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MtDNA and nDNA corroborate existence of sympatric dwarf lemur species at Tsinjoarivo, eastern Madagascar

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ABSTRACT

Madagascar is a biodiversity hotspot, well known for its endemic primates, the lemurs. Numbers of recognized lemur species have increased drastically in some genera (e.g. *Microcebus*), while field-based studies revealed low species diversity in the dwarf lemurs (genus *Cheirogaleus*). Only three (*C. medius*, *C. major*, *C. crossleyi*) of seven described species have to date been identified in field-based studies. Blanco et al. (2009) reported two sympatric *Cheirogaleus* species at Tsinjoarivo based on morphological data, one of which they attributed to *C. crossleyi* and the other of which they described as *C. sibreei*-like, or possibly a new species. Based on comparative analyses of mtDNA (*cytb*) and nDNA (*vWF*, *fiba*, *adora3*), we confirm the presence of *C. crossleyi* and show that the *C. sibreei*-like individuals form a well-defined fourth clade, basal to the three recognized species. Whereas these molecular analyses demonstrate that a non-holotype museum specimen considered by Groves (2000) to belong to *C. sibreei* does not cluster with the *C. sibreei*-like individuals from Tsinjoarivo, morphometric analysis of one Tsinjoarivo individual, the *C. sibreei* holotype from Ankeramadinika, and samples of *C. medius*, *C. major*, and *C. crossleyi* strongly suggests that the fourth (and basal) clade is indeed *C. sibreei*. Tsinjoarivo therefore becomes the only known field site harboring *C. sibreei* today. Given ongoing forest loss and fragmentation at Tsinjoarivo we can surmise that this population, critical to our understanding of the evolution of the genus *Cheirogaleus*, is also critically endangered.

1. Introduction

Madagascar, one of the world's major biodiversity hotspots (Myers et al., 2000), is well known for its enigmatic, charismatic, and endemic primates, the lemurs (Primates: Lemuriformes). Although lemur taxonomy has been the subject of scientific study for over a century, the field remains highly dynamic and much debated (Tattersall, 2007). In recent years the number of described lemur species has increased dramatically, thus changing species distributions and abundances which in the face of drastic habitat loss inevitably leads to the necessity of revising conservation priorities (Tattersall, 2007; Thalmann, 2007).

The small-bodied and nocturnal family Cheirogaleidae has experienced a pronounced increase in the number of recognized species, mostly within the mouse lemurs (genus *Microcebus*). The number of mouse lemur species has risen from two accepted species in the early 1990s (Petter et al., 1977; Tattersall, 1982) to 18 species today (Andriantompohavana et al., 2006; Kappeler et al., 2005; Louis et al., 2006, 2008; Olivieri et al., 2007; Radespiel et al., 2008; Rasoloarison et al., 2000; Schmid and Kappeler, 1994; Zimmermann et al., 1998). Field studies of the closely related and ecologically very similar dwarf lemurs of the genus *Cheirogaleus* on the other hand, have not found evidence for many locally restricted species, but instead have reported three widely occurring species (Groeneveld et al., 2009, in press; Hapke et al., 2005). The last revision of this genus, based on morphological data from museum specimens, identified seven species, of which two were

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newly described (*C. minusculus* and *C. ravus*), two were resurrected from synonymy (*C. adipicaudatus* and *C. sibreei*), one was elevated from subspecific status (*C. crossleyi*), and two were previously accepted species reconfirmed (*C. major* and *C. medius*) (Groves, 2000). The existence of living populations of some of these species is not confirmed (Mittermeier et al., 2006). *C. adipicaudatus*, *C. ravus*, *C. minusculus*, and *C. sibreei* are only known from the localities of museum specimens, although Rasolofoson et al. (2007) reported *C. ravus* and *C. sibreei* to occur in the region of Makira in northeastern Madagascar. As Blanco et al. (2009) have stated, these attributions should be viewed as provisional and need reconfirmation, since species identifications were based on census data.

Individuals belonging to two sympatric dwarf lemur species were captured in late 2006 and late 2007 at two localities in mid and high-altitude rainforests at Tsinjoarivo, 80 km south–southeast of Antananarivo (Blanco et al., 2009). One species was found in a 228 ha forest fragment at Andasivodihazo and the other in continuous rain forest 10 km to the southeast at Vatateza. Due to morphometric and dental analyses and morphological characteristics of live-caught individuals the authors concluded that the individuals present in the forest fragment at Andasivodihazo most likely represent *C. sibreei*, while those occurring in the continuous forest at Vatateza are conspecific with reference individuals from Ranomafana/Talatakely and likely represent *C. crossleyi* (Blanco et al., 2009). More recently (November 2008 and April 2009), MBB and VR recorded the presence of the Vatateza species (*C. crossleyi*) at Andasivodihazo and the fragment species at Ankadivory, an intermediate forest site located between the continuous and fragmented forests. Past surveys carried out in this region ascribed all dwarf lemurs to *C. major* (e.g. Goodman and Schütz, 1999).

In this study we present genetic data from individuals trapped in 2006 and 2007 belonging to the two sympatric species found at Tsinjoarivo and reference individuals from Ranomafana/Talatakely. We also present morphometric data for the single known skull (UADBA48311) of an individual belonging to the species at Andasivodihazo. Our goal is to determine the species identity of the two sympatric dwarf lemur species at Tsinjoarivo, using comparative genetic analyses based on multiple loci (mtDNA and nDNA) and all available previously published sequence data, as well as new and previously collected morphometric data. As such, our approach can serve as an example of how to deal with a common problem in biodiversity research on tropical vertebrates.

2. Material and methods

2.1. Tissue sample collection

From November 2006 to 2007 MBB took small tissue biopsies from ears of a total of 19 *Cheirogaleus* individuals from three sites in Madagascar (Table 1). Two of the sites are in close proximity to one another at Tsinjoarivo, 80 km south–south-east of Antananarivo. At Tsinjoarivo 10 individuals were caught in a 228 ha forest fragment at Andasivodihazo (19°41′15″S, 47°46′25″E, 1660 m) and seven individuals in continuous rain forest at Vatateza 10 km to the southeast (19°43′15″S, 47°51′25″E, 1396 m). At the third site in Ranomafana National Park (Talatakely 21°15′50″S, 47°25′08″E, 1000 m) an additional two individuals were caught. The sampling sites are marked with white circles on the map in Fig. 1. For a more detailed description of these localities and sampling procedures, see Blanco et al. (2009). The tissue samples were stored in 70% EtOH until further processing.

A total of 375 published *Cheirogaleus* sequences was incorporated into the analyses of the mtDNA and nDNA data sets (Table 2). Of these 375 sequences 354 can be linked to clearly identifiable voucher specimens (first 65 rows in Table 2), while only limited information is available for the remaining 22 sequences. For the first 17 individuals in Table 2, only short cytochrome *b* (*cytb*) fragments (246 bp) were available, since these represent museum specimens (GenBank Accession Nos.: EU825210–EU825226). Data for all markers employed in this study were available for the next 48 individuals in Table 2 (EU825227–EU825610). The 22 sequences that are not linked to a unique specimen and instead are denoted by their GenBank accession number are *cytb* fragments of variable length (307–1140 bp).

Table 1
Cheirogaleus sp. field samples included in this study.

Unique field #	Locality	Locality #
MB206	Tsinjoarivo/Andasivodihazo	42
MB207	Tsinjoarivo/Andasivodihazo	42
MB208	Tsinjoarivo/Andasivodihazo	42
MB209	Tsinjoarivo/Andasivodihazo	42
MB210	Ranomafana/Talatakely	40
MB211	Tsinjoarivo/Andasivodihazo	42
MB212	Tsinjoarivo/Vatateza	41
MB213	Tsinjoarivo/Andasivodihazo	42
MB214	Tsinjoarivo/Vatateza	41
MB215	Tsinjoarivo/Vatateza	41
MB216	Tsinjoarivo/Andasivodihazo	42
MB217	Ranomafana/Talatakely	40
MB218	Tsinjoarivo/Andasivodihazo	42
MB219	Tsinjoarivo/Andasivodihazo	42
MB220	Tsinjoarivo/Vatateza	41
MB221	Tsinjoarivo/Vatateza	41
MB222	Tsinjoarivo/Vatateza	41
MB223	Tsinjoarivo/Vatateza	41
MB224	Tsinjoarivo/Andasivodihazo	42

Unique field number identifying individuals (MB = Marina Blanco), sampling locality and number of sampling locality as marked in Fig. 1 are given.

