, and four were designated as unconfirmed candidate species based on comparative mitochondrial DNA sequence data gleaned from the literature or this study. Additionally, *C. thomasi* was resurrected. Given the widespread distribution of the genus *Cheirogaleus* throughout Madagascar, the methodology employed in this study combined all available lines of evidence to standardize investigative procedures in a genus with limited access to type material and a lack of comprehensive sampling across its total distribution. Our results highlighted lineages that likely represent new species and identified localities that may harbor an as-yet undescribed cryptic species diversity pending further field and laboratory work.

Key Words: *Cheirogaleus*, candidate species, dwarf lemurs, Madagascar, mtDNA, nuclear DNA

Introduction

Madagascar is an island of such proportions and unique natural history that it has been likened to a continent (de Wit 2003). The population of this biodiversity hotspot, exceeding 20 million people (INSTAT 2011), is ever-increasing its demand on forest resources to fulfill its needs, ranging from timber for construction to expanding agricultural lands (Durbin *et al.* 2003; Harper *et al.* 2007; Gorenflo *et al.* 2011). Unfortunately, an estimated 90% of Madagascar's endemic wildlife resides in these overtaxed forest ecosystems (Dufils 2003). The result of this is a crisis of survival for the most threatened large group of mammals on Earth, the lemurs (Schwitzer *et al.* 2014). Often referred to as the country's flagship species-group, additional research is required to properly characterize the diversity of these strepsirrhine primates.

The identification of new lineages is vital to the preservation of biodiversity. Bringing to light previously unknown species allows for more informed decisions regarding conservation funding and the designation of protected areas (DeSalle and Amato 2004). Advancements in molecular technology, combined with improvements in analytical tools and intensive field investigation, have greatly increased the number of described lemur species in less than three decades—from 36 in 1982 (Tattersall 1982) to more than 100 today (Thalmann 2007; Tattersall 2007, 2013; Mittermeier *et al.* 2008, 2010; Lei *et al.* 2012; Thiele *et al.* 2013). This taxonomic explosion has been especially notable in the family Cheirogaleidae, where

the number of recognized species in the genus *Microcebus* increased from two (Tattersall 1982) to 21 based on the evaluation of mitochondrial DNA (mtDNA) sequence fragments and morphological data (Schmid and Kappeler 1994; Zimmermann *et al.* 1998; Rasoloarison *et al.* 2000, 2013; Kappeler *et al.* 2005; Andriantompohavana *et al.* 2006; Louis *et al.* 2006, 2008; Olivieri *et al.* 2007; Radespiel *et al.* 2008, 2012). Such work has not involved detailed field study of interfertility, and instead relied largely on biogeographic inference, molecular data, and the Phylogenetic Species Concept (PSC; Eldredge and Cracraft 1980; Wheeler and Platnick 2000).

Although the genus *Cheirogaleus*, the dwarf lemurs, is closely related and ecologically similar to *Microcebus*, a comparable radiation has yet to be confirmed. The broadest circumscription of *Cheirogaleus* included seven species (Groves 2000), with more than a century lapsing between the identification of new species (Forsyth Major 1896). This comparatively low diversity may be more of an artifact of incomplete sampling than a reflection of the true state of dwarf lemur diversity, as indicated by recent genetic investigations (Hapke *et al.* 2005; Groeneveld *et al.* 2009, 2010; Thiele *et al.* 2013). An effective exploration of the evolution of *Cheirogaleus* with broader genetic sampling is warranted, but should be conducted with regard to historical specimens and literature to ensure the careful application of names to identified lineages. However, gaining a historical perspective on this genus has proved complicated (Groves 2000).

The circumscription of *Cheirogaleus* was suspect right from its inception. The first species were provisionally described by É. Geoffroy St. Hilaire (1812) based on drawings by Commerçon, which he thought to be faithful representations of lemurs seen in the field. Later study of these three illustrations indicated that they were drawn not directly from specimens, but from memory. This was evidenced by the fact that they had features uncharacteristic of this group, such as claws (Groves 2000). Thus, the initial species concepts were flawed, and the genus was vulnerable to synonymization, resurrection, lumping, splitting, and rearrangements (Wolf 1822; Smith 1833; Lesson 1840; Gray 1872; Forsyth Major 1894, 1896; Elliot 1913; Schwarz 1931; Groves 2000).

Some of the discord in *Cheirogaleus* taxonomic systems, the majority of which were published before 1900, stemmed from the paltry number of specimens available for study. A review of historical documents and museum collection databases showed that prior to the turn of the 20th century there were only about 50 specimens, many incomplete, deposited in a handful of European institutions: the Natural History Museum, London (formerly British Museum (Natural History) BMNH), Muséum National d'Histoire Naturelle (MNHN), Museum für Naturkunde Berlin (MfN, also known as ZMB), and Naturalis Biodiversity Center, formerly Rijksmuseum van Naturlijk Historie (NMNL). Although these specimens were invaluable for introducing dwarf lemurs to the world outside Madagascar, they were insufficient to accurately delimit species based on morphology and anatomy, and these difficulties were compounded by vague collection localities. Schwarz (1931) recognized these challenges and acknowledged that his narrow classification of *Cheirogaleus* was the weakest in his revision of Madagascar's lemurs.

Groves (2000), referring to Schwarz's (1931) work as oversimplified, mounted an extensive morphological study on the same museum specimens as well as on more recent additions. He designated neotypes for *C. major* and *C. medius* in order to fix the names so that other species could be recognized. Unfortunately, there is no type locality information for the *C. major* neotype, but the type locality for *C. medius* is along the Tsiribihina River, previously known as the Tsidsibon River (Goodman and Rakotondravony 1996), in western Madagascar. In addition to the two aforementioned species, Groves also accepted *C. crossleyi*, *C. adipicaudatus*, *C. sibreei*, *C. ravus*, and *C. minusculus*. The species circumscriptions from this work were valuable in laying the foundation for the genetic studies that were to follow.

Using mitochondrial Cytochrome b (cytb) sequences to investigate three morphotypes near Tolagnaro in southeastern Madagascar, Hapke *et al.* (2005) confirmed the existence of three distinct lineages corresponding to Groves's (2000) accepted species. These monophyletic clades were identified as *C. major*, *C. medius*, and *C. crossleyi* based on genetic and morphological comparisons with museum specimens (Hapke *et al.* 2005). The authors did note extensive intraspecific genetic distances, in some cases greater than that found between species of mouse lemurs, within the latter two clades. Further study was encouraged, in particular into the putative southern *C. crossleyi* population and a notable population of *C. medius* in Ankarana in northern Madagascar (Hapke *et al.* 2005).

The existence of strong mitochondrial phylogeographic structure hinted at by Hapke *et al.* (2005) within the *C. medius*, *C. major* and *C. crossleyi* groups was confirmed using an expanded dataset by Groeneveld *et al.* (2009, 2010). This was echoed by Thiele *et al.* (2013) who stressed the existence of unnamed diversity contained within these highly variable units based on the same mtDNA and nuclear sequence data. This resulted in the description of a new species, *C. lavasoensis*, corresponding to Hapke *et al.*'s (2005) divergent southern *C. crossleyi* lineage. Three other species were also proposed, but not described, and were provisionally referred to as *Cheirogaleus* sp. Ranomafana Andrambovato, *C.* sp. Bekaraoka Sambava, and *C.* sp. Ambanja (Thiele *et al.* 2013).

Although many of the species accepted by Groves have been supported, *C. adipicaudatus* and *C. ravus* were synonymized with *C. medius* and *C. major*, respectively, in genetic studies that combined historical and contemporary specimens (Groeneveld *et al.* 2009, 2010). Thus, there are currently six accepted species: *C. major*, *C. medius*, *C. crossleyi*, *C. lavasoensis*, *C. sibreei*, and *C. minusculus*. *C. minusculus* and *C. major* are considered Data Deficient according to IUCN's Red List, while the widespread and morphologically variable *C. medius* is listed as Least Concern (Andrainarivo *et al.* 2013). *C. sibreei* is listed as Critically Endangered, and *C. lavasoensis* is in a similarly dire situation, having been

provisionally named to the upcoming list of the World's 25 Most Endangered Primates 2014–2016 (R. A. Mittermeier, unpubl.). The possibility of segregating additional cryptic taxa from *C. medius* and *C. major* would result in narrower ranges for these species, and the entire genus would be in need of reassessment.

As Groves (2000) designated neotypes for *C. major* and *C. medius*, this work intends to provisionally link those names to their corresponding clades as well as to that of *C. crossleyi*. Once accomplished, clades that represent lineages distinct from those already named can be assessed. To accomplish this, in this study a general work protocol (proposed by Padial *et al.* 2010) was applied that integrates all available evidence in taxonomic practice to standardize the species delimitation process according to the Phylogenetic Species Concept (PSC; Eldredge and Cracraft 1980; Wheeler and Platnick 2000). The number and geographic breadth of *Cheirogaleus* specimens was increased by 91 individuals from throughout the genus' range and the mtDNA and nuclear sequence data sets were enlarged. Geographic regions harboring potential new species were identified and put into context with historical type specimens and localities.

Methods

Sampling collection

From 1999 to 2008, 91 *Cheirogaleus* samples were collected from 31 different localities throughout Madagascar (Table 1; Fig. 1; Appendix II(a)). Of the currently accepted species, only *C. minusculus* could not be assessed as comparable field samples from the Ambositra area could not be obtained for this study. The lemurs were immobilized with a CO_2 projection rifle or blowgun as described in Louis *et al.* (2006). Whole blood (1.0 cc/kg) and four 2 mm biopsies were collected and placed in room temperature preservative (Seutin *et al.* 1991) until transferred to the laboratory for storage at -80 °C. All collection and export permits were obtained from the Ministère de l'Environnement, de l'Ecologie et des Forêts and samples were imported to the United States with appropriate Convention for International Trade in Endangered Species (CITES) permits. We recorded the GPS coordinates to accurately identify the capture location of each animal so that it could be released where it was initially caught (Table 1). Morphometric measurements were taken on sedated animals as described in Louis *et al.* (2006) and Andriantompohavana *et al.* (2007). Museum samples listed in Appendices IIb-IId were measured as in Groves (2000).

Data generation

Genomic DNA was extracted from samples using a phenol-chloroform extraction method (Sambrook *et al.* 1989). To correlate our data with previously published molecular studies, we analyzed the following regions of the mtDNA: Cytochrome b (cytb) (Irwin *et al.* 1991); Cytochrome oxidase subunit II (COII) (Adkins and Honeycutt 1994); the displacement loop or control region (D-loop) (Baker *et al.* 1993; Wyner *et al.* 1999); a fragment of the Cytochrome oxidase subunit III gene (COIII); NADH-dehydrogenase subunits 3, 4L, and 4 (ND3, ND4L, and ND4); as well as the $tRNA^{Gly}$, $tRNA^{Arg}$, $tRNA^{His}$, $tRNA^{Ser}$, and partial $tRNA^{Leu}$ genes (PAST) (Pastorini *et al.* 2000). Three independent nuclear loci were also amplified: alpha fibrinogen intron 4 (FIBA), von Willebrand Factor intron 11 (vWF) and Cystic Fibrosis Transmembrane conductance (CFTR-PAIRB), which were the same loci used in Heckman *et al.* (2007) and Horvath *et al.* (2008). The thermocycler profile conditions were as follows: 95°C for 2 min; 34 cycles of 94°C for 30 sec, 45°C–60°C (Appendix II(e)) for 45 sec, 72°C for 45 sec; 72°C for 10 min. PCR amplifications were carried out in 25 µl reaction volumes containing 2–5 ng of total genomic DNA, 12.5 µM of each primer, 200 µM dNTPs, 10 mM Tris-HCl, 1.5 mM $MgCl_2$, 100 mM KCl (pH 8.0) and 0.5 units of BIOLASE™ Taq DNA Polymerase (Bioline USA Inc., Randolph, MA).

PCR products were confirmed, purified, and sequenced as in Lei *et al.* (2012). Additionally, PCR and sequencing primers specific for *Cheirogaleus* were designed for the cytb, COII, D-loop, PAST fragment, FIBA, vWF, and CFTR-PAIR (Appendix II(e)). Accessioned sequences were used to compare and augment the datasets to evaluate the current taxonomic knowledge of the genus *Cheirogaleus* (Hapke *et al.* 2005; Groeneveld *et al.* 2009, 2010; Thiele *et al.* 2013; see Appendix II(f)).

Phylogenetic analysis

The sequences were edited and aligned using Sequencher v4.10 (Gene Corp, Ann Arbor, Michigan). All sequences (accession numbers KM872106-KM872736) have been deposited in GenBank. MEGA v4.0 (Tamura *et al.* 2007) was used to calculate parsimony informative sites and uncorrected "p" distances for cytb, COII, D-loop, PAST fragments and three nuclear marker sequences. Based on the sequence divergence criteria of Thiele *et al.* (2013), we subdivided *C. crossleyi* into groups Crossleyi A–E, *C. major* into groups Major A–C, *C. medius* into groups Medius A–H and *C. sibreei* formed one group Csi. All genetic data were used for subsequent maximum likelihood (ML) and Bayesian phylogenetic analyses. Optimal nucleotide substitution models for each locus were chosen using the Akaike Information Criterion (AIC) as implemented in Modeltest v3.7 (Posada and Crandall 1998). All ML analyses were performed using a genetic algorithm approach in Garli v0.951 (Zwickl 2006) under the models specified by the AIC in Modeltest. Twenty-five replicates were run for each data set to verify consistency in log likelihood (ln L) scores and tree topologies. Maximum likelihood bootstrap percentages (BP) were estimated in Garli by performing 200 pseudoreplicates on all data sets. PAUP* 4.0b10 (Swofford 2001) was then used to calculate a majority-rule consensus tree for each data set and to visualize the phylogenetic trees.

