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Karyotypic Evolution in Malagasy Flying Foxes (Pteropodidae, Chiroptera) and Their Hipposiderid Relatives as Determined by Comparative Chromosome Painting

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Key Words

Chromosome painting · Hipposideridae · Madagascar · *Myotis myotis* · Pteropodidae

Abstract

Pteropodidae and Hipposideridae are 2 of the 9 chiropteran families that occur on Madagascar. Despite major advancements in the systematic study of the island's bat fauna, few karyotypic data exist for endemic species. We utilized G- and C-banding in combination with chromosome painting with *Myotis myotis* probes to establish a genome-wide homology among Malagasy species belonging to the families Pteropodidae (*Pteropus rufus* 2n = 38; *Rousettus madagascariensis*, 2n = 36), Hipposideridae (*Hipposideros commersoni* s.s., 2n = 52), and a single South African representative of the Rhinolophidae (*Rhinolophus clivosus*, 2n = 58). Painting probes of *M. myotis* detected 26, 28, 28, and 29 regions of homology in *R. madagascariensis*, *P. rufus*, *H. commersoni* s.s, and *R. clivosus*, respectively. Translocations, pericentric inversions, and heterochromatin additions were responsible for karyotypic differences amongst the Malagasy pteropodids. Comparative chromosome painting revealed a novel pericentric inversion on *P. rufus* chromosome 4. Chromosomal characters suggest a close evolutionary relationship between *Rousettus* and *Pteropus*. *H. commersoni* s.s. shared several chromosomal characters with extralimital congeners but did not exhibit 2 chromosomal synapomorphies proposed for Hipposideridae. This study provides further insight into the ancestral karyotypes of pteropodid and hipposiderid bats and corroborates certain molecular phylogenetic hypotheses.

Madagascar boasts a unique chiropteran fauna that includes 3 of the 7 families within the suborder Pteropodiformes: Hipposideridae (Old World leaf-nosed bats), Pteropodidae (Old World fruit bats), and the recently described Rhinonycteridae (formerly included in Old World leaf-nosed bats). Hypotheses concerning the evo-

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lutionary history of certain Malagasy pteropodid and hipposiderid taxa have largely remained ambiguous as phylogenies, in general, were not fully resolved at the generic and species levels for both families [Alvarez et al., 1999; Agnarsson et al., 2011; Almeida et al., 2011; Murray et al., 2011; Foley et al., 2015]. For example, based on morphology the 3 endemic Malagasy pteropodid species are classified into 2 subfamilies: *Eidolon dupreanum*, *Rousettus madagascariensis* in the subfamily Rousettinae, and *Pteropus rufus* in the subfamily Pteropodinae [Bergmans, 1997]. Molecular studies have disputed the morphologically based divisions of the Rousettinae and Pteropodinae and have posed alternative hypotheses concerning the evolutionary relationships among pteropodids. Mitochondrial and nuclear DNA-based analyses are consistent in recognizing *Rousettus* and the Indomalayan *Eonycteris* as sister taxa, to the exclusion of other rousettine genera [Giannini and Simmons, 2005; Almeida et al., 2011]. *R. madagascariensis* is considered the most derived species within this abridged rousettine clade [Almeida et al., 2011] and to be the sister species to *R. obliviosus* of the nearby Comoros Archipelago [Goodman et al., 2010], but it is unclear if these are derived from Asian or African ancestors. Pteropodinae as defined by Bergmans [1997] is polyphyletic, as it includes at least 2 clades that have evolved independently from each other [Almeida et al., 2011]. *Pteropus*, the most speciose pteropodine genus, dispersed to Madagascar from Austral-Asia/Indo-Malaysia via different Indian Ocean islands; the Malagasy species is regarded as a more recently evolved taxon [O'Brien et al., 2009; Chan et al., 2011]. *Eidolon*, a genus composed of 2 species, one from mainland Africa and the other from Madagascar, does not show close evolutionary relationships to any other genus [Almeida et al., 2011].