Sequences from *Mirza zaza*, *Microcebus berthae*, *M. murinus*, and *M. ravelobensis*, which serve as representatives of other major cheirogaleid lineages (Horvath et al., 2008), were used as outgroups to root the phylogenetic trees (Table 2; AF285530, AF285543, AF285564, AY434036, DQ003347, DQ003410, DQ003447, EF052411, EF052462, EF052508, EF052512, EF052561, EF052619, EU342234, EU342261). The localities represented are summarized in Fig. 1, with previously published *Cheirogaleus* sequences stemming from recent field samples marked with squares, and sequences stemming from tissue taken from museum specimens marked with triangles. For a detailed description of most of the included previously published sequences, see Hapke et al. (2005) and Groeneveld et al. (2009). All sequences generated for this study were deposited in GenBank under Accession Nos. GQ243443–GQ243573. A complete list of the GenBank accession numbers used in this study can be found in Supplementary material.

2.2. DNA extraction, amplification and sequencing

Genomic DNA was extracted from tissue samples using the QIAamp™ DNA Mini Kit for DNA purification (QIAGEN) following standard protocol. One mitochondrial [cytochrome *b* (*cytb*)] and three nuclear loci [adenosine receptor A3 exon 2 (*adora3*), alpha fibrinogen intron 4 (*fiba*), and von Willebrand Factor intron 11 (*vWF*)] were amplified as described in Groeneveld et al. (2009). For a little more than half of the nuclear sequences, due to multiple polymorphic sites in heterozygous individuals, PCR products were cloned into a pGEM vector (pGEM™-T EasyVector System I, Promega), averaging five clones per polymorphic sequence. Both strands of all PCR products were sequenced, using the respective primer pair used for amplification and standard vector primers M13F and M13R for the cloned products, employing the BigDye™ Terminator

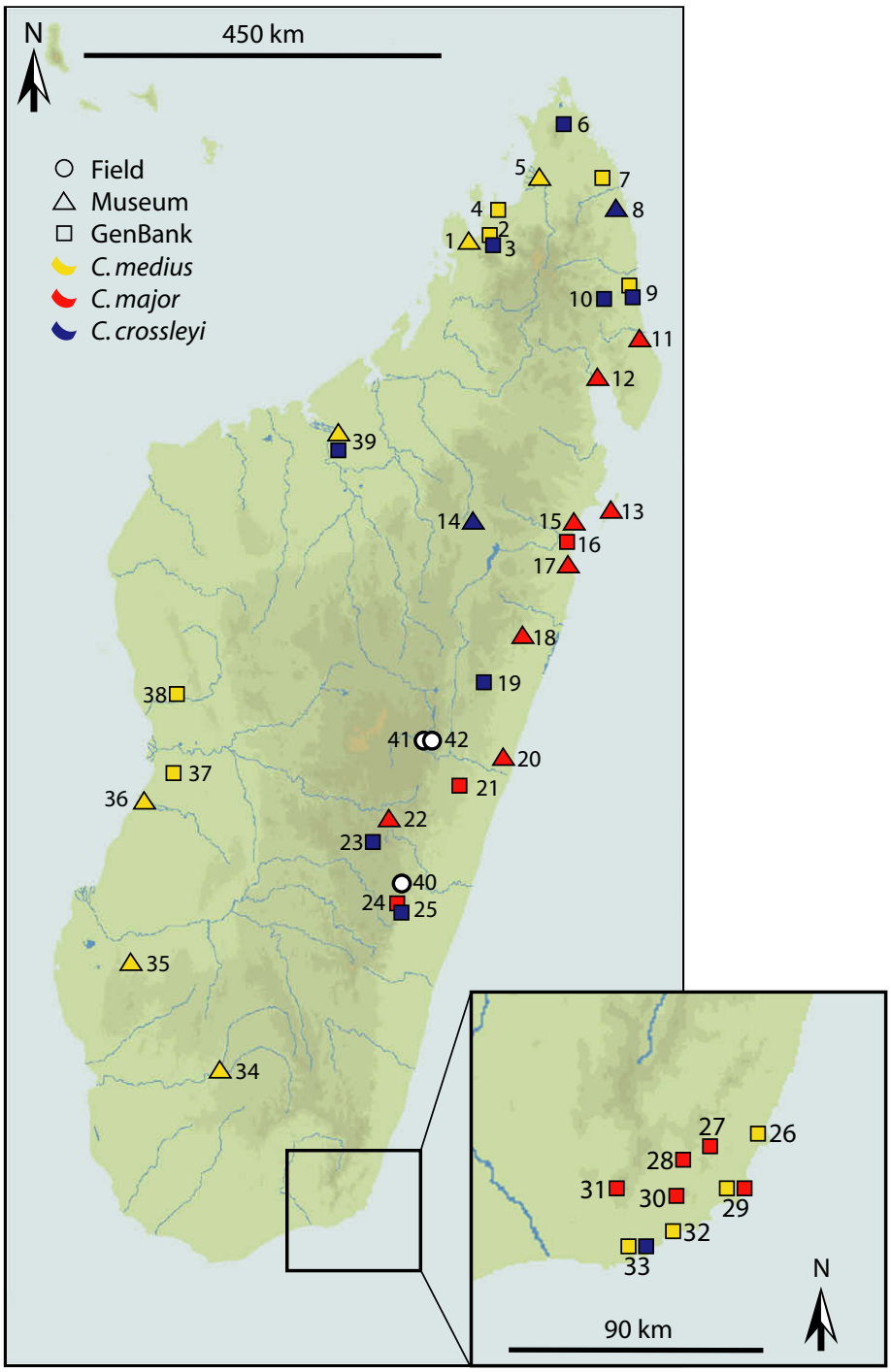

Fig. 1. Sampling localities. Sites at which was sampled for this study are marked with circles. Sampling localities of previously published data are either marked with triangles or squares. Triangles denote presumed sampling localities of museum specimens, while squares identify sampling localities of recent field samples.

v1.1 Cycle Sequencing Kit (Applied Biosystems) on an ABI Prism™ 3100-Avant-Genetic Analyzer (Applied Biosystems).

2.3. Phylogenetic tree reconstruction

Sequences were edited and aligned using Sequencher 4.7 (Gene Codes Corporation, Ann Arbor, MI, USA) and manually verified. Subsequently, sequences were collapsed into haplotypes and coding regions checked for unexpected stop codons using MacClade 4.05 (Maddison and Maddison, 2002). GenBank sequences stemming from museum specimens were collapsed into haplotypes separately, since, due to missing data, unambiguous assignment was not possible. Haplotype data sets were used for all 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 conducted using a genetic algorithm approach in Garli v0.96b8 (Zwickl, 2006). In Garli only the model specifications settings were adjusted according to the respective data set; all other settings were left at their default value. Ten replicates were run for each data set to verify consistency in log likelihood (ln *L*) scores and tree

Table 2
Published *Cheirogaleus* and outgroup samples included in this study.