Bayesian inference analyses of each data set were conducted using MrBayes v3.1.2 (Huelsenbeck *et al.* 2001; Ronquist and Huelsenbeck 2003). The model of evolution was

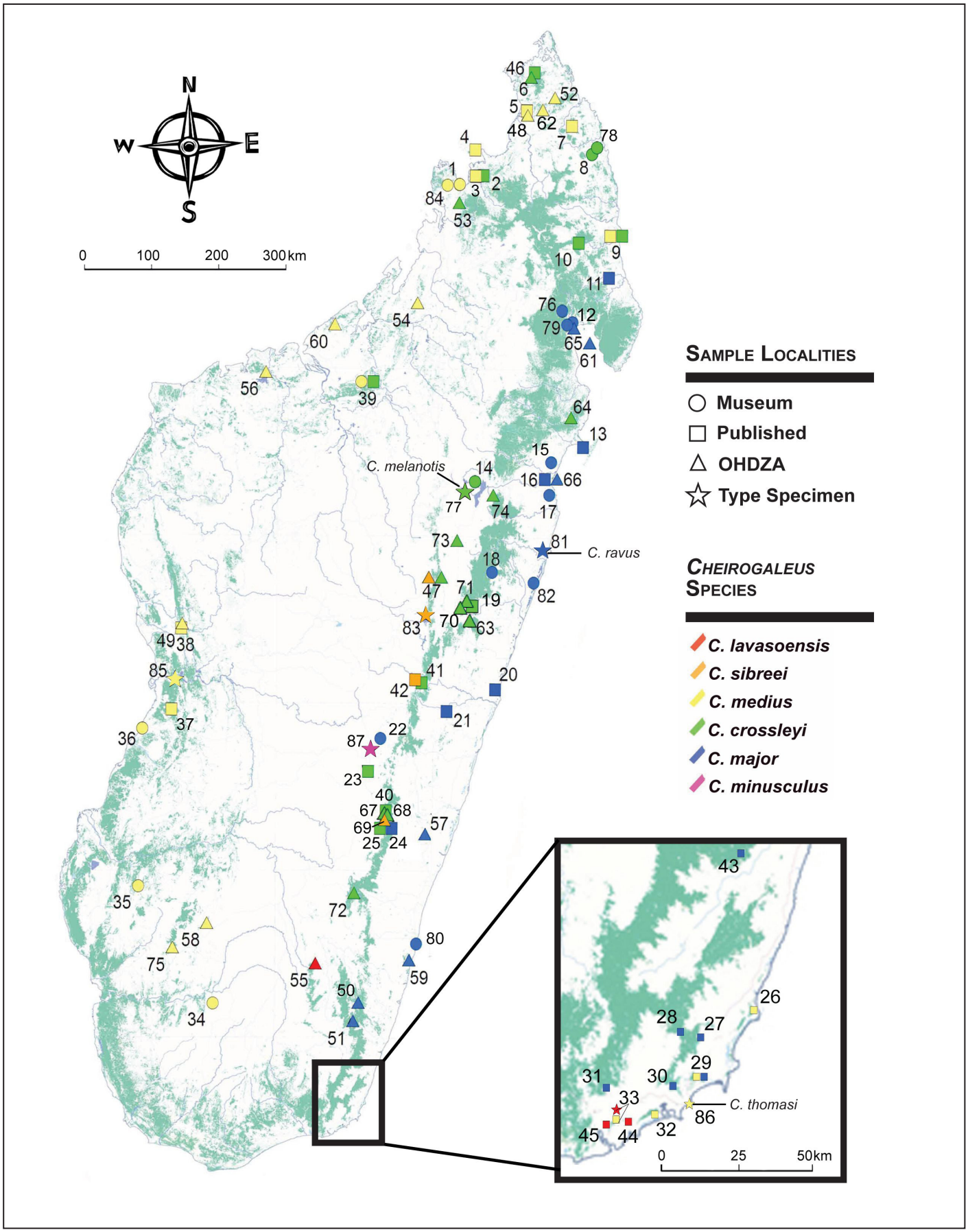

Figure 1. Map of sampling localities of the dwarf lemurs of Madagascar. Triangles represent sites sampled for this study; squares denote sampling localities of recently published field samples; circles represent presumed georeferenced sampling localities of museum specimens. Detailed information for locality sites, marked by locality number, is shown in Table 1 and Appendices II(a,d).

Table 1. Free-ranging *Cheirogaleus* samples used in this study.

ID	Original species designation	Current species designation	Location	Locality number	Latitude	Longitude	Clade
AMB5.22	*C. crossleyi*	CCS1	Montagne d'Ambre	46	-12.52731	49.17331	Crossleyi A
AMB5.23	*C. crossleyi*	CCS1	Montagne d'Ambre	46	-12.53017	49.17464	Crossleyi A
AMB5.27	*C. crossleyi*	CCS1	Montagne d'Ambre	46	-12.51722	49.17950	Crossleyi A
AMB5.28	*C. crossleyi*	CCS1	Montagne d'Ambre	46	-12.47881	49.21222	Crossleyi A
AMB5.29	*C. crossleyi*	CCS1	Montagne d'Ambre	46	-12.47922	49.21606	Crossleyi A
AMB5.30	*C. crossleyi*	CCS1	Montagne d'Ambre	46	-12.47917	49.21597	Crossleyi A
AMB5.31	*C. crossleyi*	CCS1	Montagne d'Ambre	46	-12.51083	49.19275	Crossleyi A
AMB5.32	*C. crossleyi*	CCS1	Montagne d'Ambre	46	-12.51242	49.18956	Crossleyi A
AMB5.34	*C. crossleyi*	CCS1	Montagne d'Ambre	46	-12.47822	49.21717	Crossleyi A
AMB5.35	*C. crossleyi*	CCS1	Montagne d'Ambre	46	-12.49519	49.20783	Crossleyi A
ANJZ1	*C. crossleyi*	*C. crossleyi*	Anjozorobe	47	-18.47750	47.93812	Crossleyi B
ANJZ2	*C. crossleyi*	*C. crossleyi*	Anjozorobe	47	-18.47750	47.93812	Crossleyi B
ANJZ3	*C. crossleyi*	*C. crossleyi*	Anjozorobe	47	-18.47750	47.93812	Crossleyi B
ANK5.12	*C. medius*	CCS6	Ankarana	48	-12.96631	49.13808	Medius A
ANK5.13	*C. medius*	CCS6	Ankarana	48	-12.96631	49.13808	Medius A
ANK5.14	*C. medius*	CCS6	Ankarana	48	-12.96631	49.13808	Medius A
ANK5.15	*C. medius*	CCS6	Ankarana	48	-12.96631	49.13808	Medius A
ANK5.16	*C. medius*	CCS6	Ankarana	48	-12.96631	49.13808	Medius A
ANK5.17	*C. medius*	CCS6	Ankarana	48	-12.96631	49.13808	Medius A
ANK5.18	*C. medius*	CCS6	Ankarana	48	-12.96631	49.13808	Medius A
ANK5.19	*C. medius*	CCS6	Ankarana	48	-12.96631	49.13808	Medius A
ANK5.20	*C. medius*	CCS6	Ankarana	48	-12.96631	49.13808	Medius A
ANK5.21	*C. medius*	CCS6	Ankarana	48	-12.96631	49.13808	Medius A
BEMA7.19	*C. medius*	*C. medius*	Tsingy de Bemaraha	49	-19.04525	44.77772	Medius B
BEMA7.21	*C. medius*	*C. medius*	Tsingy de Bemaraha	49	-19.04581	44.78119	Medius B
BEMA7.22	*C. medius*	*C. medius*	Tsingy de Bemaraha	49	-19.05383	44.78075	Medius B
DOG14	*C. major*	CCS4	Midongy du Sud	50	-23.52111	47.08803	Major A
DOG8.2	*C. major*	CCS4	Beharena Sagnira Midongy	50	-23.52464	47.09236	Major A
DOG8.3	*C. major*	CCS4	Beharena Sagnira Midongy	50	-23.52161	47.08717	Major A
DOG8.4	*C. major*	CCS4	Beharena Sagnira Midongy	50	-23.52064	47.09025	Major A
DONGY8.4	*C. major*	CCS4	Ampasy Midongy	51	-23.74075	47.02592	Major A
DONGY8.5	*C. major*	CCS4	Ampasy Midongy	51	-23.74272	47.03344	Major A
DONGY8.6	*C. major*	CCS4	Ampasy Midongy	51	-23.74458	47.02656	Major A
FIA5.19	*C. medius*	CCS6	Andrafiamena (Anjakely)	52	-12.91539	49.31956	Medius A
FIA5.22	*C. medius*	CCS6	Andrafiamena (Anjakely)	52	-12.91539	49.31956	Medius A
GAR8	*C. crossleyi*	CCS2	Manongarivo	53	-14.02369	48.27233	Crossleyi C
HIH7.3	*C. medius*	UCS2	Anjiamangirana	54	-15.21642	47.75189	Medius D
HIH9	*C. medius*	UCS2	Anjiamangirana (Antsohihy)	54	-15.15692	47.73311	Medius D
JOZO4.7	*C. crossleyi*	*C. crossleyi*	Anjozorobe	47	-18.46789	47.94131	Crossleyi B
JOZO4.8	*C. crossleyi*	*C. crossleyi*	Anjozorobe	47	-18.46789	47.94131	Crossleyi B
JOZO4.9	*C. crossleyi*	*C. crossleyi*	Anjozorobe	47	-18.46789	47.94131	Crossleyi B
JOZO4.10	*C. crossleyi*	*C. crossleyi*	Anjozorobe	47	-18.46789	47.94131	Crossleyi B
JOZO4.17	*C. sibreei*	*C. sibreei*	Anjozorobe	47	-18.46789	47.94131	*C. sibreei*
KAL7.7	*C. crossleyi*	*C. lavasoensis*	Kalambatritra (Sahalava)	55	-23.53672	46.53350	Crossleyi E
KIBO7.9	*C. medius*	UCS1	Tsiombikibo	56	-16.04886	45.81067	Medius C
LAKI5.18	*C. major*	CCS5	Lakia	57	-21.51558	47.91147	Major B
LAKI5.19	*C. major*	CCS5	Lakia	57	-21.51558	47.91147	Major B
LAKI5.26	*C. major*	CCS5	Lakia	57	-21.51558	47.91147	Major B
LAVA1	*C. medius*	*C. medius*	Analalava	58	-22.59242	45.13333	Medius B
LAVA45	*C. medius*	*C. medius*	Analalava	58	-22.58778	45.12803	Medius B
MAB4.9	*C. major*	CCS4	Manombo	59	-23.01228	47.73281	Major A
MAR30	*C. medius*	UCS3	Mariarano	60	-15.47992	46.69333	Medius E
MAS6.10	*C. major*	*C. major*	Masoala (Masiaposa)	61	-15.67189	49.96617	Major C
MAS6.8	*C. major*	*C. major*	Masoala (Masiaposa)	61	-15.67122	49.96375	Major C
MAS6.9	*C. major*	*C. major*	Masoala (Masiaposa)	61	-15.67150	49.96417	Major C
MATY5.31	*C. medius*	CCS6	Analamera (Ampasimaty)	62	-12.76556	49.48358	Medius A
MATY5.40	*C. medius*	CCS6	Analamera (Ampasimaty)	62	-12.76703	49.48358	Medius A
MATY5.42	*C. medius*	CCS6	Analamera (Ampasimaty)	62	-12.77136	49.48303	Medius A

Table 1. continued

ID	Original species designation	Current species designation	Location	Locality number	Latitude	Longitude	Clade
MIZA16	*C. crossleyi*	*C. crossleyi*	Maromizaha	63	-18.97375	48.46461	Crossleyi B
MIZA19	*C. crossleyi*	*C. crossleyi*	Maromizaha	63	-18.97067	48.46431	Crossleyi B
MIZA6.1	*C. crossleyi*	*C. crossleyi*	Maromizaha	63	-18.95694	48.49236	Crossleyi B
MIZA6.2	*C. crossleyi*	*C. crossleyi*	Maromizaha	63	-18.95694	48.49236	Crossleyi B
MIZA7.1	*C. crossleyi*	*C. crossleyi*	Maromizaha	63	-18.95694	48.49236	Crossleyi B
NARA8.2	*C. major*	*C. major*	Mananara-Nord (Ambavala)	64	-16.55831	49.73422	Major C
NOSY46	*C. major*	*C. major*	Nosy Mangabe	65	-15.49539	49.76256	Major C
POLO5.2	*C. major*	*C. major*	Tampolo	66	-17.28989	49.40753	Major C
POLO5.20	*C. major*	*C. major*	Tampolo	66	-17.28747	49.40858	Major C
POLO5.21	*C. major*	*C. major*	Tampolo	66	-17.28783	49.40894	Major C
RANO229	*C. crossleyi*	CCS3	Ranomafana (Talatakely)	67	-21.24833	47.42406	Crossleyi D
RANO2.95	*C. crossleyi*	CCS3	Ranomafana (Vatoharanana)	68	-21.29250	47.43842	Crossleyi D
RIR01	*C. sibreei*	*C. sibreei*	Maharira	69	-21.32367	47.40786	*C. sibreei*
TAD4.10	*C. crossleyi*	*C. crossleyi*	Mantadia	70	-18.80942	48.42731	Crossleyi B
TAD4.11	*C. crossleyi*	*C. crossleyi*	Mantadia	70	-18.80942	48.42731	Crossleyi B
TAD4.12	*C. crossleyi*	*C. crossleyi*	Mantadia	70	-18.80942	48.42731	Crossleyi B
TOR6.2	*C. crossleyi*	*C. crossleyi*	Torotorofotsy	71	-18.83658	48.34719	Crossleyi B
TORO8.11	*C. crossleyi*	*C. crossleyi*	Torotorofotsy	71	-18.77044	48.42814	Crossleyi B
TORO8.16	*C. crossleyi*	*C. crossleyi*	Torotorofotsy	71	-18.76856	48.42475	Crossleyi B
TRA8.81	*C. crossleyi*	CCS3	Andringitra (Ambarongy)	72	-22.22269	47.01889	Crossleyi D
TRA8.82	*C. crossleyi*	CCS3	Andringitra (Ambarongy)	72	-22.22292	47.01950	Crossleyi D
TVY7.12	*C. crossleyi*	*C. crossleyi*	Ambatovy	73	-18.85086	48.29256	Crossleyi B
TVY7.196B	*C. crossleyi*	*C. crossleyi*	Ambatovy	73	-18.86433	48.31136	Crossleyi B
TVY7.197	*C. crossleyi*	*C. crossleyi*	Ambatovy	73	-18.86658	48.30972	Crossleyi B
TVY7.199	*C. crossleyi*	*C. crossleyi*	Ambatovy	73	-18.87294	48.30500	Crossleyi B
TVY7.20	*C. crossleyi*	*C. crossleyi*	Ambatovy	73	-18.84797	48.29433	Crossleyi B
TVY7.200	*C. crossleyi*	*C. crossleyi*	Ambatovy	73	-18.86883	48.30975	Crossleyi B
TVY7.206	*C. crossleyi*	*C. crossleyi*	Ambatovy	73	-18.87289	48.30453	Crossleyi B
TVY7.207	*C. crossleyi*	*C. crossleyi*	Ambatovy	73	-18.87178	48.30297	Crossleyi B
TVY7.22	*C. crossleyi*	*C. crossleyi*	Ambatovy	73	-18.85017	48.29200	Crossleyi B
TVY7.33	*C. crossleyi*	*C. crossleyi*	Ambatovy	73	-18.85086	48.29256	Crossleyi B
ZAH240	*C. crossleyi*	*C. crossleyi*	Zahamena	74	-17.48917	48.74722	Crossleyi B
ZOM6.2	*C. medius*	*C. medius*	Zombitse	75	-22.88631	44.69375	Medius B

selected by using MrModeltest v2.2 (Nylander 2004). Two simultaneous Markov Chain Monte Carlo (MCMC) runs with four chains each at the default temperature were performed for 5,000,000 generations. Majority-rule consensus trees were constructed from 50,000 sample trees in PAUP* 4.0b10 for each data set (Swofford 2001). Topologies prior to –ln likelihood of equilibrium were discarded as burnin, and clade posterior probabilities (PP) were computed from the remaining trees.