The Malagasy hipposiderid fauna comprises at least 2 endemic species: *Hipposideros commersoni* s.s., and *Hipposideros cryptovalorona* [Rakotoarivelo et al., 2015; Goodman et al., 2016], taking into account the recent transfer of *Paratriaenops auritus*, *P. furculus*, and *Triaenops menamena* into the family Rhinonycteridae [Foley et al., 2015]. Until recently, intergeneric relationships amongst Hipposideridae largely remained unresolved, as molecular phylogenies were either poorly sampled or were incongruent in describing basal associations [e.g. Jones et al., 2002; Wang et al., 2003; Li et al., 2007; Agnarsson et al., 2011; Murray et al., 2012]. Debate arose from the positioning of the genera *Aselliscus* and *Hipposideros* within the hipposiderid family tree. Phylogenies derived from morphological data placed *Aselliscus* either at the root of the tree [Hand and Kirsch, 1998, 2003] or among *Hipposideros* spp. [Bogdanowicz and Owen, 1998; Hand and Kirsch, 1998]. Recent molecular phylogenies based on mitochondrial DNA (mtDNA) sequence data of Afro-Malagasy taxa showed *Hipposideros* as a lineage in the clade containing the genera *Asellia*, *Coelops*, and *Aselliscus* [Benda and Vallo, 2009]. In contrast, the nuclear DNA-based study of Foley et al. [2015] revealed *Asellia* as the most basal taxon amongst Hipposideridae to the exclusion of the genera comprising the newly erected Rhinonycteridae. This molecular study also supported the paraphyly of the genus *Hipposideros* suggested by previous morphological studies [Sigé, 1968; Legendre, 1982; Bogdanowicz and Owen, 1998; Hand and Kirsch, 1998]. According to the phylogeny of Foley et al. [2015], members of the *H. commersoni* group have closer affinities to *Aselliscus* and *Coelops* than other *Hipposideros* spp and may be best transferred to a different genus.

Karyotypic evolution may advance at a slower pace than nucleotide evolution [Murphy et al., 2004]; thus, chromosomal rearrangements are rare genomic markers capable of retracing common ancestry at different taxonomic levels [Rokas and Holland, 2000]. Chromosomal banding and chromosome painting studies of Chiroptera have implicated Robertsonian (Rb) rearrangements, inversions, and heterochromatin additions in genomic restructuring amongst pteropodids and hipposiderids [Haiduk et al., 1981; Ao et al., 2007; Mao et al., 2007, 2008, 2010; Volleth et al., 2002, 2011]. Painting studies have also identified several clade-specific chromosomal characters in support of molecular hypotheses concerning evolutionary relationships amongst Pteropodidae and Hipposideridae [Ao et al., 2007; Mao et al., 2008, 2010; Volleth et al., 2011]. These studies were based primarily on Indo-Malaysian taxa with no representatives from the Afro-Malagasy region. Hence, the cytosystematics of African taxa relative to those from Asia are poorly understood, and our understanding of karyotypic evolution within the Pteropodidae and Hipposideridae remains limited.

Relative to the families Hipposideridae and Rhinolophidae, the Pteropodidae have been the least studied using chromosome painting techniques. Only 3 species drawn from the subfamilies Cynopterinae and Rousettinae have been examined thus far [Volleth et al., 2002; Ao et al., 2007; Mao et al., 2007]. In this study, we present G- and C-banded karyotypes of Malagasy endemic pteropodids *(P. rufus, R. madagascariensis)* from 2 subfamilies and 1 hipposiderid species (*H. commersoni* s.s., endemic to Madagascar). We included an African representative of the Rhinolophidae, *Rhinolophus clivosus*, for comparative purposes as rhinolophid bats fall within the same su-