Unique identifier	Species Groeneveld et al. (2009)/museum or GenBank label	Locality	Locality #	*cytb* (bp)
MNHN CG 1932-3362	*C. major*/*C. major*	Maroantsetra	12	246
MNHN CG 1932-3364	*C. medius*/*C. adipicaudatus*	170 km east of Tulear	34	246
MNHN CG 1932-3365	*C. medius*/*C. adipicaudatus*	170 km east of Tulear	34	246
MNHN CG 1932-3365	*C. medius*/*C. adipicaudatus*	170 km east of Tulear	34	246
MNHN CG 1964-72	*C. major*/*C. ravus*	Mahambo	17	246
MNHN CG 1964-74	*C. major*/*C. ravus*	Ambodivoangy	*	246
MNHN CG1967-1655	*C. medius*/*C. medius*	Ampijoroa	39	246
Naturalis 1887:66b[a]	*C. medius*/*C. sibreei*	Baie de Passandava	1	246
Naturalis 1887:66c[a]	*C. major*/*C. major*	Madagascar	*	246
Naturalis 1887:66g[a]	*C. major*/*C. major*	Maranzettra = Maroantsetra	12	246
Naturalis 1887:66f[a]	*C. major*/*C. major*	Passumbée = Ampasimbe	15	246
Naturalis D.C. van Dam a	*C. medius*/*C. medius*	Moroundava = Morondava	36	246
Naturalis D.C. van Dam e	*C. medius*/*C. medius*	Moroundava = Morondava	36	246
NHM 1935.1.8.168	*C. medius*/*C. adipicaudatus*	Tabiky	35	246
NHM 1935.1.8.169	*C. major*/*C. major*	Maroantsetra	12	246
NHM 1939.1289	*C. major*/*C. crossleyi*	Imerina, E.	22	246
NHM 1948.160	*C. crossleyi*/*C. crossleyi*	Lake Alaotra	14	246
E1001	*C. medius*/*	Ambanja/Ambato	4	1140
E1002	*C. medius*/*	Kirindy	37	1140
E1003	*C. medius*/*	Kirindy	37	1140
E1004	*C. medius*/*	Kirindy	37	1140
MR0291	*C. medius*/*	Bekaraoka	7	1140
RMR132	*C. major*/*	Marolambo	21	1140
RMR133	*C. major*/*	Marolambo	21	1140
RMR134	*C. major*/*	Marolambo	21	1140
RMR135	*C. major*/*	Marolambo	21	1140
RMR137	*C. major*/*	Marolambo	21	1140
RMR139	*C. major*/*	Tampolo	16	1140
RMR140	*C. major*/*	Tampolo	16	1140
RMR141	*C. major*/*	Tampolo	16	1140
RMR146	*C. crossleyi*/*	Andrambovato/Oranjatsy	25	1140
RMR148	*C. major*/*	Andrambovato/Ambalavero	24	1140
RMR149	*C. major*/*	Andrambovato/Ambalavero	24	1140
RMR150	*C. medius*/*	Bemaraha	38	1140
RMR152	*C. medius*/*	Bemaraha	38	1140
RMR153	*C. crossleyi*/*	Montagne d'Ambre	6	1140
RMR155	*C. crossleyi*/*	Montagne d'Ambre	6	1140
RMR158	*C. crossleyi*/*	Montagne d'Ambre	6	1140
RMR162	*C. medius*/*	Ambanja/Benavony	2	1140
RMR164	*C. crossleyi*/*	Ambanja/Beandroana	3	1140
RMR166	*C. crossleyi*/*	Sambava	9	1140
RMR167	*C. crossleyi*/*	Sambava	9	1140
RMR168	*C. crossleyi*/*	Sambava	9	1140
RMR169	*C. medius*/*	Sambava	9	1140
RMR170	*C. medius*/*	Sambava	9	1140
RMR171	*C. crossleyi*/*	Sambava	9	1140
RMR172	*C. crossleyi*/*	Sambava	9	1140
RMR173	*C. crossleyi*/*	Sambava	9	1140
RMR174	*C. crossleyi*/*	Sambava	9	1140
RMR175	*C. crossleyi*/*	Sambava	9	1140
RMR176	*C. crossleyi*/*	Sambava	9	1140
RMR177	*C. medius*/*	Sambava	9	1140
RMR178	*C. medius*/*	Sambava	9	1140
RMR179	*C. crossleyi*/*	Manantenina	10	1140
RMR180	*C. crossleyi*/*	Manantenina	10	1140
RMR181	*C. crossleyi*/*	Manantenina	10	1140
RMR182	*C. crossleyi*/*	Manantenina	10	1140
RMR183	*C. crossleyi*/*	Manantenina	10	1140
RMR184	*C. crossleyi*/*	Manantenina	10	1140
RMR193	*C. crossleyi*/*	Ankazomivady	23	1140
RMR194	*C. crossleyi*/*	Ankazomivady	23	1140
RMR196	*C. crossleyi*/*	Ankazomivady	23	1140
RMR201	*C. major*/*	Ivorona	28	1140
RMR205	*C. major*/*	Ivorona	28	1140
RMR212	*C. major*/*	Manantantely	30	1140
AH014105	*C. major*/*C. major*	Nosy Boraha, Ile Ste. Marie	13	208 + 259 + 241
AH014106	*C. major*/*C. major*	Mahanoro	20	633 + 241
AY441457	*C. crossleyi*/*C. major*	Andasibe	19	1140
AY605903	*C. medius*/*C. medius*	Morondava CFPF	37	1140
AY605904	*C. medius*/*C. medius*	Forêt de l'Ankarana	5	933
AY605905	*C. medius*/*C. medius*	Ste. Luce	26	1140
AY605906	*C. medius*/*C. medius*	Ste. Luce	26	1140
AY605907	*C. medius*/*C. medius*	Ste. Luce, Mandena	26, 29	1140
AY605908	*C. medius*/*C. medius*	Mandena	29	1140
AY605909	*C. medius*/*C. medius*	Petriky, Lavasoa	32, 33	1140

Table 2 (*continued*)

Unique identifier	Species Groeneveld et al. (2009)/museum or GenBank label	Locality	Locality #	*cytb* (bp)
AY605910	*C. medius*/*C. medius*	Lavasoa	33	1140
AY605911	*C. major*/*C. major*	Maroantsetra	11	1140
AY605915	*C. major*/*C. major*	Toamasina/Tamatave	18	1140
AY605918	*C. major*/*C. major*	Andohavondro	31	1140
AY605919	*C. major*/*C. major*	Manantantely	30	1140
AY605920	*C. major*/*C. major*	Manantantely, Mandena	30, 29	1140
AY605921	*C. major*/*C. major*	Ivorona	28	1140
AY605922	*C. major*/*C. major*	Farafara	27	1140
AY605923	*C. major*/*C. major*	Farafara	27	1140
AY605926	*C. crossleyi*/*C. crossleyi*	Iharana/Vohemar	8	633
AY605927	*C. crossleyi*/*C. crossleyi*	Lavasoa	33	1140
EF122249	*C. crossleyi*/*C. medius*	Ampijoroa	39	307
Voucher 149	∗/*Microcebus berthae*	∗	∗	∗
Voucher 66	∗/*Microcebus ravelobensis*	∗	∗	∗
Voucher 203	∗/*Microcebus murinus*	∗	∗	∗
DLC2307	*Mirza zaza*/*Mirza coquereli*	∗	∗	∗
DUPC384F	*Mirza zaza*/*Mirza coquereli*	∗	∗	∗
RMR24	∗/*Microcebus murinus*	∗	∗	1140
RMR53	∗/*Microcebus ravelobensis*	∗	∗	1140
Jorg46	∗/*Microcebus berthae*	∗	∗	1140
U53571	*Mirza zaza*/*Mirza coquereli*	∗	∗	1140

The unique identifier either represents a field collection number or museum catalog number, or where this is not available the GenBank accession number. Species label according to Groeneveld et al. (2009) as well as species label according to museum catalogs or GenBank records, the name of the sampling locality and the locality number as used in Fig. 1 and the length of the cytochrome *b* fragment in bp are given. Asterisks denote non-availability of the data.

[a] Listed under *Cheirogaleus milii* in Jentink (1887).

topologies. Maximum likelihood bootstrap percentages (BP) were estimated in Garli by performing 1000 pseudoreplicate runs. PAUP* v4.0b10 (Swofford, 2002) was then used to calculate a majority-rule consensus tree for each data set.

Bayesian analyses were conducted using MrBayes v3.2-cvs (Huelsenbeck et al., 2001; Ronquist and Huelsenbeck, 2003). In all analyses we used four Monte Carlo Markov chains (MCMC) with a temperature of 0.05. Analyses were run for 10 million generations with tree and parameter sampling occurring every 1000 generations. Flat priors were assumed for the model parameters including the proportion of invariable sites and the gamma shape parameter of rate variation among sites. The first 25% of samples were discarded as burn-in, leaving 7501 trees per run. The adequacy of this burn-in and convergence of all parameters were assessed by examining the uncorrected potential scale reduction factor (PSRF) (Gelman and Rubin, 1992) as calculated by MrBayes v3.2-cvs (Huelsenbeck et al., 2001; Ronquist and Huelsenbeck, 2003), which should approach 1 as runs converge and visual inspection of the trace of the parameters across generations using the software Tracer v1.4.1 (Rambaut and Drummond, 2008). Moreover, AWTY (Nylander et al., 2008) was used to check whether posterior clade probabilities were also converging. Posterior probabilities (PP) for each split and a phylogram with mean branch lengths were calculated from the posterior density of trees using MrBayes v3.2-cvs (Huelsenbeck et al., 2001; Ronquist and Huelsenbeck, 2003). Phylogenetic trees were visualized using FigTree v1.2.1 (Rambaut, 2009).

Uncorrected "*p*" distances were calculated for the *cytb* data set using PAUP* v4.0b10 (Swofford, 2002). Missing data, including gaps and ambiguity codes, were inferred by distributing them proportionately to unambiguous changes.

Statistical parsimony haplotype networks were constructed for each nuclear locus using the program TCS version 1.21 (Clement et al., 2000). A 95% connection limit was used and gaps were treated as missing data.

The genealogical sorting index (gsi) was calculated across nuclear gene trees to quantify the degree of exclusive ancestry of the mtDNA-defined clades. Ten thousand permutations were carried out via the web interface at http://www.genealogicalsorting.org/ (Cummings et al., 2008).