We implemented the coalescent-based Bayesian species tree inference method using the software *BEAST (Drummond and Rambaut 2007; Heled and Drummond 2010) (an extension of BEAST v1.8.0). This software also implements a Bayesian MCMC analysis, and is able to co-estimate species trees and gene trees simultaneously. ‘‘Species tree’’ was used in the sense of Heled and Drummond (2010) here and in the following to distinguish this method from other analyses of combined data. For comparison to Thiele *et al*. (2013), we randomly selected one individual from each *Cheirogaleus* lineage to create two datasets: nuclear and a combined nuclear and mtDNA data set. Monophyly constraints were applied to the *Cheirogaleus* ingroups. The split between *Cheirogaleus* and *Microcebus* was used as a calibration point for divergence time estimates with a normal prior (mean = 23.0 Ma, Standard deviation = 2.4 Ma) on the divergence time of the root node to the species trees in all analyses, which was based on Horvath *et al.* (2008) and Thiele *et al.* (2013). Analyses were performed based on each locus in the *Cheirogaleus* dataset. Separate substitution models for each locus were utilized (HDZ dataset: GTR+G, COII: GTR+I+G, cytb: HKY+I+G, DLP: GTR+I+G, PAST: HKY, CFTR: HKY+G, FIBA: HKY + G, vWF: HKY + G; Combined dataset: GTR+I+G, cytb: HKY+G, FIBA: HKY+G, vWF). The input file was formatted with the BEAUti utility included in the software package, using the same partition scheme of the concatenated analysis.

Although *BEAST does not require the inclusion of outgroups for rooting purposes, *Microcebus ravelobensis* was incorporated in the analysis. The *BEAST analysis was conducted using a relaxed uncorrelated lognormal clock model, a random starting tree, and a speciation Yule process as the tree prior. Each run comprised 100,000,000 generations sampled every 10,000th generation. The post-burnin samples from the

two independent runs were combined with a burnin of 10% for both datasets. Convergence of the MCMC was assessed by examining trace plots and histograms in Tracer v1.6 after obtaining an effective sample size (ESS) greater than 200 for all model parameters (Rambaut and Drummond 2009). A maximum clade credibility tree was generated using the program TreeAnnotator v1.8.0 provided in the BEAST package, with a burnin of 1000 (10%) and visualized in FigTree v1.3.1 (Drummond and Rambaut 2007; Rambaut 2009).

As described in Davis and Nixon (1992) and Louis *et al.* (2006), we used MacClade 3.01 (Maddison and Maddison 1992) and MEGA v4.0 (Tamura *et al.* 2007) in a diagnostic search to designate evolutionarily significant units (ESU) for the *Cheirogaleus* species using a population aggregate analysis (PAA) of the sequence data. With the sequential addition of each individual without an *a priori* species designation, a PAA distinguishes attributes or apomorphic characters according to the smallest definable unit (Davis and Nixon 1992; Louis *et al.* 2006).

To further corroborate the validity of each ESU, we implemented a system to categorize and assemble all lines of evidence from the available ecological and genetic data. Thus, deep genealogical lineages of *Cheirogaleus* were classified based on framework by Vieites *et al.* (2009), Padial *et al.* (2010) and Ratsoavina *et al.* (2013). First, the currently valid species names were assigned to lineages based on diagnostic morphological characters, taxonomy, and assignment of sequences from populations close to or at type localities when known. Second, based on the amount of evidence available from other data sets, unnamed lineages were classified as confirmed candidate species (CCS) or unconfirmed candidate species (UCS). The lineages referred to as CCS are strongly supported by morphological, genetic, and biogeographic evidence and most likely represent distinct species that were not previously scientifically named. The lineages that were denoted as UCS require additional evidence, thus the taxonomic status remains unclear.

Results

Sequence data

A concatenated mtDNA dataset with cytb, D-loop and PAST fragments was assembled only with data from the 91 field samples collected for this study (Fig. 1, Table 1) as the sequence information on all of these fragments was not available for samples used in previous studies. This yielded 4,826 bp of aligned data that contained 1,550 variable sites and 1,440 parsimony informative sites (Table 2). The complete cytb sequences of this study were aligned with the 124 *Cheirogaleus* cytb accessioned sequences from GenBank, which resulted in a total set of 98 haplotypes defined through 384 variable sites. The 48 *Cheirogaleus* COII published sequences from GenBank were aligned with sequences from this study resulting in 191 variable sites defining 55 haplotypes.

The concatenated nucDNA datasets from 91 field samples amounted to 2,337 bp, which contained 163 variable sites and 120 parsimony informative sites (Table 2). There were four bp insertions at site 377–380 (TGAT) in the CFTR-PAIRB fragment of *C. sibreei*. In the vWF alignment, there were two individuals carrying alleles with a deletion of 242 bp from the Medius B clade which were collected in Zombitse and Analalava. Combining the FIBA and vWF published sequences from GenBank and sequences of this study resulted in a data set of 208 sequences. There were 45 variable sites among 606 bp of FIBA fragment sequences. The 795 bp vWF fragment had 108 variable sites. In addition, there were 11 individuals carrying alleles with a deletion of 242 bp, all of which are from either Medius B or Medius G (Groeneveld *et al.* 2010). There are 21 individuals carrying alleles with a deletion of 19 bp, all of which were from Medius A and F distributed in northern Madagascar except for one sample from Tsingy de Bemaraha (Medius B) (Groeneveld *et al.* 2010). There were three bp deletions at sites 200–202 (CAT) and two bp insertions at sites 610–611 (AG) in the vWF fragment of *C. sibreei*.

The three mitochondrial data sets best fit a GTR+I+G model according to AIC for both ML and Bayesian analyses except the D-loop, cytb, COII and PAST data sets with TVM+I+G for ML analyses (Table 2). The vWF locus was found to best fit an HKY+I+G model for both ML and Bayesian analyses, while the CFTR-PAIRB+FIBA+vWF data set best fit a GTR+I+G model for both ML and Bayesian analyses. A TVM+I+G model was favored for the FIBA locus (analyzed under a GTR+I+G model in Bayesian phylogenetic analyses).

Genetic distances

The uncorrected p-distances of the four mtDNA and three nucDNA sequence alignments were presented in Appendices II(g–m). In mtDNA sequence alignments, distances between 18 *Cheirogaleus* clades ranged from 0.021 to 0.142 in cytb (Appendix II(g)), from 0.021 to 0.149 in PAST (Appendix II(h)), from 0.045 to 0.224 in D-loop (Appendix II(i)) and from 0.016 to 0.126 in COII (Appendix II(j)). Distances between the five most closely related clades ranged from 0.021 to 0.042 in cytb, from 0.021 to 0.044 in PAST, from 0.038 to 0.054 in D-loop and from 0.016 to 0.035 in COII. The greatest intra-clade distances were 0.014 in cytb, 0.011 in PAST, 0.029 in D-loop, and 0.019 in COII. Based on genetic distance, we subdivided *Cheirogaleus crossleyi* into clades Crossleyi A–E; *C. medius* into Medius A–H; and *C. major* into Major A–C. *Cheirogaleus sibreei* formed one group (Table 1).

In nucDNA sequence alignments, distances between 18 *Cheirogaleus* clades ranged from 0.000 to 0.011 in CFTR-PAIRB (Appendix II(k)), from 0.000 to 0.007 in FIBA (Appendix II(l)) and from 0.000 to 0.016 in vWF (Appendix II(m)). The distances between clades of *C. crossleyi* were negligible, as were the distances between clades of *C. major* and *C. medius*.

Phylogenetic analyses

Based on the phylogenetic inference from the Bayesian and ML analyses of the four mtDNA sequence alignments,

Table 2. Data sets and nucleotide substitution models.

Data set	AL	No.S	No.H	No.VS/No.PIS	ML[b]	Bayesian[b]
D-Loop+cytb+COII+PAST	4826	91	77	1550/1440	TVM+I+G	GTR+I+G
cytbGB	1140	216	98	384/348	GTR+I+G	GTR+I+G
COIIGB	684	139	55	191/170	GTR+I+G	GTR+I+G
CFTR-PAIRB+FIBA+vWF	2337	91	[a]	163/120	GTR+I+G	GTR+I+G
FIBAGB	606	208	[a]	45/30	TVM+I+G	GTR+I+G
vWFGB	795	208	[a]	108/80	HKY+I+G	HKY+I+G

Note: AL, Alignment length including outgroup; No.S, Number of sequences in data set excluding outgroup; No.H, number of haplotypes excluding outgroup; [a]only for mitochondrial DNA; No.VS and No. PIS, number of variable site and number of parsimony informative sites, respectively, excluding outgroup; [b]Nucleotide substitution models for each data set.

four major *Cheirogaleus* subgroups were represented, which correspond to the four species *C. crossleyi*, *C. major*, *C. medius* and *C. sibreei* (Figs. 2–3; Appendix I(a)). All of these subgroups were strongly supported (ML BP = 100 and Bayesian PP > 0.99). Cytb was used by all the previously published data, and the results of analyses did not vary based on data type, so for expediency we will use cytb for subsequent analyses and discussions.

Cheirogaleus sibreei formed a distinct clade with high support values (ML BP = 100 and Bayesian PP = 1.00), which contains mtDNA haplotypes from Tsinjoarivo (Vatateza), Anjozorobe and Maharira in Ranomafana National Park. There are more than 180 km of continuous high altitude forest between Tsinjoarivo and Maharira and 130 km of continuous high altitude forest between Tsinjoarivo and Anjozorobe, expanding the possible known range of this species. Additional research in this corridor could provide confirmation of a continuous extended range.

The *C. crossleyi* subgroup contained five distinct clades (Crossleyi A–E) with high support values (ML BP > 99 and Bayesian PP = 1.00). Crossleyi A was composed of mtDNA haplotypes from the northern tip of Madagascar (Montagne d'Ambre, localities 6 and 46). Crossleyi B contained haplotypes from eastern Madagascar (from Tsinjoarivo to Zahamena) and Iharana, a site whose exact locality was unknown in northern Madagascar but may be Vohemar (Falling Rain Genomics, Inc. 2014). A sample from Ampijoroa (locality 39) in western Madagascar was also included, but only 300 bp of data were available, making its placement in the tree possibly a result of missing data rather than a reflection of its true relationship. Crossleyi C had haplotypes from northern Madagascar (localities 3, 9, 10, and 53). Crossleyi D was composed of mtDNA haplotypes from southeastern Madagascar (localities 40, 67, 68 and 71). Crossleyi E contained mtDNA haplotypes from the southeastern tip of Madagascar (localities 33, 44, and 45) and one from Kalambatritra. Uncorrected p-distances based on the complete mtDNA cytb sequence data were calculated and presented in Appendix II(g). The genetic distances were from 5.6–8.1% between Crossleyi A and Crossleyi B–E. Compared with Crossleyi B and Crossleyi A, C–E, there were 4.2–8.3% sequence divergence. Similarly, there are 4.2–7.7%, 6.0–8.2%, 7.7–8.3% between Crossleyi C and Crossleyi A–B, D–E, between Crossleyi D and Crossleyi A–C, E, between Crossleyi E and Crossleyi A–D, respectively.

The *C. major* subgroup included three distinct clades (Major A–C). Major A was strongly supported (ML BP = 99 and Bayesian PP = 1.00) and was composed of mtDNA haplotypes from southeastern Madagascar (Localities 27–31, 43, 44, 50, 51 and 59). Major B had a ML BP value of 86 and a Bayesian PP of 0.89, including haplotypes from central-eastern Madagascar (Localities 21, 24 and 57). Major C had a ML BP value of 78 and a Bayesian PP of 0.90, containing mtDNA haplotypes from central-eastern and northeast Madagascar (Localities 11, 13, 16, 18, 61 and 64–66). The genetic distances in the complete cytb fragment (Appendix II(g)) were from 3.2–3.6% between Major A and Major B–C. Compared with Major B and Major A and C, there was 2.2–3.2% sequence divergence. Similarly, there was 2.2–3.6% sequence divergence between Crossleyi C and Crossleyi A–B.

The *C. medius* subgroup included eight distinct clades (Medius A–H). Medius C, D, E, F and H have single localities such as Tsiombikibo, Anjiamangirana, Mariarano, Sambava and Ambanja, respectively. Medius B was strongly supported (ML BP = 95 and Bayesian PP = 1.00), which contained mtDNA haplotypes from Zombitse to Tsingy de Bemaraha (Localities 37, 38, 49, 58 and 75). Medius G was highly supported (ML BP = 100 and Bayesian PP = 1.00), composed of mtDNA haplotypes from the southeastern tip of Madagascar (Localities 26, 29, 32, and 33). Medius A formed a distinct clade with a high support value (ML BP = 96 and Bayesian PP = 1.00), with mtDNA haplotypes from Ankarana to Andrafiamena (Localities 5, 7, 26, 29, 32, and 33). The genetic distances of the complete cytb fragment (Appendix II(g)) were from 4.7–8.0% between Medius A and Medius B–H. Compared with Medius B and Medius A and C–H, there was 2.1–7.2% sequence divergence. Similarly, there was 3.1–7.7% sequence divergence between Crossleyi G and Crossleyi A–F and H.