Table 1. Chiropteran species investigated in this study

Species name and abbreviation	Locality	GPS coordinates	Sex	2n	FN	Accession number
Rousettus madagascariensis (RMA)	Grotte d'Anjohibe, Province de Mahajanga, Madagascar	15.614°S, 46.928°E	♂	36	66	FMNH 209106
Pteropus rufus (PRU)	Captive in Ambovondramanesy village, near Berivotra, Province de Mahajanga, Madagascar	15.900°S, 46.575°E	♂	38	68	UADBA 43751
Pteropus rufus (PRU)	Captive in Ambovondramanesy village, near Berivotra, Province de Mahajanga, Madagascar	15.900°S, 46.575°E	♂	38	68	UADBA 43763
Hipposideros commersoni s.s. (HCO)	Grotte d'Anjohibe, Province de Mahajanga, Madagascar	15.614°S, 46.928°E	♂	52	60	FMNH 209110
Hipposideros commersoni s.s. (HCO)	Réserve Spéciale d'Ankarana, Province d'Antsiranana, Madagascar	12.942°S, 49.055°E	♀	52	60	FMNH 213588
Rhinolophus clivosus (RCL)	Ferncliffe Nature Reserve, Pietermaritzburg, South Africa	29.550°S, 30.320°E	♀	58	60	DM 12005

DM = Durban Natural Science Museum; FMNH = Field Museum of Natural History, Chicago; UADBA = Université d'Antananarivo, Département de Biologie Animale.

perfamily as hipposiderids (Rhinolophoidea) and share certain chromosomal characters [Volleth et al., 2002; Ao et al., 2007; Mao et al., 2008]. Using chromosome painting with *Myotis myotis* (MMY) as the reference, we establish homology among the Malagasy species relative to their congeners. Secondly, utilizing chromosomal characters identified from published chromosomal maps of extralimital taxa, we infer phylogenomic relationships among Malagasy pteropodids and their hipposiderid and rhinolophid relatives. Thirdly, we tested for the presence of previously described synapomorphic characters proposed for Pteropodidae and Hipposideridae within the genomes of their Malagasy representatives. Our comparative analyses allowed us to revisit recent molecular-based hypotheses concerning evolutionary relationships among Pteropodidae and Hipposideridae.

Materials and Methods

Specimens and Chromosome Preparation

The 4 species used in this study were collected from wild populations on Madagascar and in South Africa (table 1). Specimens were identified using external morphological characteristics (e.g. forearm length) and/or echolocation characteristics [Monadjem et al., 2010; Goodman 2011]. On the basis of molecular genetic analyses conducted by A. Rakotoarivelo and S. Willows-Munro, the 2 specimens of *H. commersoni* (FMNH 209110 and 213588) used in the current study represent *H. commersoni* s.s. rather than a cryptic species currently being described as new to science [Goodman et al., 2016]. Metaphases were obtained from bone marrow preparations or were harvested from actively growing fibroblast cell lines that were established from tail and/or wing membrane biopsies using the methods of Volleth et al. [2009]. G-banding with trypsin followed Seabright [1971], and C-banding of Malagasy taxa used barium hydroxide according to a modified method of Sumner [1972].

Cross-Species Chromosome Painting (Zoo-FISH)

Flow sorted whole chromosome probes comprising 21 autosomes and the X chromosome of *M. myotis* were used for painting [Ao et al., 2006]. They remain the only set of chiropteran probes that have been painted reciprocally to human chromosomes [Volleth et al., 2011], thus allowing comparison of our data to *Homo sapiens* (HSA) syntenic homologies. *Myotis* probes were labelled with biotin-16-dUTP or digoxigenin-11-dUTP (Roche Molecular Chemicals) using DOP-PCR [Telenius et al., 1992] and hybridized to metaphases of the 4 species investigated in this study following procedures previously described [Richards et al., 2010]. Biotin-labelled *Myotis* probes were detected using Cy3-labelled streptavidin (1:500 dilution, Amersham), and Dig-labelled probes were detected with FITC-conjugated sheep anti-dig (1:500 dilution, Amersham). Slides were counterstained with DAPI for 5 min and mounted in antifade (Vectashield, Vector). FISH images were captured using the Genus System version 3.7 (Applied Imaging Corp., Newcastle, UK) with a CCD camera mounted on an Olympus BX 60 epifluorescence microscope. Hybridization signals were assigned to specific chromosomes identified by using inverted DAPI banding patterns.