2.4. Skeletal sample collection and analysis

We measured 19 cranial and 13 dental traits on one available skull and mandible from the fragmented forest at Tsinjoarivo (UADBA48311), the holotype of *Cheirogaleus sibreei* (NHD ZD 1897.9.1.160), and 60 individuals belonging to *C. major* ($n = 16$), *C. medius* ($n = 31$), and *C. crossleyi* ($n = 13$), respectively. The identities of the individuals in the latter three groups were previously confirmed using a combination of genetic and morphometric data (Groeneveld et al., 2009, in press). The cranial and dental variables used here are described in Rasoloarison et al. (2000). The skull belonging to the individual from Tsinjoarivo was described in Blanco et al. (2009) under the provisional number UADBA-TFFP-001; it is housed at the Université d'Antananarivo (Département de Biologie Animale). A specimen from northwestern Madagascar identified by Groves (2000) as belonging to *C. sibreei* but shown via genetic analysis to belong to *C. medius* (Groeneveld et al., 2009) was omitted from our analysis, as our interest is in determining whether the fragment population from Tsinjoarivo matches the *C. sibreei* holotype (and not other individuals that may have been previously misidentified). The 32 morphometric variables were first entered into a Principal Components Analysis of their correlation matrix. Because this analysis demonstrated that the four species separate cleanly even when group membership is not assigned, the data were then subjected to Discriminant Function Analysis to determine the phenetic affinities of the specimen from Tsinjoarivo. We first used the whole set of 32 variables, and then the subset of the 19 cranial variables, with the Tsinjoarivo individual treated as an ungrouped case. Statistical analyses were performed using SPSS 14 (SPSS, Inc., Chicago, IL, USA).

3. Results

3.1. Haplotype data

Complete mitochondrial *cytb* sequences (1140 bp) were generated for the 19 field samples included in this study. Among the 19 samples, there were 190 variable sites defining seven haplotypes. These were aligned with the 59 published *cytb* haplotypes derived from museum and field specimens. Of the 59 haplotypes,

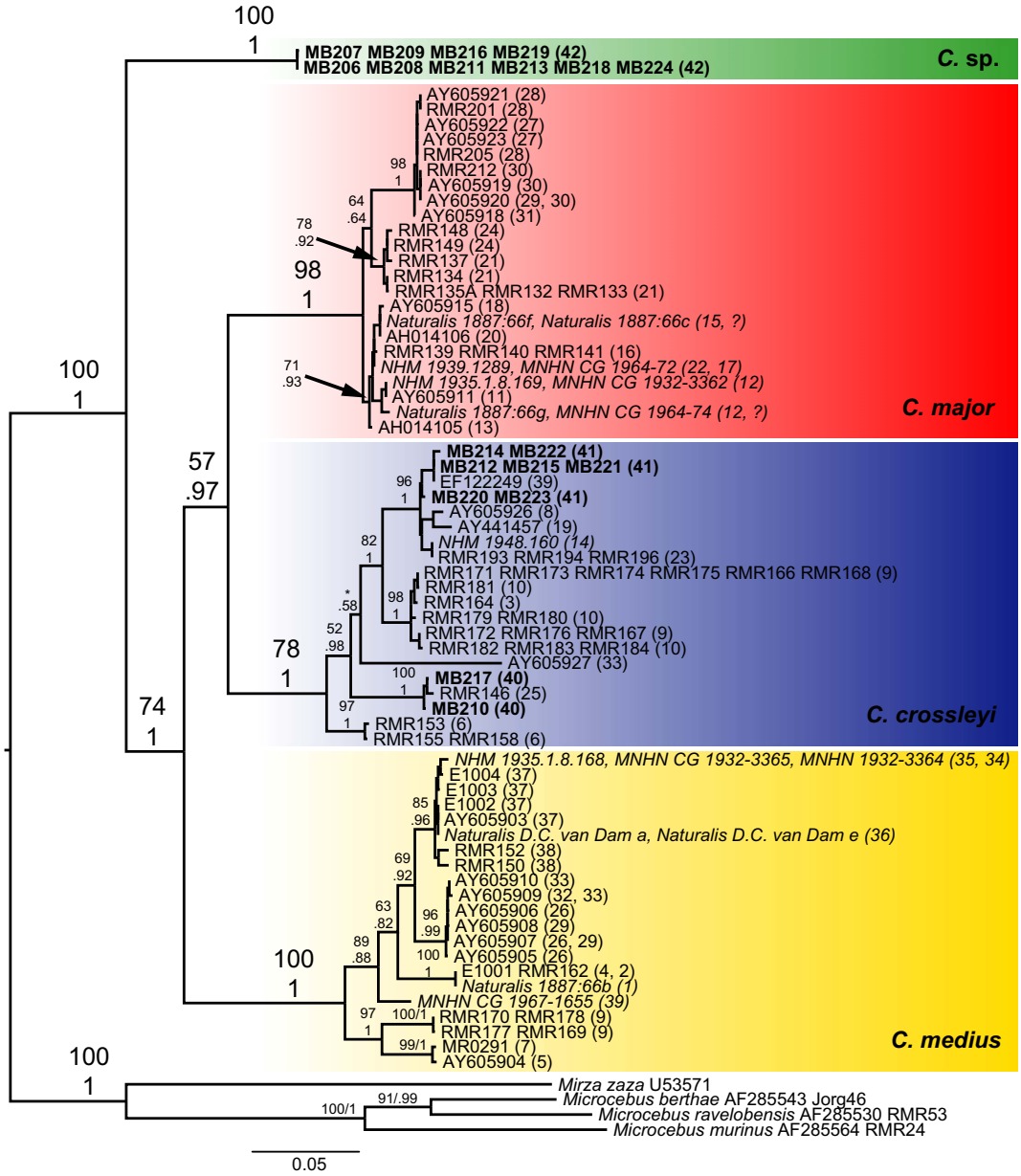

Fig. 2. Maximum likelihood phylogram based on mtDNA. ML phylogram based on an alignment of complete *cytb* haplotype sequences (1140 bp) from new field samples (bold), published sequences derived from museum specimens (italic), and published sequences derived from recent field samples (regular). Tip labels contain the individual field collection number (MB, MR, E, RMR), the GenBank accession number or the museum catalog number of sequences within haplotype. The sampling locality, where a haplotype was found, is given in parentheses as marked in Fig. 1. GenBank haplotypes may occur in more than one locality. ML bootstrap values and Bayesian posterior probabilities are depicted above the branches. Asterisks denote support values below 50 or 0.50.

nine haplotypes consisted of 246 bp (museum specimens) and five haplotypes ranged from 307 to 933 bp in length, while all other haplotypes consisted of the complete *cytb* fragment. The resulting final alignment (1140 bp) consisted of 66 haplotypes defined by 360 variable sites and contained no indels.

For the nuclear markers both alleles were unambiguously scored in all individuals with the following exceptions: no reliable sequence was generated for individual MB223 for the intronic *fiba* fragment and therefore this individual was excluded from the *fiba* data set. One character was scored as ambiguous in individual MB219 in the exonic *adora3* fragment and in the intronic *vWF* fragment. For each of the three nuclear loci 96 published *Cheirogaleus* sequences (described in Groeneveld et al., 2009) were available and included in all analyses.

The 36 *fiba* alleles generated for this study collapsed into 15 haplotypes defined by 22 variable sites. Together with the 96 published *Cheirogaleus* sequences the final *fiba* alignment of 606 bp consisted of 63 haplotypes defined by 50 variable sites. Among the 38 *vWF* alleles there were 44 variable sites defining 20 haplotypes. When combined with the 96 published *Cheirogaleus vWF* sequences, a final alignment of 794 bp consisting of 70 haplotypes defined by 113 variable sites results. The 38 *adora3* alleles collapsed into 14 haplotypes defined by 12 variable sites. In combina-

Table 3
Uncorrected "*p*" distances for *Cheirogaleus* species based on an alignment of complete *cytb* haplotype sequences (1140 bp).

	C. medius	*C. major*	*C. crossleyi*	Clade 4
C. medius	0.041			
C. major	0.122	0.023		
C. crossleyi	0.135	0.117	0.045	
Clade 4	0.129	0.121	0.128	0.001

tion with 96 published *Cheirogaleus* sequences the alignment of 370 bp comprised 37 haplotypes defined by 27 variable sites.

The mitochondrial *cytb* data set best fit a general time reversible (GTR) model with a proportion of invariable sites (I) and gamma distributed rate heterogeneity (Γ) according to AIC. A K81uf+I+Γ model was favored for the *fiba* locus, (analyzed under a GTR+I+Γ model in Bayesian phylogenetic analyses). The *vWF* locus was found to best fit an HKY+I+Γ model and the *adora3* locus a TrN+I model (Bayesian: GTR+I).