Based on Figure 4, all mtDNA published sequences from museum samples of *C. major* were clustered in clade Major C. The mtDNA published sequence from a museum sample of *C. crossleyi* was included in clade Crossleyi B. The mtDNA published sequences from museum samples of *C. medius* were placed in clade Medius B. A mtDNA published sequence from a single museum sample (#1967-1655) of *C. medius* was placed in clade Medius E, which is geographically close to

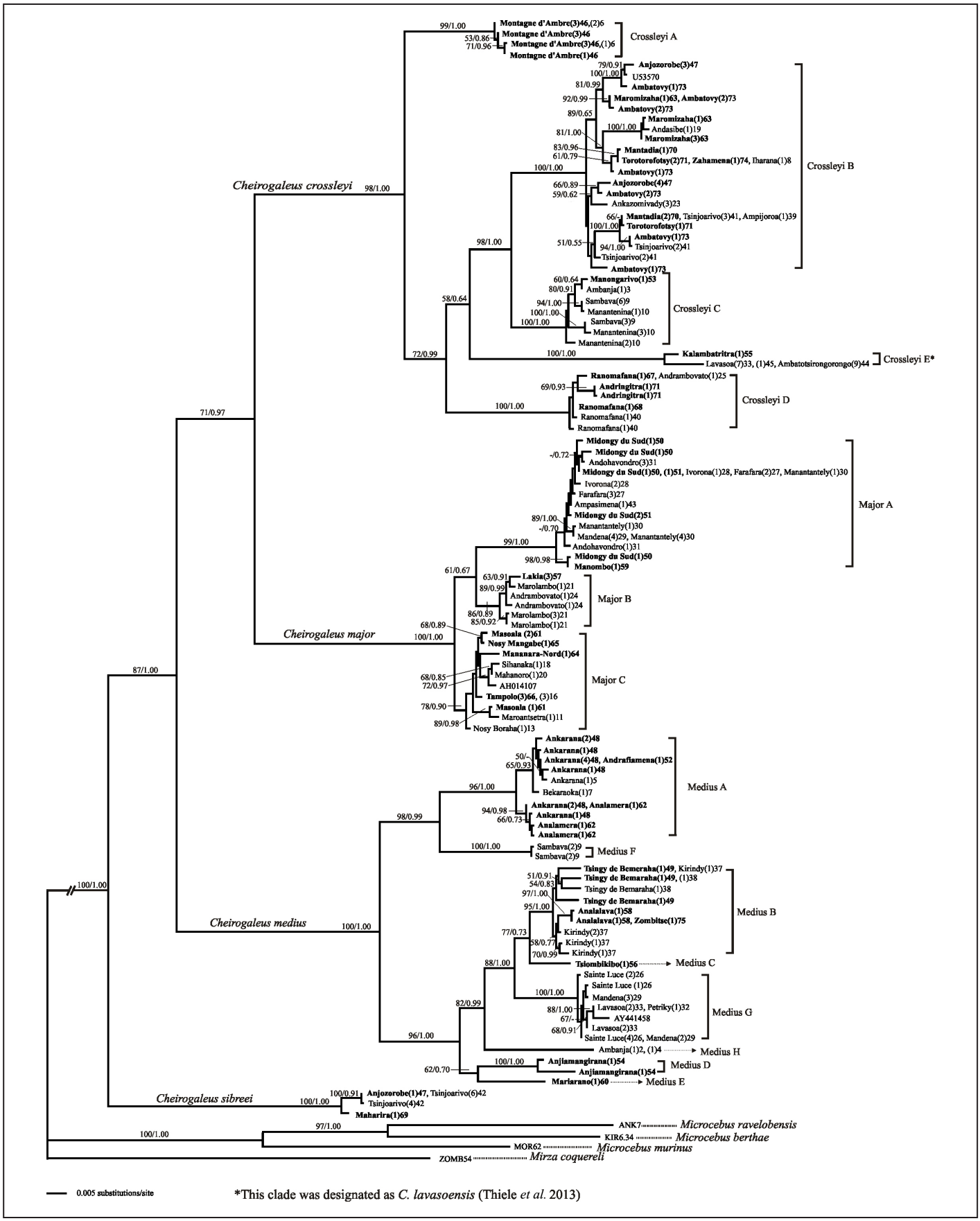

Figure 2. Phylogenetic relationships between *Cheirogaleus* species inferred from the maximum likelihood and Bayesian approaches of the complete cytb sequence data (1140 bp) generated from the 225 *Cheirogaleus* individuals with four out-group taxa. New field samples were labeled in bold. Numbers on branches represent maximum likelihood values followed by posterior probability support. Tip labels include locality, followed by number of individuals carrying the haplotype in brackets, then the locality numbers.

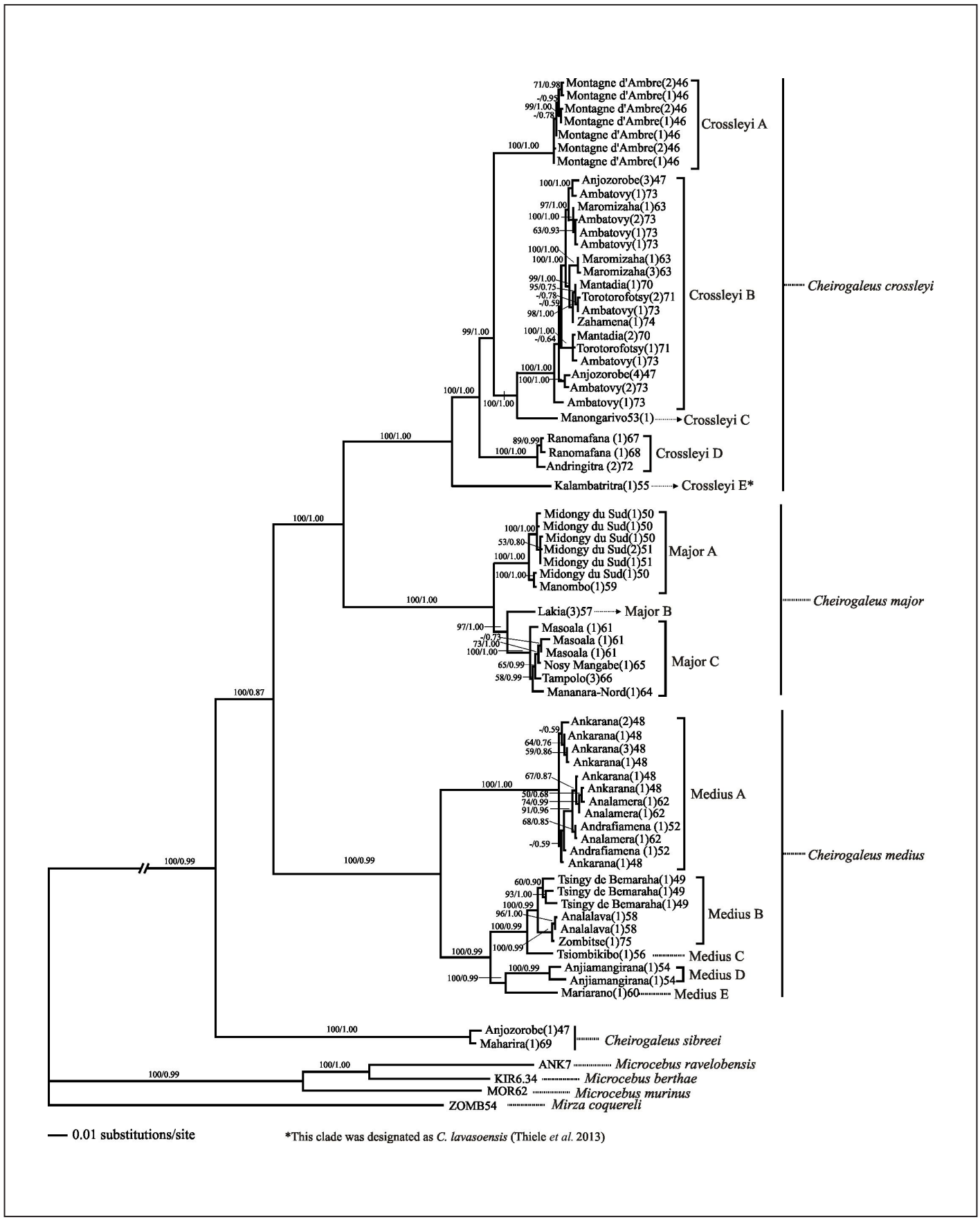

Figure 3. Phylogenetic relationships between *Cheirogaleus* species inferred from the maximum likelihood and Bayesian approaches of D-loop, cytb, COII and PAST combined sequence data (4826 bp) generated from the 91 *Cheirogaleus* individuals with four out-group taxa. Numbers on branches represent maximum likelihood values followed by posterior probability support. Tip labels include locality, followed by number of individuals carrying the haplotype in brackets, then the locality numbers.

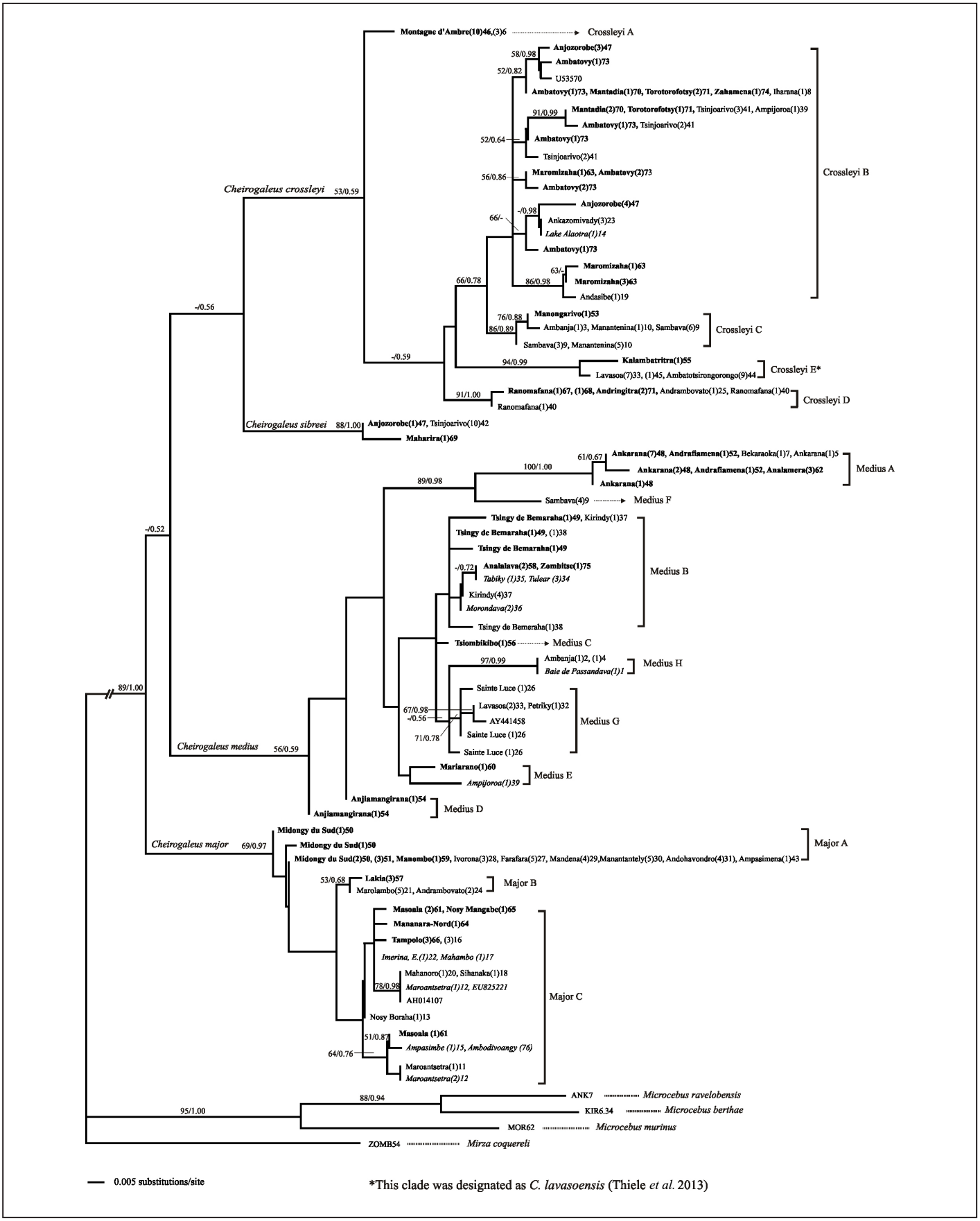

Figure 4. Phylogenetic relationships between *Cheirogaleus* species inferred from the maximum likelihood and Bayesian approaches of the partial cytb sequence data (246 bp) generated from the 242 *Cheirogaleus* individuals with four out-group taxa. Sequences generated from new field samples were labeled in bold and published sequences derived from museum specimens were presented in italic. Numbers on branches represent maximum likelihood values followed by posterior probability support. Tip labels include locality, followed by number of individuals carrying the haplotype in brackets, then the locality numbers.

its sister taxon (Fig. 1). A mtDNA published sequence from another single museum sample (#1887:66b) of *C. medius* was placed in clade Medius H, which is geographically close to its sister taxa (Fig. 1).

Based on the phylogenetic inference from the Bayesian and ML analyses of the three nucDNA sequence alignments, four major *Cheirogaleus* subgroups were strongly supported (ML BP = 100 and Bayesian PP > 0.98), which were congruent to phylogenetic analyses based on mtDNA data (Fig. 5; Appendices I(b–c)). However, in contrast to forming distinct clades and strong phylogeographic structures and harboring extremely divergent haplotypes as in the mtDNA data set, only Medius A formed a clade with distinct subdivisions. There were no distinct clades, and alleles were shared among populations, even with a geographic distance of more than 900 km (Fig. 5; Appendices I(b–c)). The incongruence may be due to ancient introgression, incomplete lineage sorting, or insufficient nucDNA data.

In the two Bayesian species tree analyses, ESS for all factors was greater than 200. *Cheirogaleus crossleyi*, *C. major*, *C. medius* and *C. sibreei* formed strongly supported monophyletic groups (Fig. 6). The relationships among subgroups were incongruent between analyses.

Population aggregate analyses

The results of the PAA of all the sequence data were presented in Appendices II(n–t). In the clade Crossleyi A, there were four diagnostic sites in cytb, nine in PAST, five in D-loop and two in COII. In the clade Crossleyi D, there were six diagnostic sites in cytb, 13 in PAST, two in D-loop and one in COII. In the clade Major A, there were three diagnostic sites in cytb, eight in PAST, none in D-loop and two in COII. In the clade Major C, there were two diagnostic sites in cytb, two in PAST, one in D-loop and none in COII. In the clade Medius A, there were five diagnostic sites in cytb, 36 in PAST, 13 in D-loop and one in COII. In the clade Medius B, there were three diagnostic sites in cytb, one in PAST, none in D-loop and none in COII. In the clade Medius G, there were four diagnostic sites in cytb. For these clades, there were no diagnostic sites found in the three nuclear gene sequence data sets.

Morphometric data

The mean and standard deviation of the morphometric data for each clade of dwarf lemurs are presented in Appendix I(d), and Appendices II(b–c, u) (see Table 4). No extensive quantitative and comparative analyses were conducted on the morphometric data because of numerous factors such as small sample sets, independent data sets, multiple data collectors, the variance between live individuals versus processed museum vouchers, along with seasonal and age differences of individual dwarf lemurs. Therefore, morphometric information was provided as supplemental data only.