Chromosome Nomenclature

The G-banded karyotypes of *R. madagascariensis* (RMA) and *P. rufus* (PRU) were arranged according to the scheme for *R. le-*

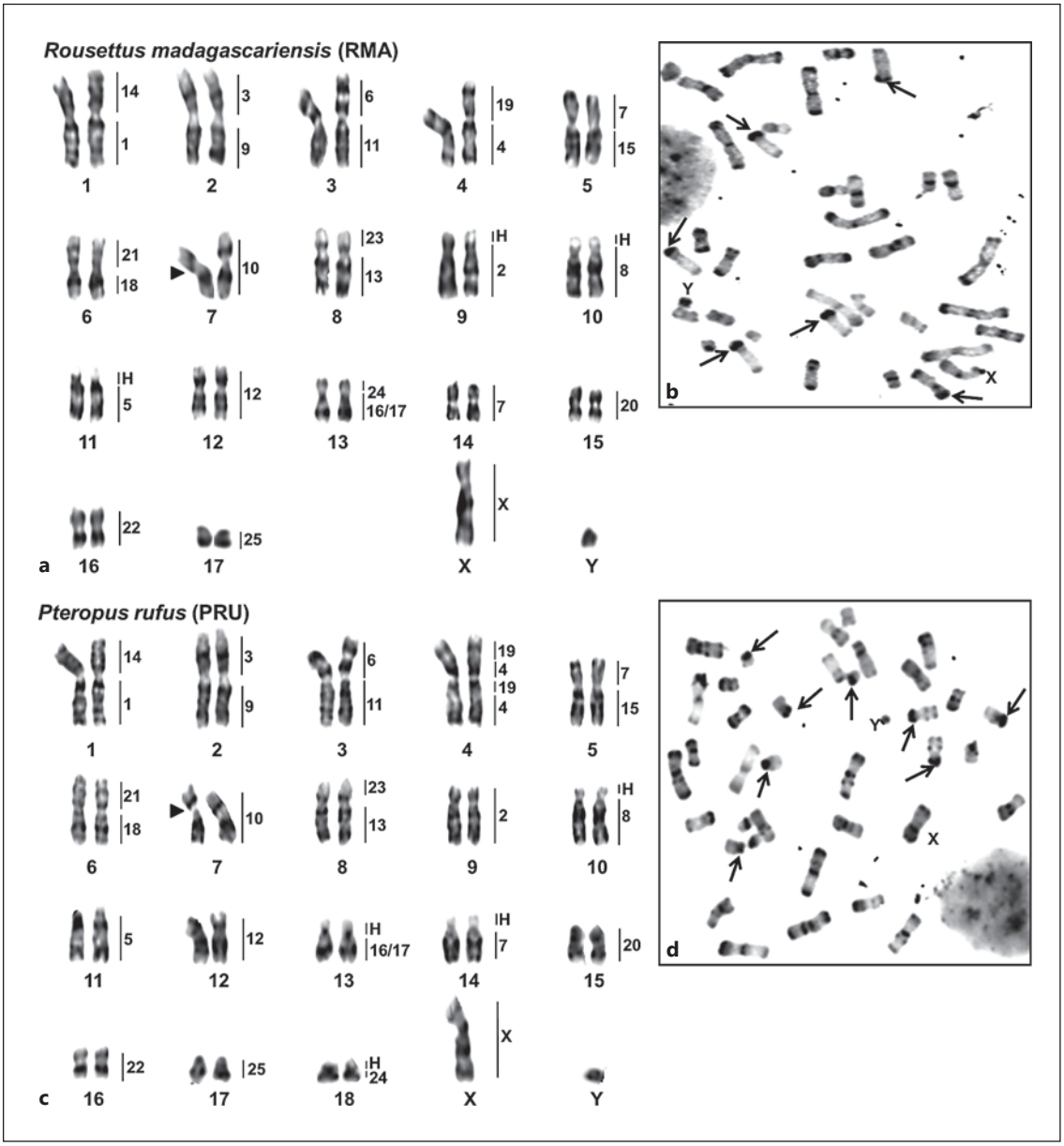

Fig. 1. G-banded karyotypes of *R. madagascariensis* (RMA) (**a**) and *Pteropus rufus* (PRU) (**c**). Chromosomal homologies to *M. myotis* chromosomes are indicated on the right to each chromosome pair. C-banded metaphase spreads of *R. madagascariensis* (**b**) and *P. rufus* (**d**). Arrows indicate C-positive heterochromatic short arms present in the Pteropodidae karyotypes. The gonosomes are indicated by X and Y. Arrows indicate secondary constrictions.

schenaultii by Mao et al. [2007], with chromosomes arranged and numbered according to size. The karyotypes of *H. commersoni* s.s. (HCO) and *R. clivosus* (RCL) followed the scheme of *R. mehelyi* used by Volleth et al. [2002], whereby bi-armed chromosomes are numbered first. To the best of our knowledge, no comprehensive chromosome banding and chromosome painting data exists for the 3 Malagasy species presented herein.