3.2. Phylogenetic relationships—mtDNA

Bayesian and ML analyses resulted in congruent trees with four main clades (Fig. 2), three of which represent *C. medius*, *C. major*

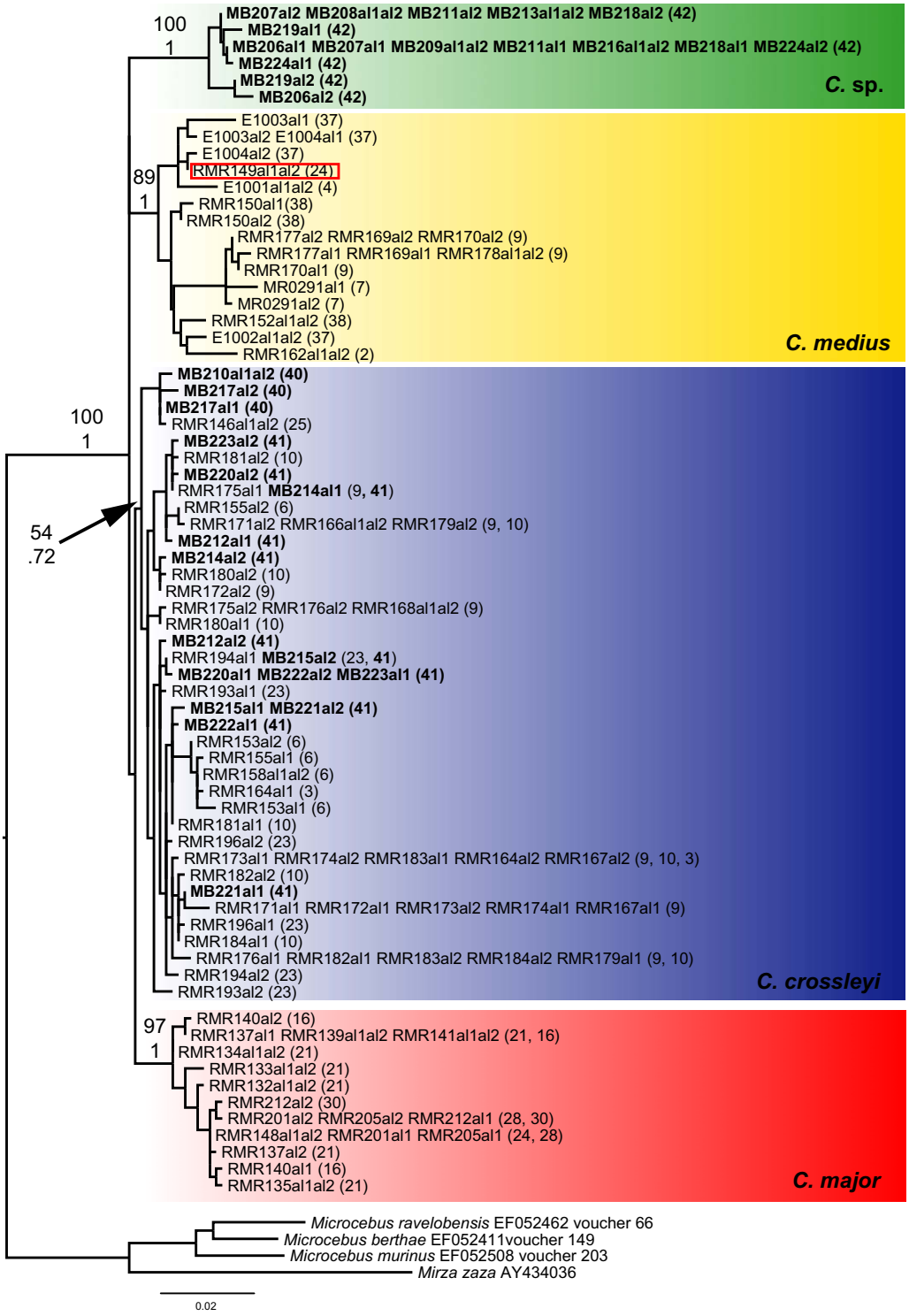

Fig. 3. Maximum likelihood phylogram based on nuclear *vWF* locus. ML phylogram based on an alignment of partial *vWF* haplotype sequences (795 bp) from new field samples (bold) and published sequences derived from recent field samples (regular). Tip labels contain the individual field collection number (MB, MR, E, RMR) of sequences within haplotype. The sampling locality, where a haplotype was found, is given in parentheses as marked in Fig. 1. ML bootstrap values and Bayesian posterior probabilities are depicted above the branches.

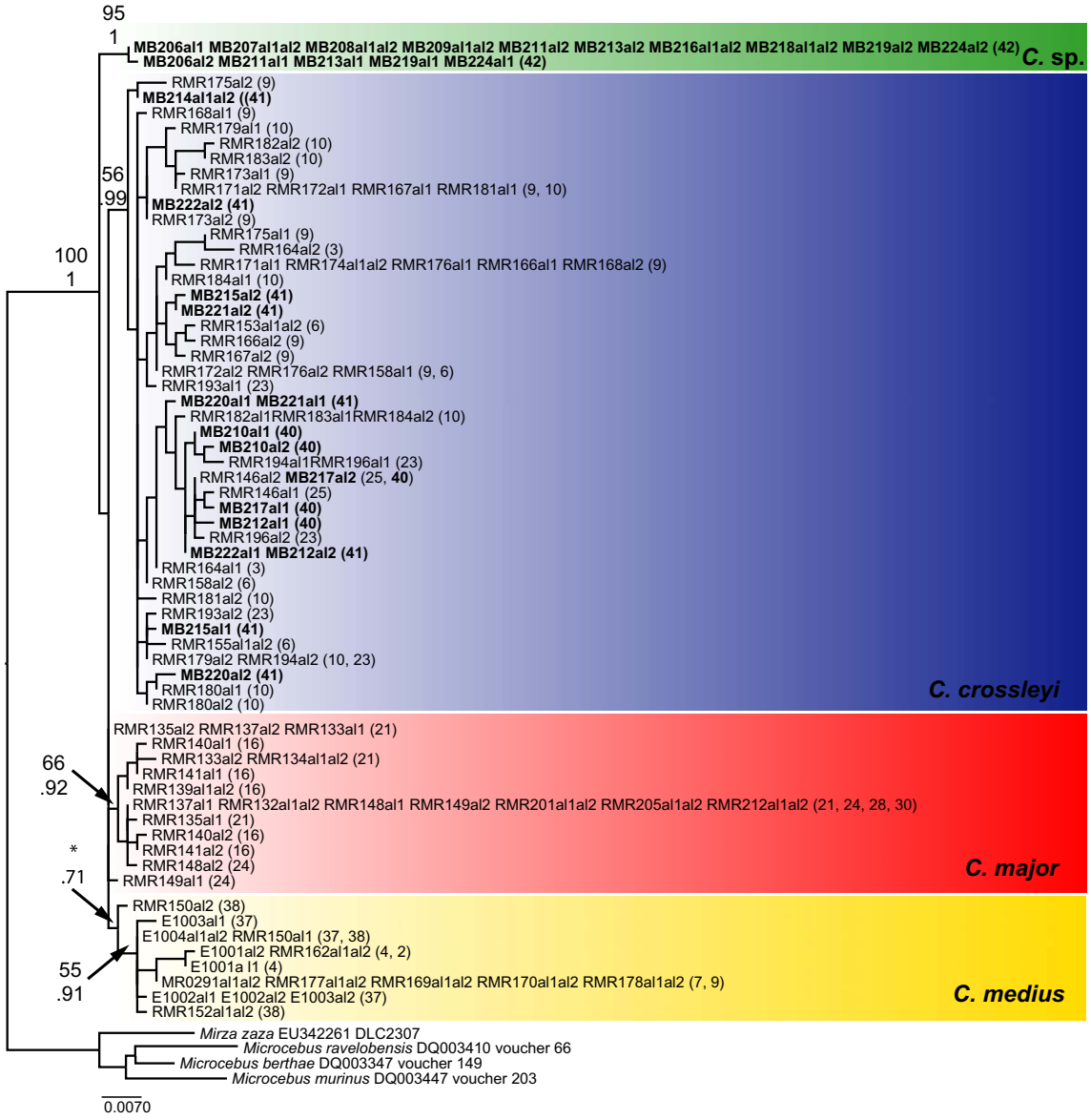

Fig. 4. Maximum likelihood phylogram based on nuclear *fiba* locus. ML phylogram based on an alignment of partial *fiba* haplotype sequences (606 bp) from new field samples (bold) and published sequences derived from recent field samples (regular). Tip labels contain the individual field collection number (MB, MR, E, RMR) of sequences within haplotype. The sampling locality, where a haplotype was found, is given in parentheses as marked in Fig. 1. ML bootstrap values and Bayesian posterior probabilities are depicted above the branches. Asterisks denote support values below 50 or 0.50.

and *C. crossleyi* (Groeneveld et al., 2009; Groves, 2000). The fourth main clade (clade 4), which is strongly supported (ML BP = 100 and Bayesian PP = 1), consists of two haplotypes found exclusively in individuals from Tsinjoarivo/Andasivodihazo (locality 42). All sequences generated from individuals from Tsinjoarivo/Vatateza (locality 41) and Ranomafana/Talatakely (locality 40) are within the clade representing *C. crossleyi*. More specifically, the sequences generated from the two individuals from Ranomafana/Talatakely (locality 40) form a well-supported clade (ML BP = 100 and Bayesian PP = 1) with a sequence from a *C. crossleyi* individual from Andrambovato/Oranjatsy (locality 25) and the three haplotypes generated from individuals from Tsinjoarivo/Vatateza (locality 41) are placed in a clade consisting of haplotypes of individuals from Ankazomivady (locality 23), Andasibe (locality 19), Iharana/Vohemar (locality 8), Ampijoroa (locality 39) and Lake Alaotra (locality 14) (ML BP = 96 and Bayesian PP = 1).