Taxonomy of Cheirogaleus

Combining the information from previous studies and the new results obtained here, the taxonomy of *Cheirogaleus* was elucidated, including six nominal species of *Cheirogaleus* (excluding *C. minusculus*), seven CCS, and four UCS. The described species and undescribed forms, and the associated morphological and geographical data assessed in this study are summarized in Tables 3 and 4. The geographical distribution of accepted species, CCS and UCS in the genus *Cheirogaleus* are presented in Figure 7. Localities of museum specimens were georeferenced when possible for historical information on distributions; see Appendix II(d) for institutes of deposit, localities and determination histories.

Discussion

Species concepts

Increasingly powerful computational and laboratory tools have made ever more complex genomic analyses (Baker 2010) possible and pushed the boundaries of species definitions outside the realm of Mayr's (1942) Biological Species Concept (BSC). The BSC states that sympatric reproductive isolation is the hallmark of a species. The PSC (Eldredge and Cracraft 1980; Wheeler and Platnick 2000) grew out of the early work of Hennig (1965) and provides a methodology for species description more suitable to the era of genomics, allowing new species to be described based on fixed variations in sequence data, and proposing the monophyly of a species as a criterion. Descriptions of new lemur species have partly relied on this concept to justify the elevation of often phenotypically similar animals to species status (Louis *et al.* 2006; Radespiel *et al.* 2012; Rasoloarison *et al.* 2013; Thiele *et al.* 2013). Relying on fixed genetic characters as markers has now become an accepted methodology for the delineation of new species (Schuh and Brower 2009; Louis and Lei 2014).

Historical and contemporary taxonomy

Genetic analyses indicate that the morphologically variable and widespread species, *C. major*, *C. medius* and *C. crossleyi*, harbor previously uncharacterized diversity (Thiele *et al.* 2013). The recent description of *C. lavasoensis* addressed this in part, but resulted in a polyphyletic *C. crossleyi* at odds with the PSC (Thiele *et al.* 2013). To support the continued recognition of this new species, there must be agreement on which lineages represent *C. crossleyi*, *C. major* and *C. medius sensu stricto*. To address this need, we link these names to their respective clades and provide additional support for *C. sibreei* and *C. lavasoensis*, which were already corroborated with genetic evidence (Groeneveld *et al.* 2009, 2010; Thiele *et al.* 2013). Summaries of genetic and historical data are provided in the species descriptions (see below). The remaining unnamed lineages complemented with sufficient evidence can now be elevated to species status.

Cheirogaleus does not appear to have undergone as large of a radiation as *Microcebus*, but our molecular analyses indicate that the number of described species is still well below the probable total (Schmid and Kappeler 1994; Zimmermann *et al.* 1998; Rasoloarison *et al.* 2000, 2013; Kappeler *et al.* 2005; Louis *et al.* 2006; Olivieri *et al.* 2007; Radespiel *et al.*

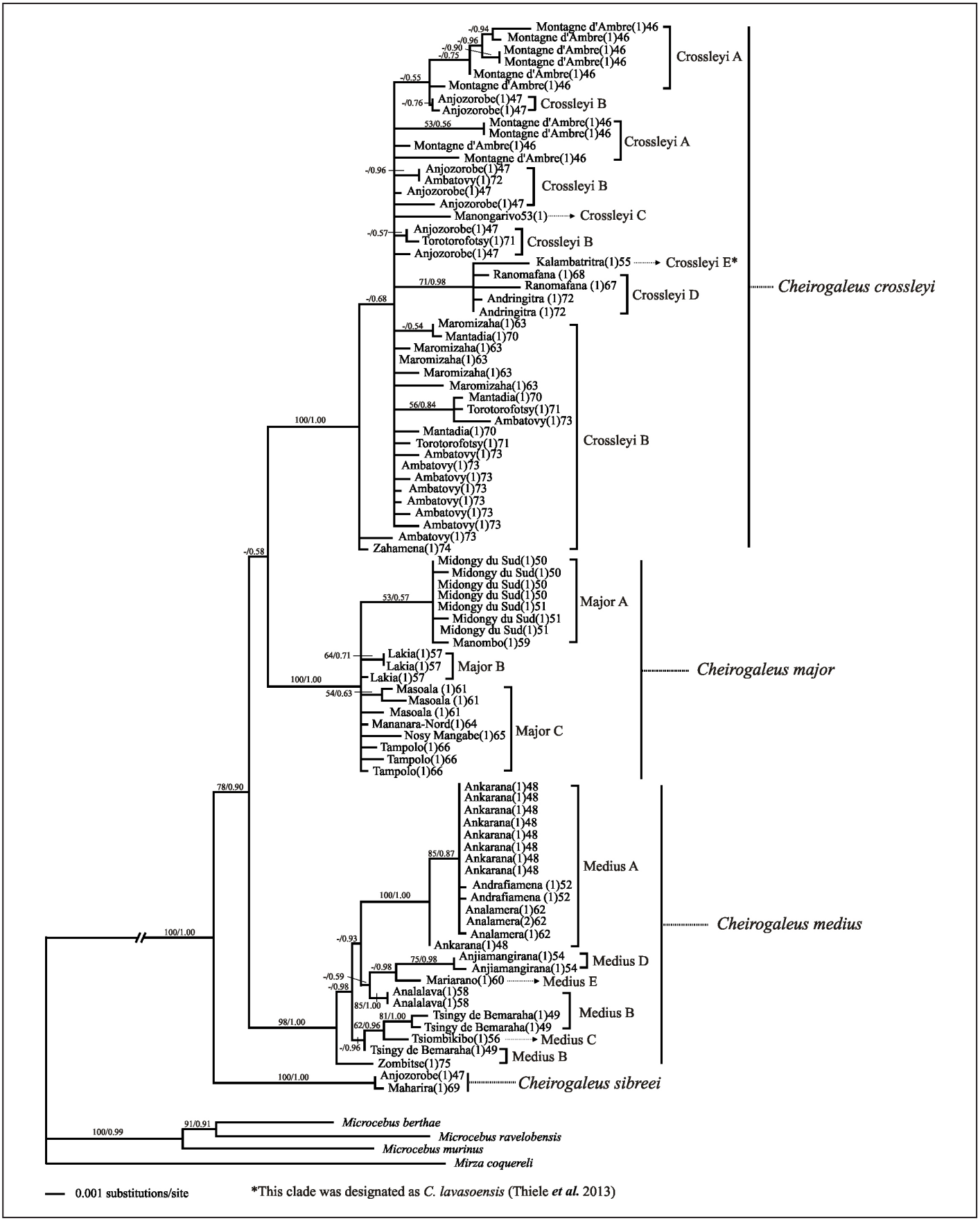

Figure 5. Phylogenetic relationships between *Cheirogaleus* species inferred from the maximum likelihood and Bayesian approaches of CFTR-PAIRB, FIBA, and vWF combined sequence data (4826 bp) generated from the 91 *Cheirogaleus* individuals with four out-group taxa. Numbers on branches represent maximum likelihood values followed by posterior probability support. Tip labels include locality, followed by the number of individuals carrying the haplotype in brackets, then the locality numbers.

2008, 2012). We followed the designation criteria of earlier studies (Vieites *et al.* 2009; Padial *et al.* 2010) and adopted the nomenclature of Ratsoavina *et al.* (2013) to distinguish between lineages that require additional information to confirm species status (UCS) and those that currently have sufficient evidence to be described as species (CCS). This study of Malagasy leaf-tailed geckos (genus *Uroplatus*) is particularly pertinent to our work with *Cheirogaleus*, as both lineages contain widespread phenotypically similar taxa with large mtDNA sequence divergence between species.

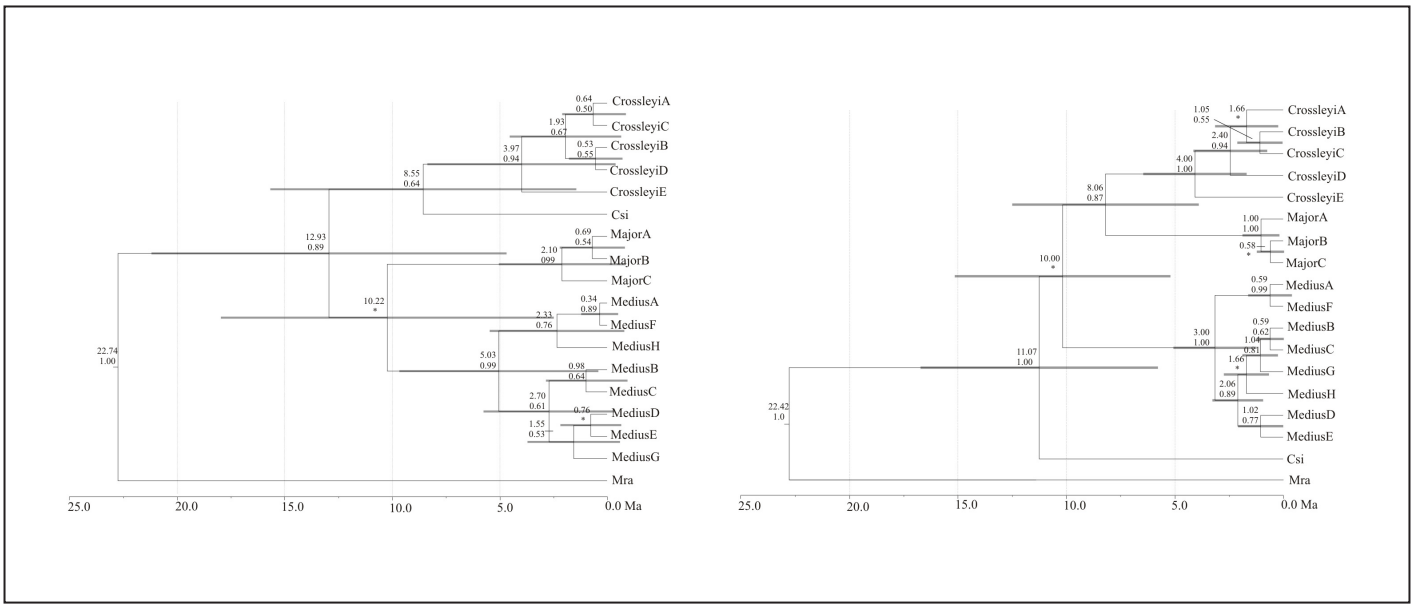

Figure 6. Maximum clade credibility phylogeny of the genus *Cheirogaleus* inferred by the *BEAST species tree analyses of nuclear genes (A) and a combined nuclear gene and mtDNA datasets (B) with *Microcebus ravelobensis* (Mra) as outgroup. Node labels: estimated divergence time (Ma) and posterior probabilities (≥ 0.5; * stands for < 0.5). Node bars indicate the 95% interval of divergence time estimates with posterior probabilities.

Table 3. History of accepted *Cheirogaleus* species included in published genetic investigations and the most recent morphological study (Groves 2000) correlated with clades identified in this study. New candidate species are also identified. Notations: n.i. = not included or not explicitly mentioned in the respective paper; CCS = confirmed candidate species; USC = unconfirmed candidate species.

Species	Clade	Hapke *et al.* (2005)	Groeneveld *et al.* (2009, 2010)	Thiele *et al.* (2013)	This study
C. sibreei	*C. sibreei*	n.i.	*C. sibreei*	*C. sibreei*	*C. sibreei*
C. ravus	n.i.	n.i.	*C. major*	n.i.	*C. major*
C. minusculus	n.i.	n.i.	n.i.	n.i.	*C. minusculus*[a]
C. crossleyi	Crossleyi A	*C. crossleyi*	*C. crossleyi*	*C. crossleyi*	CCS1
	Crossleyi B	*C. crossleyi*	*C. crossleyi*	*C. crossleyi*	*C. crossleyi*
	Crossleyi C	*C. crossleyi*	*C. crossleyi*	*C. crossleyi*	CCS2
	Crossleyi D	*C. crossleyi*	*C. crossleyi*	*C.* sp. Ranomafana Andrambovato	CCS3
C. lavasoensis	Crossleyi E	*C. crossleyi*	*C. crossleyi*	*C. lavasoensis*	*C. lavasoensis*
C. major	Major A	*C. major*	*C. major*	*C. major*	CCS4
	Major B	*C. major*	*C. major*	*C. major*	CCS5
	Major C	*C. major*	*C. major*	*C. major*	*C. major*
C. medius	Medius A	*C. medius*	*C. medius*	*C.* sp. Bekaraoka Sambava	CCS6
	Medius B	*C. medius*	*C. medius*	*C. medius*	*C. medius*
	Medius C	n.i.	n.i.	n.i.	UCS1
	Medius D	n.i.	n.i.	n.i.	UCS2
	Medius E	n.i.	n.i.	n.i.	UCS3
	Medius F	n.i.	*C. medius*	*C.* sp. Bekaraoka Sambava	CCS7
	Medius H	n.i.	*C. medius*	*C.* sp. Ambanja	UCS4
C. adipicaudatus	Medius G	n.i.	*C. medius*	*C. medius*	CCS8

[a]Data Deficient

The identification of seven CCS and four UCS vastly expands the possible circumscription of *Cheirogaleus* (Table 3). The distribution of proposed taxa resembles that of the nocturnal *Lepilemur* group (Louis *et al.* 2006), with numerous pockets of diversity in the North, Northwest 1, and Northwest 2 biogeographic regions marked by the presence of rivers that appear to act as gene flow barriers (Louis and Lei in press). In contrast, speciation in southern Madagascar may be driven more by the convoluted intersection of three biogeographic regions, Central Highlands, West 2 and East 2, associated with rapidly shifting climatic and geological characteristics across a short geographic distance. In this area, near the city of Tolagnaro (Ft. Dauphin), there are three *Cheirogaleus* species, all of which may be sympatric (Fig. 7).

Five clades demonstrated sufficient genetic differentiation (PAA) via our use of multiple genetic analyses, along with sufficient geographic distance or barriers (ascertained by examining maps of Madagascar) from other species to warrant their elevation as four new and one resurrected species. Within the Crossleyi group, CCS1, found in proximity to Montagne d'Ambre, was elevated to full species status as *Cheirogaleus* **species nova 1**. CCS3 has been elevated to species status as *C.* **species nova 2.** Of the Major subgroups, CCS4 has been elevated to species status as *C.* **species nova 3.** CCS6 from the Medius lineage has been elevated to species status as *C.* **species nova 4**. Additionally, we resurrected *C. thomasi*, described by Forsyth Major (1894) as *Opolemur thomasi*, for CCS8. This species was initially described from Tolagnaro (Ft. Dauphin) by Forsyth Major (1894), but synonymized with *C. medius* by Schwarz (1931). Our study indicates the presence of an unnamed lineage here, and based on the principle of priority in species naming of the International Code of Zoological Nomenclature (ICZN), the available name is *C. thomasi* (see below).