Phylogenomic Comparisons Using Chromosomal Characters

For a more meaningful interpretation of phylogenomic relationships among taxa, we integrated our results with the published comparative maps of an additional 7 Pteropodiformes taxa [Volleth et al., 2002; Ao et al., 2007; Mao et al., 2007, 2008]. We identified chromosomal characters based on G-banded comparisons and *M. myotis* homology. We followed Volleth and Eick [2012] in

distinguishing between Rb fusion products and non-Rb products. The chromosomal characters were overlaid on a phylogenetic tree adapted from the DNA sequence-based phylogenetic reconstructions of Eick et al. [2005], Almeida et al. [2011], and Foley et al. [2015].

Results

Pteropodidae – Karyotypes and Zoo-FISH

R. madagascariensis has a karyotype with 2n = 36, FN = 66 (fig. 1a). The chromosome complement comprises 7 large metacentrics (pairs 1–7), 4 medium-sized submetacentrics (pairs 8–11), 5 pairs of small metacentrics (pairs 12–16), and the single acrocentric pair 17. A secondary constriction appears to be present on the short arm near the centromere of pair 7. The X chromosome is a large submetacentric and the Y is the smallest chromosome and largely heterochromatic. Pairs 9–11 have short arms comprised mostly of heterochromatin (fig. 1b), and all chromosomes contained heterochromatin in the pericentromeric and telomeric regions.

The karyotype of *P. rufus* (2n = 38, FN = 70; fig. 1c) is characterized by 11 pairs of metacentrics, 6 pairs of submetacentrics, 1 pair of acrocentric chromosomes, a large submetacentric X chromosome, and a small acrocentric Y chromosome. Chromosomal pair 7 appears to display a secondary constriction. C-banding analysis revealed the presence of heterochromatic short arms in pairs 10, 13, 14, and 18 (fig. 1d). Heterochromatin was present in the pericentromeric and telomeric regions of all chromosomes, and intercalary heterochromatic bands were detected in at least 3 pairs of bi-armed chromosomes.

The complete suite of *M. myotis* probes successfully hybridized to both pteropodid species, resulting in 26 and 28 regions of homology detected in *R. madagascariensis* and *P. rufus*, respectively (fig. 1a, c). Eight chromosomal pairs of *R. madagascariensis* (1–6, 8, and 13) corresponded to 2 MMY probes whereas only 7 *P. rufus* autosomal pairs (1–6, 8) were highlighted by 2 MMY probes. Hybridization patterns in the 2 pteropodid species differed due to the fusion of chromosomes homologous to MMY16/17 + 24 in RMA13, and the retention of MMY16/17 and 24 as separate chromosomes in *P. rufus* (fig. 2a). Comparative painting analyses of the pteropodids, involving MMY probes 19 and 4, revealed a pericentric inversion in PRU4 in comparison to RMA4, undetectable using G-banding patterns alone (fig. 2b).