Relationships among the four main clades based on mtDNA data are poorly supported and best viewed as unresolved. However, it should be noted that the fourth clade, consisting of haplotypes from individuals from Tsinjoarivo/Andasivodihazo (locality 42), was found in a basal position in ML and Bayesian analyses of mtDNA data. Uncorrected "*p*" distances among the four main clades were quite similar with an average ranging from 11.7% to 13.5% between the three named species and an average of 12.1% between *C. major* and clade 4, 12.8% between *C. crossleyi* and clade 4, and 12.9% between *C. medius* and clade 4 (Table 3).

3.3. Phylogenetic relationships—nDNA

ML and Bayesian analyses of the individual nuclear loci resulted in generally congruent topologies containing the four main clades identified by analyses of mtDNA. ML phylograms based on *vWF* and *fiba* haplotype sequences are shown in Figs. 3 and 4. Both phylograms consist of four main clades corresponding to the mtDNA clades with two exceptions: in the vWF phylogram an individual identified as *C. major* is placed in the *C. medius* clade and in the *fiba*

phylogram the *C. major* individuals do not form a resolved clade. Clade 4 is well supported (*vWF*: ML BP = 100 and Bayesian PP = 1, *fiba*: ML BP = 95 and Bayesian PP = 1) and found in a basal position in both phylograms. As found for mtDNA, all haplotypes from Tsinjoarivo/Vatateza (locality 41) and Ranomafana/Talatakely (locality 40) are placed in the clades representing *C. crossleyi*. The phylogram for the *adora3* locus is not shown due to its low resolution and a statistical parsimony haplotype network is presented instead (Fig. 5). The haplotype networks for the *vWF* and *fiba* loci are not shown, since they do not provide further insights. The *adora3* haplotype network resolves a clade of *C. crossleyi* haplotypes. All haplotypes from Tsinjoarivo/Vatateza (locality 41) and Ranomafana/Talatakely (locality 40) are found among the *C. crossleyi* haplotypes, which is congruent with analyses of mtDNA and the nuclear *fiba* and *vWF* loci. The remaining haplotypes correspond to *C. medius*, *C. major* and clade 4. There is shared polymorphism among *C. medius* and *C. major*, as well as *C. major* and clade 4.

The ensemble gsi across the three nuclear loci was 1 for *C. crossleyi*, demonstrating complete monophyly for this group, 0.78 for *C. major*, 0.85 for *C. medius*, and 0.93 for clade 4 ($p = 0.0001$ for all four statistics). The degree of exclusivity of clade 4 thus lies within the range of the labeled species.

3.4. Phenetic clues—Principal Components and Discriminant Function Analysis of morphometric data

A Principal Components Analysis of the correlation matrix for 32 craniodental variables measured on 62 individuals was able to capture 84.1% of the variance in two factors (the first accounting for 76.4% of the variance, and the second an additional 7.8%; see Supplementary material for a list of the variables and descriptive statistics for the four species). A plot of individual scores on these two axes (Fig. 6) shows that *C. medius*, *C. crossleyi*, and *C. major* separate well. The holotype for *C. sibreei* does not cluster with any of the latter three; rather, it falls between *C. medius* (with low scores

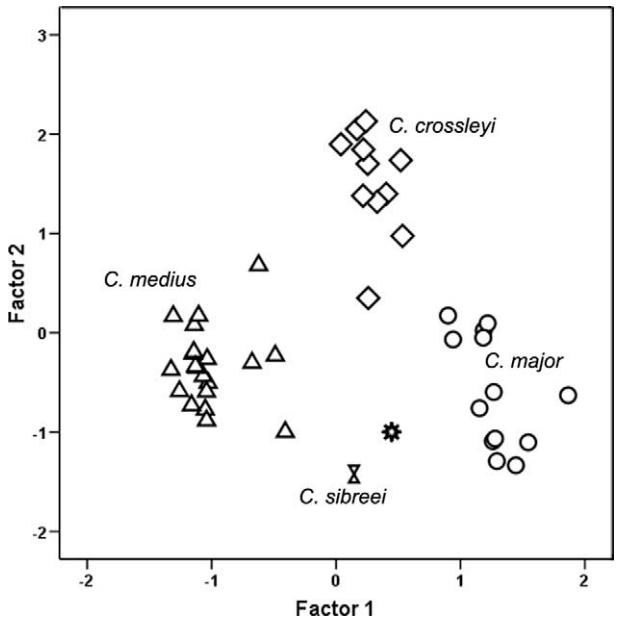

Fig. 6. Principal Components Analysis of the correlation matrix of 32 cranial and dental variables. The skull from Tsinjoarivo (UADBA48311) is represented by an asterisk. See text for further explanation.

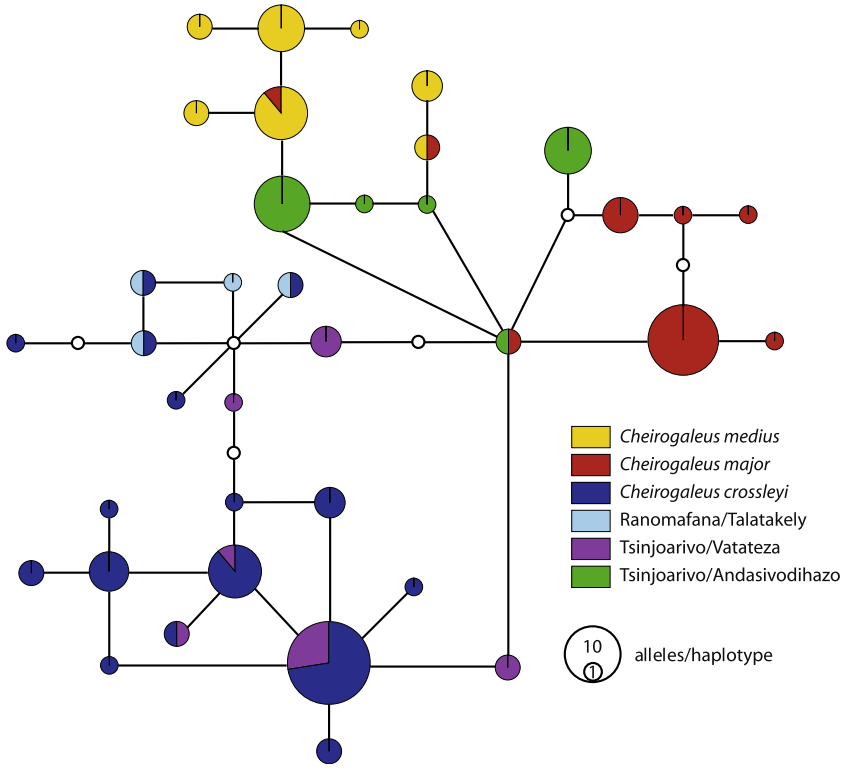

Fig. 5. Haplotype network based on nuclear *adora3* locus. Statistical parsimony haplotype network depicting genealogical relationships among 37 haplotypes of the *adora3* locus, from new field samples and published sequences derived from recent field samples. Previously published haplotypes are coded according to species labels as assessed in Groeneveld et al. (2009), while new field samples are coded according to their sampling locality. The sizes of the circles representing haplotypes reflect the number of sequences that share a haplotype. Inferred intermediate haplotypes, either not sampled, or extinct, are represented by small non-colored circles. Further details linking the haplotypes to specimens can be found in Supplementary material. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

on Factor 1) and *C. major* (with high scores on Factor 1). Factor 2 distinguishes *C. crossleyi* (with positive scores) from all others, particularly *C. sibreei*. It is noteworthy that UADBA48311 from Tsinjoarivo, with its intermediate score on Factor 1 and negative score on Factor 2, falls nearest the holotype of *C. sibreei* (Fig. 6). All variables are significantly positively correlated with Factor 1 (which reflects skull size to a large degree), but Factor 2 is a contrast vector distinguishing individuals with relatively small anterior teeth (incisors, canines, second, and third premolars) and relatively large values for the temporal line and parietal width measurements (positive scores) from those with the opposite suite of characteristics (i.e., relatively large anterior teeth, and small values for the temporal line and parietal width measurements). Factor 2 separates *C. crossleyi* (with high positive scores) from all others, including the holotype of *C. sibreei* and the individual from Tsinjoarivo (with strongly negative scores).