In the case of CCS2, 5, and 7 additional sampling and physical examinations from wild populations need to be

Table 4. Summary of preliminary morphometric data and collection localities of species and candidate *Cheirogaleus* species, with information merged for male and female adult specimens (juveniles were excluded). Data are preliminary, and details will be reported in forthcoming revisions. W: weight, HC: head crown, BL: body length, TL: tail length; ( ) number of genetic samples.

Species and candidate species	Morphological characters				Altitude range (m)	Collection localities	Specimens examined
	W (kg)	HC (cm)	BL (cm)	TL (cm)			
C. sibreei	0.23±0.00 0.27±0.04[a]	7.0±1.4 -	15.4±1.2 -	23.1±0.6 23.5±1.3[a]	1128–1660	Tsinjoarivo (Andasivodihazo), Anjozorobe, Maharira	2 (12)
C. minusculus	-	-	-	-	1678	Ambositra	-
CCS1	0.31±0.04	5.9±0.3	17.6±0.8	26.3±2.1	541–1073	Montagne d'Ambre	9 (13)
C. crossleyi	0.33±0.07	6.0±0.7	18.6±1.4	26.5±2.2	856–1535	Ambatovy, Andasibe, Anjozorobe, Ankazomivady, , Mantadia, Maromizaha, Torotorofotsy, Tsinjoarivo, Zahamena,	26 (43)
CCS2	0.32±0.10	5.7±0.2	16.8±2.0	26.6±1.5	18–303	Ambanja, Manantenina, Manongarivo, Sambava,	8 (9)
CCS3	0.41±0.12 0.37±0.04[a]	6.3±0.6 -	20.1±3.8 -	27.7±2.8 27.7±1.3[a]	754–999	Andrambovato, Andringitra (Ambarongy), Ranomafana (Talatakely), Ranomafana (Vatoharanana),	4 (5)
C. lavasoensis	0.27+0.00 0.27+0.02[b]	6.9±0.0 -	16.0±0.0 -	24.9±0.0 25.1±0.1[b]	300–1223	Petit Lavasoa, Ambatotsirongorongo, Grand Lavasoa, Kalambatritra (Sahalava)	1 (18)
CCS4	0.46±0.13	6.4±0.5	19.3±2.0	28.4±1.2	17–789	Ambatotsirongorongo, Ampasimena, Andohavondro, Farafara, Ivorona, Manantantely, Mandena, Manombo, Midongy du Sud,	8 (31)
CCS5	-	-	-	-	85–763	Lakia, Marolambo, Andrambovato	0 (10)
C. major	0.34±0.13 0.35+0.03[b]	6.0±0.9 -	19.7±2.8 -	28.1±2.7 28.9±1.8[b]	4–682	Mahanoro, Mananara-Nord, Maroantsetra, Masoala, Nosy Boraha, Nosy Mangabe, Sihanaka, Tampolo,	5 (13)
CCS6	0.09±0.03	3.9±0.5	11.6±2.0	14.4±2.1	10–292	Ankarana, Andrafiamena, Analamera, Bekaraoka	4 (16)
C. medius	0.23±0.06	4.9±0.3	13.8±0.6	20.2±2.4	60–801	Analalava, Kirindy, Tsingy de Bemeraha, Zombitse	6 (11)
UCS1	0.15±0.00	4.5±0.0	12.0±0.0	12.2±0.0	15	Tsiombikibo	1 (1)
UCS2	0.23±0.03	5.1±0.5	15.8±0.6	23.5±2.5	59–346	Anjiamangirana	2 (2)
UCS3	0.17±0.00	4.4±0.0	15.9±0.0	21.5±0.0	53	Mariarano	1 (1)
CCS7	-	-	-	-	18	Sambava	0 (4)
UCS4	-	-	-	-	0–35	Ambanja	0 (2)
CCS8	-	-	-	-	9–320	Sainte Luce, Lavasoa, Petriky	0 (18)

[a]Blanco *et al.* (2009); [b]Thiele *et al.* (2013); -means data deficient

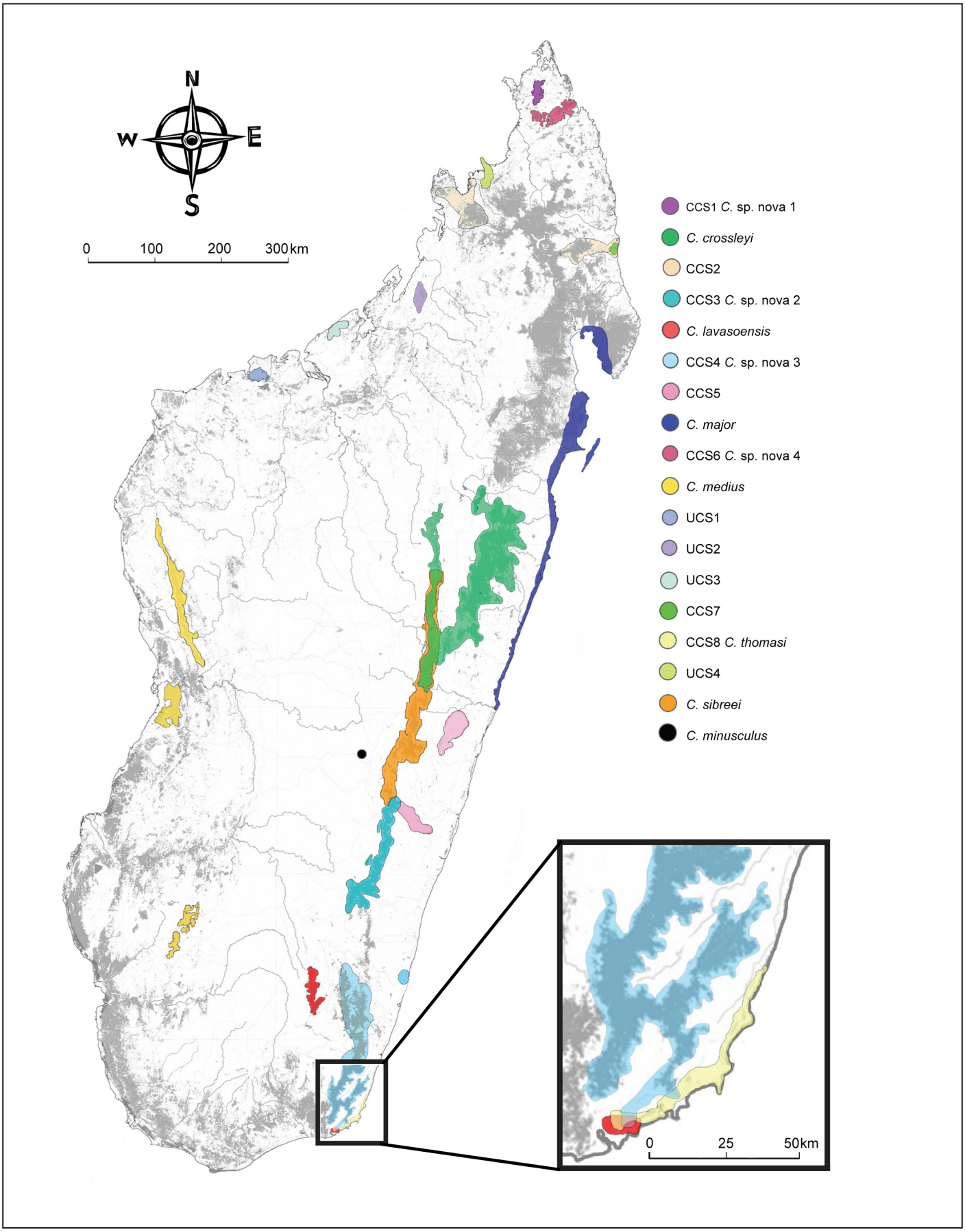

Figure 7. Proposed distributions of the dwarf lemurs of Madagascar. Geographic distribution of designated species, CCS, and UCS in the genus *Cheirogaleus*, with suspected ranges denoted by colors.

conducted to scientifically name these lineages with full confidence. Further, our CCS were all identified by previous studies as members of recognized species groups. A large amount of evidence for these three CCS is extant, but complicating factors exist in proposing a scientific name at this time. CCS2, for instance, is known from 17 genetic samples in northern Madagascar from the east and west coasts (localities 3, 9, 10, and 53). These collection localities represent very different habitats and are in separate biogeographic zones (Louis and Lei in press). Without additional fieldwork in forests between these locales, it is not possible to be certain of the monophyly of CCS2 until additional sampling is completed.

In the case of UCS1–4, we strongly suspect the possibility of independent species due to genetic and geographical factors, but lack the evidence at present to elevate them to species status. Furthermore, temporal climatic variation resulting in the expansion and contraction of forest also contributed to these speciation events (Wilmé *et al.* 2006). UCS1, for instance, is known from one specimen examined at Tsiombikibo (Locality 56) in western Madagascar. Genetic data collected from this individual, coupled with the geographic distance from other *C. medius* populations, indicates a probable but unconfirmed candidate species. UCS2 is known from two individuals sampled at Anjiamangirana (Locality 54), another isolated habitat separate from other *C. medius* populations. UCS3 is known from one individual examined and sampled at Mariarano (Locality 60). Only UCS4 was recognized in a previous study; UCS4 is known from two genetic samples collected at Ambanja (Localities 2 and 4). Groeneveld *et al.* (2009) identified UCS4 as *C. medius*, while Thiele *et al.* (2013) identified UCS4 as a probable new species, *C.* sp. Ambanja, but declined to complete the identification with a formal taxonomic name. Additional field and laboratory work is needed to confirm the status of UCS1–4.

All four of these UCS are endemic to northwestern Madagascar, where rivers serve as barriers that isolate populations already under intense pressure from deforestation and other human activities such as hunting, and may be driving speciation. It is particularly notable that a previous study (Louis *et al*. 2006) identified the northwestern part of Madagascar as the region of highest overall species richness for the sportive lemurs (Lepilemuridae). This species richness, with river boundaries a probable contributing factor, appears to be present in *Cheirogaleus* as well.

The elevation of a large number of new lemur species in a relatively short period of time has drawn some criticism and calls for a return to the BSC or a more strict application of the PSC (Tattersall 2007, 2013). We contend that the genetic and geographic evidence justify the elevation of these four new species. Madagascar's geography, including varying altitudes and river barriers, encourage speciation (Louis *et al.* 2006). Increasingly fragmented habitats have left populations isolated, and this situation may further contribute to the speciation events that result in new lineages (Quinn and Harrison 1988). Our identification of four new *Cheirogaleus* species and the probable existence of numerous others are indicative of the work that remains to be done in Madagascar to prevent the ongoing loss of that island's amazing biodiversity.

Species groups of Cheirogaleus

Four species groups in this genus are identifiable as follows:

1. *C. crossleyi* group

External characters: Characterized by a dark facial mask, consisting of broad black or blackish-grey, usually somewhat angular, rings around the eyes, extending broadly anteromedially to join with the intensely black muzzle. The ears are black and furred inside and out. The general color of the head continues as a lighter strip between the eye-rings and their anteromedial continuations as far as the muzzle. The white or whitish area of the throat continues to the cheeks and muzzle, contrasting somewhat with the color of the face. Dorsal side of the body and posterior of the head reddish-grey. Underside and inner aspects of the limbs white or light grey, forming a sharp border with the color of the upperside, and extending well up on the sides of the neck and onto the cheeks.

Skull: Facial skeleton low and straight; a broad interorbital space, not markedly constricted in the middle; orbits looking more laterally; orbital margins not, or bluntly, raised, the upper rims low, not interrupting the dorsal outline of the skull, and the inferior orbital margins hardly anterior to superior margins; orbits looking at about 45° from the front, their rims in a single plane. Lateral walls of the nasals smoothly continuing the upwardly converging slopes of the maxillae. The posterior margin of the palate distinctly curved forward; vomer not strongly prolonged backward, lateral pterygoids not enlarged; bullae relatively small. The lateral margin of the pyriform aperture is somewhat concave in lateral view; the braincase is low, suddenly steeply descending posteriorly (Appendix I(d)).

Dentition: Toothrows straight or nearly so, not or only slightly incurved posterior to M2, evenly converging anteriorly; incisor row only slightly curved, incisors slightly project forward; canine short, barely curved and not much protruding above level of P2, and with small distal cusp; P2 relatively low-crowned, barely protruding above level of P3, and separated from both canine and P3 by short diastemata; molar cusps low; P2 and P3 slender, buccolingually compressed; P4 constricted between buccal cusps and lingual cusp; upper molars square; M3 relatively small, but not reduced in structure, its lingual margin nearly symmetrically crescentic.

Cheirogaleus crossleyi (Grandidier, 1870). *Rev. Zool. pur et appliquée* 22: 49.

Chirogalus crossleyi Grandidier, 1870

Chirogale melanotis Forsyth Major, 1894

Summary: We propose that the clade identified as Crossleyi B represents Grandidier's *C. crossleyi*. This clade includes the museum specimen identified as 1948.160 (BMNH) collected 30 miles northeast of Lac Alaotra (Fig. 4). The characteristic yellow fur on the face (Groves 2000) is visible on

an individual from Zahamena (Fig. 8). A type specimen was previously unknown for *C. crossleyi*, but Groves recently discovered it in the collections of the Museum of Comparative Zoology at Harvard University from the Grandidier collection from the Forest of Antsianaka near Lac Alaotra (Viette 1991).

Holotype: MCZ 44952, adult female, skin and skull; of *melanotis*, BM 70.5.5.24, adult male, skin and skull.

Type locality: Forest of Antsianaka; of *melanotis*, Vohima.

Distribution: Known from Zahamena in the north down through Tsinjoarivo in the south in forests along the central high plateau.

Vernacular names: Crossley's dwarf lemur, furry-eared dwarf lemur, Matavirambo or Tsitsihy.