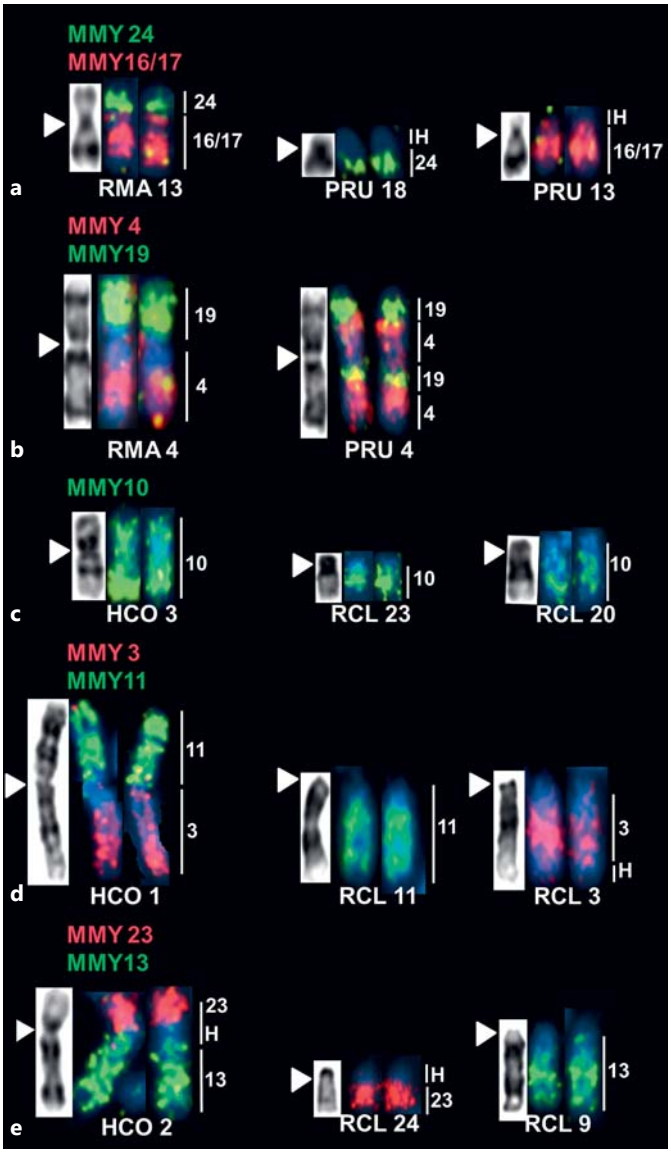

Fig. 2. The results of FISH with *Myotis* (MMY) chromosomal probes onto metaphase chromosomes of *R. madagascariensis* (RMA), *P. rufus* (PRU), *H. commersoni* s.s. (HCO), and *R. clivosus* (RCL). Paints MMY16/17 and 24 revealed a fission and heterochromatic addition in *P. rufus* (**a**). An inversion differentiating *R. madagascariensis* from *P. rufus* was detected using paints MMY4 and 19 (**b**). Hybridization of MMY10 to HCO3 and RCL20 and 23 indicated the fission of MMY10 in the genome of *R. clivosus* (**c**). MMY11 and 3 hybridized to a single chromosomal pair in *H. commersoni* s.s. and 2 separate autosomes in *R. clivosus* (**d**). MMY13 and 23 were retained on a single chromosomal pair in *H. commersoni* s.s. and as 2 separate chromosomes within the genome of *R. clivosus* (**e**). Arrowheads indicate the position of centromeric regions. Chromosomes were counterstained using DAPI, while MMY3, 4, 16/17 and 23 were labelled with biotin and MMY10, 11, 13, 19 and 24 were labelled with Dig paints.