Because Principal Components Analysis separated species of *Cheirogaleus* without *a priori* group assignment, we felt justified in using Discriminant Function Analysis to further explore the affinities of the individual from Tsinjoarivo. Taxonomic group assignments were made for *C. major*, *C. crossleyi*, *C. medius*, and *C. sibreei*; the individual from Tsinjoarivo was ungrouped. An analysis using the full set of variables yielded three highly significant functions with 100% post hoc classification success; the ungrouped Tsinjoarivo individual was classified unequivocally as a member of the *C. sibreei* group. We then ran a Discriminant Function Analysis of the 19 cranial variables taken alone to reduce the total number of variables used in the analysis. The results were the same: all three functions were significant (the first two strongly so), post hoc classification success was 100%, and the ungrouped individual was classified unequivocally as belonging to *C. sibreei*. The first two functions (Fig. 7) captured 96.2% of the variance (70.1% for Function 1, Wilks' Lambda $p < 0.001$; an additional 26.1% for Function 2, Wilks' Lambda $p < 0.001$). Individual scores on Function 1 were most strongly correlated with Skull height, Greatest skull length, and Palate length. Scores on Function 2 were most strongly positively correlated with Temporal line and Parietal width, and most strongly negatively correlated with Nasal width, Condylobasal length, and Bizygomatic width. The holotype of *C. sibreei* and the individual (UADBA48311) from Tsinjoarivo can be distinguished from all others by their moderate scores on Function 1 combined with strongly negative scores on Function 2.

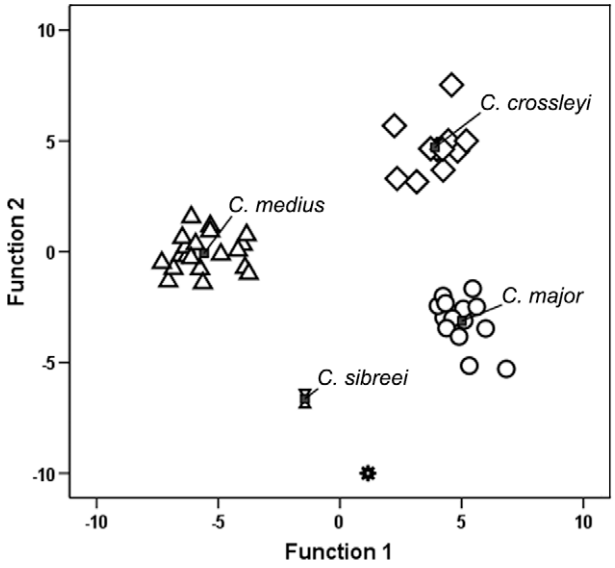

Fig. 7. Discriminant Function Analysis of 19 cranial variables. Again the skull from Tsinjoarivo (UADBA48311) is represented by an asterisk.

4. Discussion and conclusion

Blanco et al. (2009) discovered two sympatric dwarf lemur species at Tsinjoarivo, where previously only *C. major* was presumed to occur. Based on morphological data the authors concluded that the two species most likely represent *C. crossleyi* (at Vatateza, locality 41) and *C. sibreei* (at Andasivodihazo, locality 42). However, Blanco et al. (2009) also considered the possibility, which they could not exclude, that the *C. sibreei*-like individuals might represent a new species.

Analyses of mtDNA and nDNA loci in this study clearly showed that the individuals sampled at Vatateza, as well as the reference individuals from Ranomafana/Talatakely (locality 40) can be attributed to *C. crossleyi*, while the individuals from Andasivodihazo form a well-supported fourth main clade, basal to the clades representing *C. medius*, *C. major*, and *C. crossleyi*, in all loci except *adora3*.

4.1. Individuals from Talatakely and Vatateza

The individuals from Talatakely and Vatateza can be attributed to *C. crossleyi* based on analyses of mtDNA and nDNA loci, which is congruent with the conclusion drawn by Blanco et al. (2009) based on morphological data.

The haplotypes of two individuals from Ranomafana/Talatakely cluster closely with the haplotypes of a *C. crossleyi* individual from Andrambovato/Oranjatsy (locality 25) in all loci, which can be explained by their close provenance (~50 km). The *cytb* haplotypes of the individuals from Vatateza cluster with haplotypes found in *C. crossleyi* individuals from a wide geographic range: Ampijoroa (locality 39), Iharana/Vohemar (locality 8), Andasibe (locality 19), Lake Alaotra (locality 14), and Ankazomivady (locality 23) (Fig. 2). This cannot be corroborated by nuclear loci, as these loci either show no resolution within the main clades, or the existing structure is not strongly supported.

4.2. Individuals from Andasivodihazo

The individuals from Andasivodihazo (locality 42) cannot be attributed to *C. medius*, *C. major* or *C. crossleyi*, as they form a fourth well-supported clade in mtDNA and nDNA loci and can thus be viewed as a separately evolving lineage (Figs. 2–4). Only the nuclear exonic *adora3* fragment showed no clear resolution of the four main clades. The shared polymorphism among *C. major* and clade 4 (Andasivodihazo) individuals in the *adora3* locus (Fig. 5) could be an artifact due to the ambiguous character in the sequence MB219. The ensemble gsi across the three nuclear loci demonstrated clear exclusivity of the mtDNA-defined clade 4, composed of the Andasivodihazo individuals.

The genetic data presented here are congruent with morphometric and dental analyses, as well as morphological characteristics such as pelage coloration and female genitalia in recognizing the individuals from Andasivodihazo as a distinct lineage (Blanco et al., 2009).

Under the General Lineage Concept of species (de Queiroz, 1998, 2005) a species is a separately evolving lineage identified through multiple independent lines of concordant data. This is the case for the individuals from Andasivodihazo: they are morphologically distinct (see also Blanco et al., 2009) and clearly demonstrate divergence in mtDNA and nDNA loci. We therefore conclude that the individuals from Andasivodihazo are distinct from *C. medius*, *C. major*, and *C. crossleyi* and thus represent a fourth species.

4.3. Which species do the Andasivodihazo individuals represent?

In making their initial species attributions, Blanco et al. (2009) compared pelage coloration and dental morphology of individuals

ascribed to *Cheirogaleus medius*, *C. major*, *C. crossleyi*, and *C. sibreei*. Unfortunately, museum collections hold only two skulls (NHM ZD 1897.9.1.160; Naturalis 1887:66b) and four skins (NHM ZD 1897.9.1.160; Naturalis 1887:66b, 1887:66a; ZMB 71434) attributed to *C. sibreei*. Since the NHM does not allow invasive sampling of holotype material, including dental molding and sampling for molecular analyses, prior molecular and dental analyses included only one individual (Naturalis 1887:66b) attributed to *C. sibreei*, which was not the type specimen (Blanco et al., 2009; Groeneveld et al., 2009). A recent analysis of morphometric data (Groeneveld et al., in press) demonstrated incongruity within the group of museum specimens previously attributed to *C. sibreei*. The Naturalis specimen grouped with *C. medius* morphometrically (as it had genetically), but the holotype of *C. sibreei* did not. The authors suggested that museum specimens labeled as *C. sibreei* are likely not conspecific. Whether the holotype represents a distinct evolutionary group was held to be inconclusive.

We can now affirm that a living population that closely matches the holotype morphometrically also stands apart from all other *Cheirogaleus* genetically. We therefore conclude that the population of dwarf lemurs at Tsinjoarivo/Andasivodihazo is indeed *C. sibreei*, and that *C. sibreei* is a valid species of the genus *Cheirogaleus*. A detailed description of these individuals is provided in Blanco et al. (2009).

4.4. Implications for biogeography and conservation

Tsinjoarivo occupies a transitional biogeographic position: although it is found within Madagascar's eastern rain forest corridor, it is unusual in its geographic setting. First, Tsinjoarivo is part of a relatively small forest block isolated between two major river barriers (Onive to the south and Mangoro to the east), which constitute range boundaries for several lemurs (Mittermeier et al., 2006). Second, it reaches some of the highest elevations known for eastern rainforests (its eastern boundary starts at ~1200 m, but its western boundary reaches ~1650 m). Although much of Tsinjoarivo forest straddles the eastern escarpment separating Madagascar's eastern lowlands from the high central plateau, the western edge of Tsinjoarivo forest is ~20 km west of this escarpment and is topographically continuous with the central plateau; it might be better referred to as "central plateau forest". While there are a few other rainforests of similar altitude and position (e.g. Andringitra, Marojejy, Tsaratanana), they are rare, and isolated from one another by intervening lower-altitude forests.