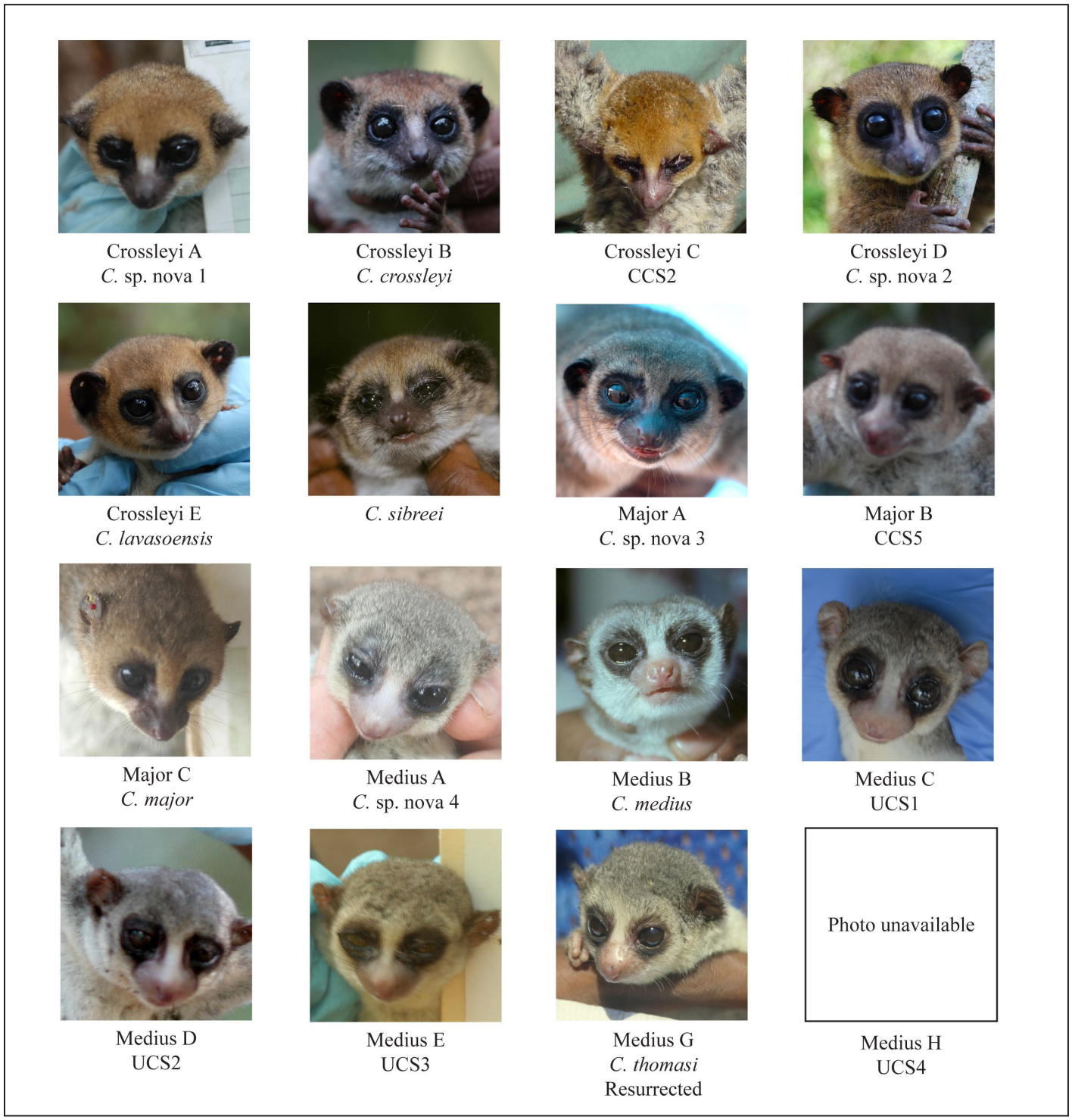

Figure 8. Photographs of living specimens in the genus *Cheirogaleus*. A photograph was not available for Medius H UCS4.

***Cheirogaleus* sp. nova 1.** New species

Formerly CCS1; identified as a subclade of *C. crossleyi* by Thiele *et al*. (2013). See Table 3.

***Cheirogaleus* sp. nova 2.** New species

Formerly CCS3; identified as *Cheirogaleus* sp. Ranomafana Andrambovato by Thiele *et al.* (2013). See Table 3.

Cheirogaleus lavasoensis Thiele, Razafimahatratra & Hapke, 2013. *Mol. Phylogenet. Evol.* 69: 605.

Holotype: IFA AH-X-00-181, DNA and tissue from an adult male, subsequently released (Thiele *et al.* 2013).

Type locality: Madagascar, Region Anosy, Lavasoa Mountains, a forest fragment locally named Bemanasy, on the southern flank of Petit Lavasoa, S 25.080894, E 46.762151, at 300 m above sea level (Thiele *et al.* 2013).

Diagnosis: Intensely reddish coloration on the head; relatively long, wide ears; higher facial skeleton and more reduced third upper molars than other members of the group.

Description: Relatively small in size, with a deeper face; upper third molars small.

Distribution: From Kalambatritra (this study) in the north down to three small forest fragments on the southern slopes of the Lavasoa Mountains (Thiele *et al.* 2013).

Vernacular name: Lavasoa Dwarf Lemur.

***Cheirogaleus crossleyi* group, other potential species**

1) Potential species from Bongolava (no currently existing specimens available for study): Thalmann (2007) and personal communication to C. P. Groves. The photos show a very dark species of the *crossleyi* group, with very large, intensely black eye-rings which leave only a very narrow interorbital space and narrow space between them and the ears. The skull measurements given by Thalmann (2007) indicated an extremely small size, which contradicted external measurements, suggesting further investigation is necessary.

2) CCS2: Representatives of this candidate species were sampled from the east and west coasts in the north of Madagascar (Thiele *et al.* 2013). This lineage was genetically distinct, but before it can be confidently described, the forests between the disjunct collection localities need to be sampled to confirm or exclude gene flow.

2. *C. major* group

External characters: Facial mask much less developed, eye-rings more rounded than in *C. crossleyi* group, and less broadly connected to the (usually dark) grey muzzle. Interorbital strip short and broad. Ears somewhat darker than head, but thinly haired. Body and head lighter reddish-grey. Underparts light grey or white, but this color not sharply marked off from that of upper parts.

Skull: Facial skeleton short, high, straight; interorbital space narrow; orbital margins (not the rims themselves) bluntly raised; inferior orbital margins well anterior to superior margins; orbits looking at about 45° from the front, their rims in a single plane; orbit enlarged, slightly interrupting the dorsal outline of the skull, extending inferiorly below the level of the zygomatic arch; lacrimal region concave in front of the orbital margin and below the posterior nasals; lateral margin of the pyriform aperture usually straight in lateral view; the nasal tip short, hardly extending anterior to the pyriform aperture margin in lateral view; rostrum bluntly rounded anteriorly, and premaxillae somewhat prolonged forward; the nasals somewhat raised above the maxillae, their lateral walls rising at an angle above the maxillary planes; postorbital constriction deep; temporal lines well-expressed; braincase relatively higher, falling away steeply behind. Posterior margin of the palate much less concave than in *C. crossleyi* group; the vomer still less prolonged than in the latter, but the basisphenoid with a strong median longitudinal ridge; lateral pterygoids small, not flared; bullae small, their inferior margin about level with alveolar line.

Dentition: Toothrows mainly straight but curved inward posterior to M2; incisors less forwardly projecting than in *C. crossleyi* group; no canine/P2 diastema, but variably one between P2 and P3; canine thick, curved, but lacking much or any development of distal cusp; P2 and especially P3 broader than in *C. crossleyi* group; P4 oblong in shape; P3 hardly projecting above P4; upper molars square; molar cusps low, bulbous; M3 fairly small in size but not reduced in structure, its lingual margin symmetrically crescentic.

Cheirogaleus major É. Geoffroy Saint-Hilaire, 1812. *Ann. Mus. Hist. Nat. Paris* 19: 172.

Lemur commersonii Wolf, 1822 (Renaming of *Cheirogaleus major* É. Geoffroy Saint-Hilaire)

Cheirogaleus milii É. Geoffroy Saint-Hilaire, 1828

Cheirogaleus typicus Smith, 1833

Mioxicebus griseus Lesson, 1840

Summary: We propose that the clade identified as Major C represents *C. major sensu* Groves (2000). Unfortunately, there is no type locality for this species, represented by a neotype in the Paris Museum, a specimen that is also the holotype of *Cheirogaleus milii* which was named by É. Geoffroy Saint-Hilaire (1828) on the basis of an individual presented to the Paris Menagerie by Pierre Bernard Milius, Governor of Réunion, and described from life by F. Cuvier (1821). Steven Goodman suggested to C. P. Groves (in litt.) that, at this period, French entry to Madagascar would most likely have been via Tamatave (now Toamasina), so the specimen would most plausibly have been obtained from that vicinity, or between there and Antananarivo. Numerous museum specimens (BMNH: 1939.1289, 1935.1.1or 8.169; MNHN: 1932-3362, 1964.72, 1964.74; NMNL: 1887:66c, 1887:66f, 1887:66g) were included in this clade based on cytb sequences (Fig. 4).

Types: Holotype of *milii* and neotype of *major* (and, by implication, of *commersonii* and *griseus*), MNHN148; holotype of *typicus*, BM 37.9.26.77.

Type locality: Of *major, commersonii, milii* and *griseus*, probably either Toamasina (formerly Tamatave) or between there and Antananarivo; of *typicus*, "Madagascar".

Distribution: Narrow coastal range along the east coast, from Masoala in the north down to Mahanoro River in the south. This littoral habitat is the most threatened in all of Madagascar (Consiglio *et al.* 2006; Watson *et al.* 2010).

Vernacular name: Greater dwarf lemur.

***Cheirogaleus* sp. nova 3.** New species

Formerly CCS4; See Table 3.

***Cheirogaleus major* group, other potential species**

1) *Cheirogaleus ravus* Groves, 2000. *Int. J. Primatol.* 21: 960: Although synonymized by Groeneveld *et al.* (2009) based on a partial dataset that did not include the type specimen, this species may represent a distinct lineage. It seems evident that Groves (2000) referred many specimens to this species when described; the type specimen, BM 88.2.18.3, from Toamasina, is unusual, with its very grey color (iron-grey with brownish tones), its short tail with a white tip, braincase less steeply falling away behind, and small M3. The field team has not found any specimen resembling this description. Some of the other specimens referred to *C. ravus* in the type description (Groves 2000) show some, but not all of the putative diagnostic features, for example, an unusually grey color. Therefore, *C. ravus* may be either a distinct species, or simply a highly distinctive morph of *C. major*.

2) CCS5: Representatives of this species were collected from three localities, Lakia (this study), Marolambo and Andrambovato (Groeneveld *et al.* 2009). Additional morphological information is required before this species can be described and additional field work is recommended between these disjunct localities.

3. *C. medius* group

External characters: Facial mask poorly developed, eye rings rounded, thin, with barely marked thin lines connecting them to the lateral muzzle; muzzle pinkish-grey. Ears thinly haired, not darker than head. Face contrastingly lighter than the general color of the head. Upperside of the body and head light or medium grey, with tendency for a short dark dorsal stripe and whitish extremities. Underside and inner aspect of the limbs sharply marked-off white, this color extending well up onto the flanks, and sending a striking white "collar" up onto the sides of the neck, leaving often a fairly narrow strip of body color on the upper side of the neck.

Skull: Facial skeleton shorter, higher than other groups, becoming convex above the level of the infraorbital foramen; orbits rounded, so that the interorbital space is constricted in the middle, and lateral rims of the orbits turned forward; orbital rims strongly raised; inferior orbital margins well anterior to superior, but the lateral rim is more antero-inferiorly directed, meeting the upper margin of the zygomatic arch at a very acute angle; upper orbital rim slightly interrupting the dorsal outline of the skull. Rostrum narrows anteriorly but its lateral walls somewhat rounded; lateral margin of the pyriform aperture concave in lateral view; nasals somewhat raised above the maxillae, their lateral walls rising at an angle above the maxillary planes. Temporal lines hardly expressed; postorbital constriction is deep; the braincase very low, flat. Posterior margin of the palate strongly concave forward, situated less far behind M3; vomer strongly raised, and prolonged backwards between the pterygoids; lateral pterygoid plates enlarged, flaring; bullae large, constricting basioccipital between them; bullae inflated, they protrude below the alveolar line.

Dentition: Toothrows somewhat converging anteriorly, then more strongly curved inward anterior to the canines, and slightly curved inward posterior to M2; incisors less forwardly projecting than in the *C. major* group; canines very long, slender, but barely curved, with a small distal cusp; diastema present between canine and P2, and between P2 and P3; P2 and P3 more rounded, less compressed, with considerable lingual pillars; P2 pointed, high-crowned, projecting well above P3; P4 triangular; molar cusps high and pointed; upper molars more rounded lingually, with a larger protocone; M3 triangular, its distolingual margin reduced.

Cheirogaleus medius É.Geoffroy Saint-Hilaire, 1812. *Ann. Mus. Hist. Nat. Paris* 19: 172.

Chirogalus adipicaudatus Grandidier, 1868

Chirogalus samati Grandidier, 1868

Summary: We propose that the clade identified as Medius B represents *C. medius sensu* Groves (2000). The neotype locality was vaguely described as the Tsidsibon River, which, according to Goodman and Rakotondravony (1996), is currently known as the Tsiribihina River, in west-central Madagascar. Numerous museum specimens were included in this clade based on cytb sequences (1935.1.8.168, 1932-3364, 1932-3365, cat. a/ van Dam a., cat. e/ van Dam e. [Morandava]; Fig. 4). This species is documented from near Toliara, north to Tsingy de Bemaraha. This area, spanning multiple biogeographic regions (Louis and Lei in press), requires additional field work and, based on speciation patterns in other organisms (Louis *et al.* 2006; Ratsoavina *et al.* 2013), will likely reveal new *Cheirogaleus* taxa.

Types. Holotype of *samati* and neotype of *medius*, MNHM 162; of *adipicaudatus*, unknown.

Type localities: of *medius* and *samati*, Tsidsibon River; of *adipicaudatus*, Tulear (Toliara).

Distribution: In western Madagascar, individuals sampled from Tsingy de Bemaraha down to Zombitse. Known from Tsingy de Bemaraha National Park and Zombitse Vohibasia National Park.

Vernacular name: Fat-tailed dwarf lemur.

Cheirogaleus thomasi (Forsyth Major, 1894). *Novitates Zoologicae* 1: 20.

Opolemur thomasi Forsyth Major, 1894

Formerly, CCS8; *C. adipicaudatus* of Groves (2000), in part.

Type: BM 91.11.30.3, skin and skull.

Type locality: Fort Dauphin.

Distribution: In the southeastern extreme of Madagascar, from St. Luce to Petriky.

Notes: Groves (2000) applied the name *C. adipicaudatus* to what is in effect this species, which does not (*contra* Groves) extend throughout the "spiny desert" country of the south of Madagascar.

Vernacular name: None known. Suggest Thomas' dwarf lemur.