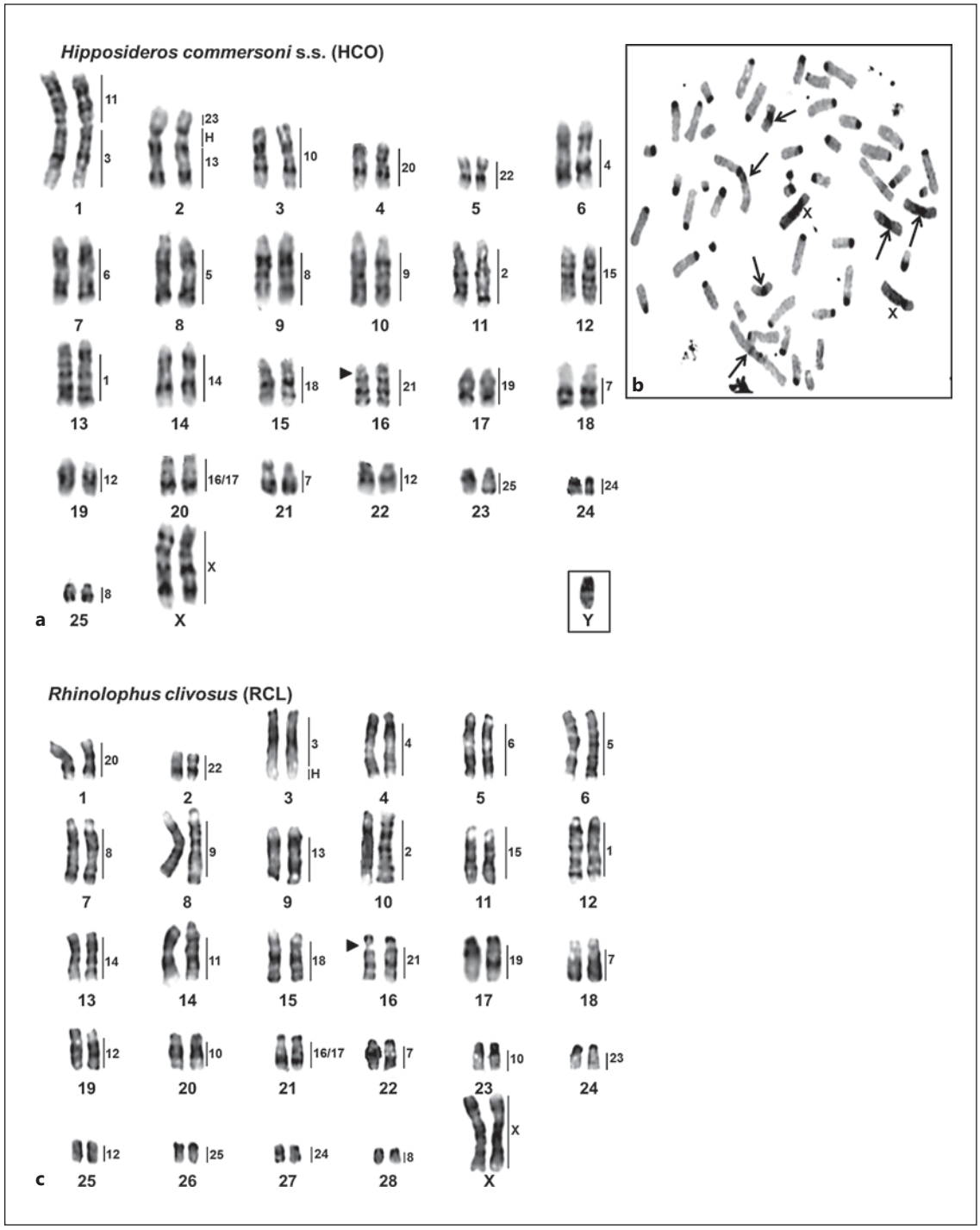

Fig. 3. G-banded karyotypes of *Hipposideros commersoni* s.s. (HCO) (**a**) and *Rhinolophus clivosus* (RCL) (**c**). Chromosomal homologies to *M. myotis* chromosomes are indicated on the right to each chromosome pair. C-banded metaphase spread of the Malagasy *H. commersoni* s.s. (**b**) is provided. The gonosomes are indicated by X and Y. Arrows indicate secondary constrictions.