Three localities have been sampled at Tsinjoarivo: Andasivodihazo (~1660 m, high altitude, western), Ankadivory (~1474 m, central), and Vatateza (~1390 m, lower altitude, eastern). Considering the original sampling (in late 2006 and 2007) and subsequent trapping (November 2008 and April 2009), *C. crossleyi* has been found at Andasivodihazo and Vatateza, while *C. sibreei* has been found at Andasivodihazo and Ankadivory. This suggests that the two species overlap geographically, but while *C. crossleyi* appears more broadly distributed, *C. sibreei* may be restricted to the western half of Tsinjoarivo forest, and perhaps to forests above ~1400 m. If these habitat preferences are typical, this species' range might turn out to be the smallest of its genus; if it is ecologically limited to high altitudes, its range is likely to be restricted to one or more high-altitude subpopulations along the western margin of the eastern rainforest corridor.

The exact location of Ankeramadinika and the altitude at which the type specimen of *C. sibreei* was found were not recorded by Forsyth Major (1896) when he described the species only as "one day's journey east" of Antananarivo. However, Mullens (1875) had visited the site two decades earlier and recorded its altitude as 4620 feet (1408 m). Similarly, a vertical section profile provided by "Capitaine X" (1901) shows Ankeramadinika on the central plateau at an altitude higher than Antananarivo (and thus over 1400 m). The latter author provided a graphic description of the journey between Antananarivo and points east, including Ankeramadinika; he depicted Ankeramadinika as lying near the edge of the cliff of Angavo. From Ankeramadinika westward (~40 km) to Antananarivo, the journey was relatively easy but, until a military road was constructed negotiating the steep escarpment to the east, the journey east of Ankeramadinika was arduous. More recently, Jenkins and Carleton (2005) estimated the geographic coordinates for this site on the basis of their reconstructed itinerary for Forsyth Major from 1894 to 1896. Given that Jenkins and Carleton's (2005) coordinates do not match Mullens's measured altitude records, we judge their itinerary to require some correction.

In conclusion, two aspects of the known range of *Cheirogaleus sibreei* suggest that its range might be extremely restricted. First, this species is so far known only from high-altitude sites. Second, the two known localities (Ankeramadinika and Tsinjoarivo) for *C. sibreei* are only ~100 km apart, and the range of *C. sibreei* is (so far) restricted by the Onive and Mangoro rivers, i.e., Tsinjoarivo, Anjozorobe, and the corridor joining them. This area has less than 2000 km^2 of forest remaining and the majority of this is lower altitude (e.g. below 1350 m) and likely occupied by *C. crossleyi*. This suggests that the actual habitat available to *C. sibreei* may be much less. The region of Ankeramadinika is today largely deforested; indeed "Capitaine X" (1901) described concessions to exploit over 900 ha of forest in Ankeramadinika over a century ago—i.e., at the time General Gallieni traveled there (~1897).

Unfortunately, the location of the Tsinjoarivo population has left it extremely vulnerable to extinction. While the rugged, eastern (lower) parts of Tsinjoarivo forest (near the escarpment) remain reasonably undisturbed due to their inaccessibility, the higher western part, which is flatter and continuous with the high plateau, has been invaded by substantial human settlement from the plateau. In Andasivodihazo and surrounding regions, human population density is high, forest cover has been reduced to ~50%, and remaining forests are highly fragmented and disturbed (Irwin, 2006). Although this forest is slated for future protection by the Malagasy government, it remains "Classified Forest", and effectively lacks on-the-ground protection. Lemurs are extremely vulnerable to habitat disturbance, including illegal selective logging, hunting, fires, invasive plants and animals (including dogs which hunt mammals and birds), and reduced gene flow due to habitat patchiness. Further, if other subpopulations are discovered in similar marginal geographic settings close to human habitation, they are likely to suffer from similar pressures. Not only does this small population represent the only confirmed living population of *C. sibreei*, but as the clade's basal member, this species is likely the most evolutionarily distinct. Its extinction would cause a disproportionately large loss of genetic and ecological diversity. Finally, the loss of this population would affect our ability to test hypotheses regarding the evolutionary history of the genus *Cheirogaleus*.

Although hypotheses regarding mechanisms of species diversification have been tested for the much more speciose mouse lemurs— close relatives of the dwarf lemurs (Wilmé et al., 2006; Yoder and Heckman, 2006)—no comparative studies exist for the genus *Cheirogaleus*, partly because extensive molecular sampling has only recently become available. It is intriguing that three of the four *Cheirogaleus* clades (*C. medius*, *C. major*, *C. crossleyi*) are broadly distributed and occupy a variety of forests from highlands to lowlands, and from eastern to western Madagascar, while the fourth and phylogenetically ancestral clade, *C. sibreei*, appears to be restricted to forest remnants of the central high plateau. *C. medius* is found from several localities in the extreme Southeast (localities 26, 29, 32, 33) along the western coast (localities 37, 38, 2, 4) up to the northern tip of the island (locality 7) and down

the eastern coast to Sambava (locality 9); usually at elevations below 200 m. *Cheirogaleus major* is known from several localities in the extreme Southeast (localities 27–31), along the east coast (localities 24, 21) up north at least to Tampolo (locality 16), with the highest recorded elevation of ~700 m at Marolambo (locality 21). *Cheirogaleus crossleyi* is also found from the extreme Southeast at Lavasoa (locality 33), along the eastern escarpment (localities 25, 23, 19) and northeastern coast (localities 9, 10) up north to Montagne d'Ambre (locality 6) and then down to Ampijoroa on the west coast (localities 3, 39), with an extensive altitudinal range from coastal sites up to ~1700 m (locality 23) (Groeneveld et al., 2009, in press; Hapke et al., 2005).

A number of hypotheses regarding mechanisms of species diversification for Madagascar have been proposed recently. Wilmé et al. (2006) hypothesized that during the periods of cooler and drier climate in the Quaternary, river catchments with sources at low elevations (<2000 m) were zones of isolation and led to locally endemic species, while rivers with sources at higher elevations acted as zones of retreat and dispersal. This model predicts that local endemics will occur at lower elevations separated by major rivers, whereas widely distributed species will be found across the island and thus also at higher elevations, possibly demonstrating an eastern–western connectivity. Rivers as barriers play a central role in the 'watershed hypothesis' and have traditionally been the focus of attention in explaining biogeographic patterns of lemurs (Craul et al., 2007; Ganzhorn et al., 2006; Goodman and Ganzhorn, 2004; Martin, 1972, 1995; Olivieri et al., 2007; Pastorini et al., 2003; Wilmé et al., 2006).

Pearson and Raxworthy (2009) challenged this view and proposed an alternative hypothesis in which diversification is driven by climatic gradients. They predicted areas of local endemism using 'climate clusters' based on 19 bioclimatic variables. Since 'climate clusters' are heavily influenced by altitude, the identified areas of local endemism follow elevational isolines and are oriented roughly perpendicular to those identified by Wilmé et al. (2006). Both models were shown to be congruent with species distributions of different taxa, such as lemurs, day geckos, leaf-tailed geckos, and chameleons. Pearson and Raxworthy (2009) thus argue that there may not be a single explanation for patterns of endemism across taxa or even within taxonomic groups, but rather multiple mechanisms may have driven diversification. Furthermore, we cannot be sure that current distributions are not the product of substantial postspeciation range shifts (Losos and Glor, 2003).

The distribution of *Cheirogaleus* species is not entirely congruent with the predictions of either the 'watershed hypothesis' (Wilmé et al., 2006) or the 'current climate hypothesis' (Pearson and Raxworthy, 2009). In particular, rivers shown to act as barriers in other lemur genera with similar life history traits do not seem to play a role in dwarf lemurs. Nonetheless, the tendency of *C. crossleyi* to be generally found at elevations higher along the eastern escarpment than *C. major* (notwithstanding *C. crossleyi*'s occurrence at some coastal sites in the southeast and northeast and northwest), and of *C. sibreei* to be further restricted to elevations above 1400 m is more readily explained by the 'current climate hypothesis' than the 'watershed hypothesis'. This stands in contrast to the closely related mouse lemurs (genus *Microcebus*), whose distributions have been shown to be congruent with the 'watershed hypothesis' (Wilmé et al., 2006; but see *M. tavaratra* in Pearson and Raxworthy, 2009) Future studies, including more complete field sampling and integration of molecular analysis with topographic (including paleotopographic) and paleoclimatic data should allow testing of other possible mechanisms of species diversification (see Vences et al., 2009) and should allow us to determine which model or models best explain the current distribution of dwarf lemur species.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2010.03.004.

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