***Cheirogaleus* sp. nova 4.** New species

Formerly CCS6; in part *C.* sp. Bekaraoka Sambava Thiele *et al.* (2013). See Table 3.

***Cheirogaleus medius* group**: other potential species

1) UCS1: Known from only one individual from one locality, Tsiombikibo. Further investigation of this western, genetically distinct lineage is highly recommended as this geographical area is bounded on its eastern side by the Mahavavy Sud River, which has been shown to be an effective genetic barrier for the genus *Lepilemur* (Louis *et al.* 2006).

2) UCS2: Known from only one individual from one locality, Anjiamangirana. Further investigation of this western genetically distinct lineage is highly recommended as this geographical area is bounded by the Mahajamba and Sofia rivers, which have been shown to be effective genetic barriers for the genus *Lepilemur* (Louis *et al.* 2006).

3) UCS3: Known from only one individual from one locality, Marirano. Further investigation of this western genetically distinct lineage is highly recommended as this geographical area is bounded by the Sofia and Betsiboka rivers, which have been shown to be effective genetic barriers for the genus *Lepilemur* (Louis *et al.* 2006).

4) CCS7: Known from four samples from Sambava (Groeneveld *et al.* 2009). This northeastern lineage is the same as that identified as CmeB (Thiele *et al.* 2013) as part of the provisionally named *Cheirogaleus* sp. Bekaroka Sambava. Further field work in this diverse region is necessary to confidently describe this species.

5) UCS4: Known only from four individuals from one locality, Ambanja (Groeneveld *et al.* 2009). This northwestern lineage is the same as that identified as CmeC (Thiele *et al.* 2013) as part of the provisionally named *Cheirogaleus* sp. Ambanja. Further field work in this geographical area is recommended as it is bounded by the Mahavavy Nord and Sambirano rivers, which have been shown to be effective genetic barriers for the genus *Lepilemur* (Louis *et al.* 2006).

4. *C. sibreei* group

External characters: Eye-rings variable, usually grey-black, and less broadly connected to the dark grey muzzle than in *C. crossleyi* group. Ears dark but not black, thinly haired. Interorbital facial strip comparatively broad. Body and head medium grey, with strongly marked deep brown dorsal stripe, and tail tip darkened. Underside and inner aspect of limbs, and underside of basal part of the tail, white, sharply marked off from the color of upperside, and extending well up on the flanks and neck.

Skull: Facial skeleton short, low, slightly convex; orbits somewhat of *medius* type, but less marked; interorbital space narrow; inferior orbital margins not markedly anterior to the superior margins, so orbit looking fairly forward, its dorsal rim very slightly interrupting the dorsal outline of the skull; postorbital constriction not so marked; nasals well raised above the maxillae, even more so than in the *medius* group; rostrum straight-sided, then suddenly converging anteriorly; premaxillae suddenly and strongly converging to a point; lateral margin of the pyriform aperture strongly concave in lateral view; nasal tip very long. Braincase steeply descending posteriorly, but shorter than in the *major* group. Bullae large, protruding well below the alveolar line; temporal lines well expressed. Vomer not prolonged backward, basisphenoid not ridged, lateral pterygoid plates not flared; posterior palatal margin strongly concave; bullae greatly enlarged.

Dentition: Toothrows straight and converging forward until P2 level, when they run parallel until anterior to the canines; incisors not projecting; canines noticeably large, long and slender and with a distal cusp, like the *medius* group; P2 and especially P3 and P4 larger than the *major* group, but similar in shape; P3 somewhat raised, with diastema both mesial and distal to it; molar cusps high, upper molars very rounded lingually; M3 very triangular in form, its distolingual margin a simple straight edge.

Cheirogaleus sibreei (Forsyth Major, 1896). *Ann. Mag. Nat. Hist.* 6th series 18: 325.

Chirogale Sibreei Forsyth Major, 1896

Summary: *Cheirogaleus sibreei* has been consistently supported as a monophyletic species (Groeneveld *et al.* 2009, 2010; Thiele *et al.* 2013), and does not currently require additional taxonomic work. This lineage would, however, benefit from further field studies. The type locality of *C. sibreei* is Ankeramadinika, but this name is no longer used. In Mrs. Standing's short essay from 1904 on her missionary work titled "The F.F.M.A. Sanatorium, Ankeramadinika, Madagascar," she mentions that this village was abandoned and clearly describes its location as being near Ambatolaona, which agrees with Forsyth Major's comment of being one day's journey east of Antananarivo. The first extant population of *C. sibreei* was recently documented south of Ankeramadinika in Tsinjoarivo and was sympatric with *C. crossleyi* (Blanco *et al.* 2009; Groeneveld *et al.* 2010). Not only are these species sympatric, they were documented occupying a single tree hole in Anjozorobe that had four individuals identified as *C. crossleyi* and one as *C. sibreei* (E. E. Louis Jr., pers. obs.).

Type: BM 97.9.1.160, skin and skull

Type locality: Ankeramadinika

Distribution: Along the central high plateau from Anjozorobe Protected Area in the north through Tsinjoarivo down to Ranomafana National Park in the south.

Vernacular name: Sibree's dwarf lemur.

***Cheirogaleus sibreei* group**: other potential species

1) *Cheirogaleus minusculus* Groves, 2000. *Int. J. Primatol*. 21: 960. This species seems closest to *C. sibreei*, with the same dorsal stripe, relatively restricted eye rings, a grey muzzle, and dark, thinly haired ears. The type is much smaller than *C. sibreei*, with a higher and more rounded braincase, the facial skeleton is not convex, the palate is broader, and the upper third molars very reduced; the tail tip appears to be white. *Cheirogaleus minusculus*, known only from the type locality of Ambositra (Groves 2000), is still Data Deficient and requires intensive field and laboratory investigation to confirm its taxonomic status.

Acknowledgments

We thank the Madagascar National Parcs and Ministere de'l Environnement et de Forets for sampling permission. We are most grateful to the Ahmanson Foundation, the Theodore F. and Claire M. Hubbard Family Foundation, the Primate Action Fund / Conservation International, the Margot Marsh Biodiversity Foundation, and the National Geographic Society, for financial assistance. Colin Groves thanks Eileen Westwig, Judy Chupasko, Larry Heaney, Paula Jenkins, Chris Smeenk, Frieder Meier and Cecile Callou for access to museum specimens under their care. In particular, thanks go to the Harvard Museum of Comparative Zoology for photographs from their collection. We also thank A. Hapke for photographs of *C. thomasi.* We also want to acknowledge the office and field staffs of the Madagascar Biodiversity Partnership for their excellence in collecting the samples from the *Cheirogaleus* safely, returning them to their forest habitat. Thank you to an anonymous reviewer for incisive and very helpful suggestions.

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Authors' addresses:

Runhua Lei[*], **Cynthia L. Frasier**, **Adam T. McLain**, **Justin M. Taylor**, **Carolyn A. Bailey**, **Shannon E. Engberg**, **Azure L. Ginter**, Grewcock Center for Conservation and Research, Omaha's Henry Doorly Zoo and Aquarium, 3701 South 10th St, Omaha, NE 68107, USA, **Richard Randriamampionona**, Madagascar Biodiversity Partnership, Lot VO 12bis A, Manakambahiny, Antananarivo 102, Madagascar, **Colin P. Groves**, School of Archaeology and Anthropology, Australian National University, Canberra, ACT 0200, Australia, **Russell A. Mittermeier,** Conservation International, 2011 Crystal Drive, Suite 500, Arlington, VA 22202, USA**,** and **Edward E. Louis Jr.**, Grewcock Center for Conservation and Research, Omaha's Henry Doorly Zoo and Aquarium, 3701 South 10th St, Omaha, NE 68107, USA, and Madagascar Biodiversity Partnership, Lot VO 12bis A, Manakambahiny, Antananarivo 102, Madagascar. [*]*Correspondence to*: Grewcock Center for Conservation and Research, Omaha's Henry Doorly Zoo and Aquarium, 3701 South 10th St, Omaha, NE 68107, USA, e-mail: <leir@omahazoo.com>.

Received for publication: 28 October 2014
Revised: 16 December 2014

The following appendices to this publication are available online at <http://www.madagascarpartnership.org/home/mbps_scientific_publications>, and can be downloaded.

Appendix I

(a). Appendix I(a). Phylogenetic relationships between *Cheirogaleus* species inferred from the maximum likelihood and Bayesian approaches of the complete COII sequence data (684 bp) generated from 134 individuals with four out-group taxa. New field samples were labeled in bold. Numbers on branches represent maximum likelihood values followed by posterior probability support. Tip labels include locality, followed by number of individuals carrying the haplotype in brackets, then the locality numbers.

(b). Appendix I(b). Phylogenetic relationships between *Cheirogaleus* species inferred from the maximum likelihood and Bayesian approaches of the partial vWF sequence data (792 bp) generated from 208 individuals with four out-group taxa. Sequences generated from new field samples were labeled in bold and published sequences derived from museum specimens were presented in italics. Numbers on branches represent maximum likelihood values followed by posterior probability support. Tip labels include locality, followed by number of individuals carrying the haplotype in brackets, then the locality numbers.

(c). Appendix I(c). Phylogenetic relationships between *Cheirogaleus* species inferred from the maximum likelihood and Bayesian approaches of the partial FIBA sequence data (606 bp) generated from 208 individuals with four out-group taxa. Sequences generated from new field samples were labeled in bold and published sequences derived from museum specimens were presented in italics. Numbers on branches represent maximum likelihood values followed by posterior probability support. Tip labels include locality, followed by number of individuals carrying the haplotype in brackets, then the locality numbers.

(d). Appendix I(d). Skulls of species in the genus *Cheirogaleus* used in morphometric comparisons.

Appendix II

(a). Appendix II(a). Table S1 Sample localities of *Cheirogaleus*.

(b) Appendix II(b). Table S2 Cranial and dental (maxillary) measurements of *Cheirogaleus* taxa.

(c) Appendix II(c). Table S3 External metrics of *Cheirogaleus* taxa. HB = head+body length, HF = hindfoot length. Measurements from the literature of the types of *major*/*milli* and *typicus* are given for comparative purposes.

(d) Appendix II(d). Table S4 *Cheirogaleus* specimens deposited at the following institutions: American Museum of Natural History, New York (AMNH), Natural History Museum, London (BMNH), Field Museum of Natural History, Chicago (FMNH), Institut für Anthropologie, Johannes Gutenberg-Universität Mainz, Germany (IFA), Museum of Comparative Zoology, Harvard (MCZH), Muséum National d'Histoire Naturelle, Paris (MNHN), Museum für Naturkunde - Leibniz Institute for Evolution and Biodiversity Science (MfN/ZMB), and Naturalis Biodiversity Center (formerly Rijksmuseum van Naturlijk Historie – NMNL). Spelling of localities is consistent with records associated with specimens and does not necessarily correspond to modern spellings; latitude and longitude were estimated *post hoc* except for those at IFA. Specimens verified as *Cheirogaleus* were arranged by species and clade when possible and then by locality. An abbreviated history of determinations was included for examined specimens. Unverified specimens in italics refer to catalog numbers in institutional databases identified as *Cheirogaleus*, but were not confirmed by the authors.

(e). Appendix II(e). Table S5 Primers used in this study.

(f). Appendix II(f). Table S6 Accession numbers of published *Cheirogaleus* sequences from Genbank (NCBI).

(g). Appendix II(g). Table S7 Genetic distance matrix for mtDNA cytb sequence data between and within clades of *Cheirogaleus*.

(h). Appendix II(h). Table S8 Genetic distance matrix for mtDNA PAST fragment sequence data between and within clades of *Cheirogaleus*.

(i) Appendix II(i). Table S9 Genetic distance matrix for mtDNA D-loop sequence data between and within clades of *Cheirogaleus*.

(j) Appendix II(j). Table S10 Genetic distance matrix for mtDNA COII sequence data between and within clades of *Cheirogaleus*.

(k) Appendix II(k). Table S11 Genetic distance matrix for nucDNA CFTR-PAIRB sequence data between and within clades of *Cheirogaleus*.

(l) Appendix II(l). Table S12 Genetic distance matrix for nucDNA FIBA sequence data between and within clades of *Cheirogaleus*.

(m) Appendix II(m). Table S13 Genetic distance matrix for nucDNA VWF sequence data between and within clades of *Cheirogaleus*.

(n) Appendix II(n). Table S14 Diagnostic nucleotide sites from the mtDNA cytb Pairwise Aggregate Analysis (PAA) of *Cheirogaleus*. No.PAA stands for number of diagnostic nucleotide sites.

(o) Appendix II(o). Table S15 Diagnostic nucleotide sites from the mtDNA PAST fragment Population Aggregate Analysis (PAA) of *Cheirogaleus*. No.PAA stands for number of diagnostic nucleotide sites.

(p) Appendix II(p). Table S16 Diagnostic nucleotide sites from the mtDNA D-loop Population Aggregate Analysis (PAA) of *Cheirogaleus*. No.PAA stands for number of diagnostic nucleotide sites.

(q) Appendix II(q). Table S17 Diagnostic nucleotide sites from the mtDNA COII fragment Population Aggregate Analysis (PAA) of *Cheirogaleus*. No.PAA stands for number of diagnostic nucleotide sites.

(r) Appendix II(r). Table S18 Variable and diagnostic nucleotide sites (shaded) from the nucDNA CFTR-PairB Population Aggregate Analysis (PAA) of *Cheirogaleus*. No.PAA stands for number of diagnostic nucleotide sites.

(s) Appendix II(s). Table S19 Variable and diagnostic nucleotide sites (shaded) from the nucDNA FIBA Population Aggregate Analysis (PAA) of *Cheirogaleus*. No.PAA stands for number of diagnostic nucleotide sites.

(t) Appendix II(t). Table S20 Variable and diagnostic nucleotide sites (shaded) from the nucDNA vWF Population Aggregate Analysis (PAA) of *Cheirogaleus*. No.PAA stands for number of diagnostic nucleotide sites.

(u) Appendix II(u). Table S21 Morphometric data (mm) collected from sedated *Cheirogaleus* individuals. Clades were designated based on mtDNA sequence data (Figure 2). Morphological data is missing, HC: head crown, BL: Body Length, TL: Tail Length, F-Tb: Front Thumb (forelimb), F-UR: Front Ulna/radius, F-Hd: Front Hand, F-LD: front longest digit (Forelimb), F-H: Front Humerus, H-T: Hind Tibia, H-LD: hind longest digit (Hindlimb), H-Ft: Hind foot, H-Tb: Hind Thumb (Hindlimb). H-F: Hind Femur, UC: Upper Canine, LC: Lower Canine, RTL: Right Testes Length, RTW: Right Testes Width, LTL: Left Testes Length, LTW: Left Testes Width.