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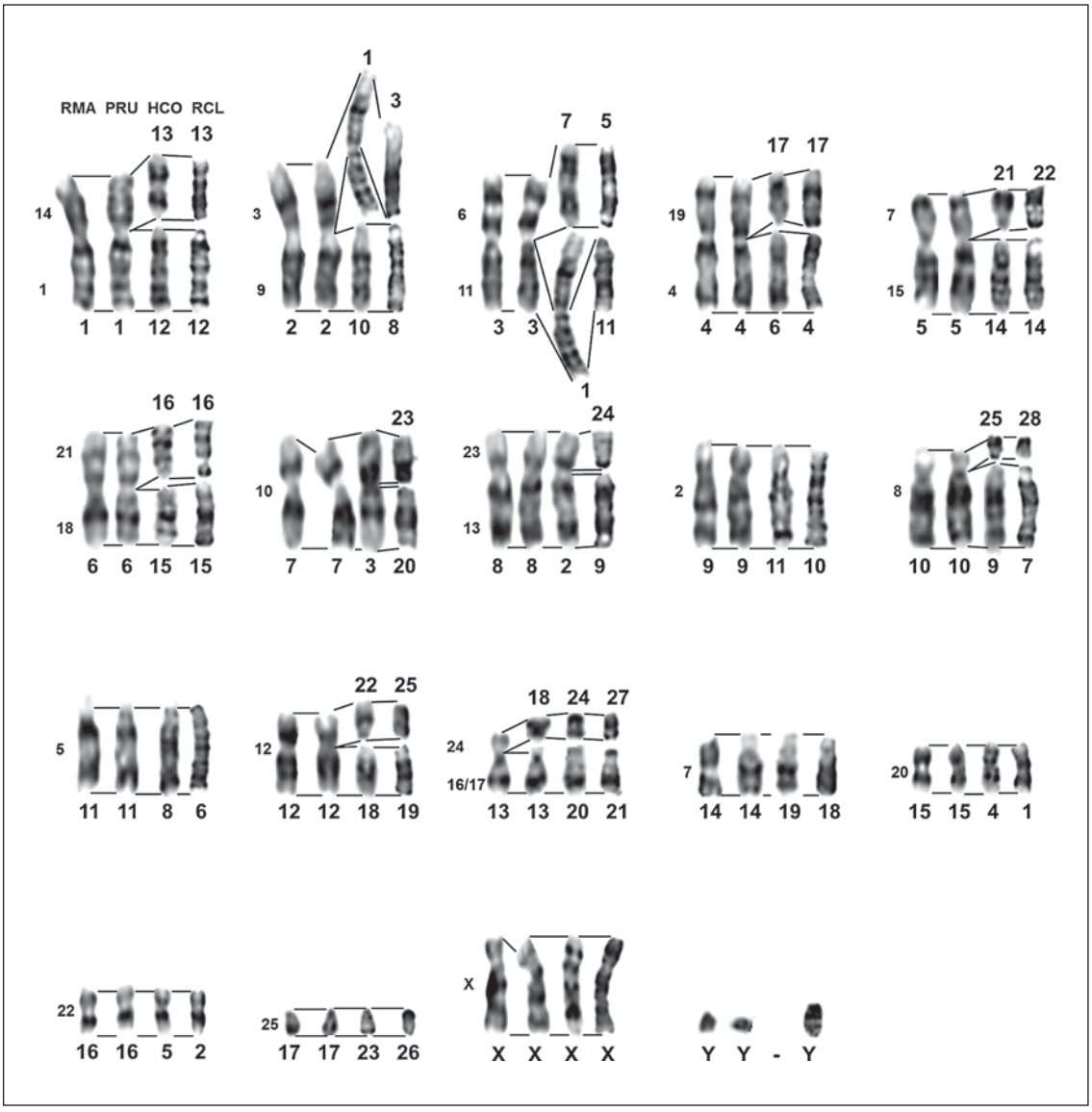

Fig. 4. Genome-wide chromosomal homologies among Afro-Malagasy pteropodid, hipposiderid and rhinolophid bats as directed by *M. myotis* (MMY) chromosome painting probes and G-banding comparison. Chromosome numbers are provided below or above the chromosomes/chromosomal segments of each species. Chromosomal homologies to MMY chromosomes are indicated on the left. *R. madagascariensis* (RMA), *P. rufus* (PRU), *H. commersoni* s.s. (HCO), *R. clivosus* (RCL).

Hipposideridae and Rhinolophidae – Karyotypes and Zoo-FISH

The chromosomal complement of *H. commersoni* s.s. (2n = 52, FN = 60; fig. 3a) comprised mostly acrocentric chromosomes with the exception of pairs 1–5 and the X chromosome. The bi-armed chromosomes consist of a large metacentric (pair 1), a medium-sized submetacentric (pair 2), and 3 pairs of medium- to small-sized metacentrics (pairs 3–5). The X chromosome is a large submetacentric with intercalary bands of heterochromatin (fig. 3a, b). The Y chromosome is acrocentric, comprising of approximately two-thirds heterochromatin. Heterochromatin was concentrated in autosomal centromeres, with the exception of pairs 1–3 where heterochromatin appeared to extend beyond the pericentromeric region (fig. 3b).

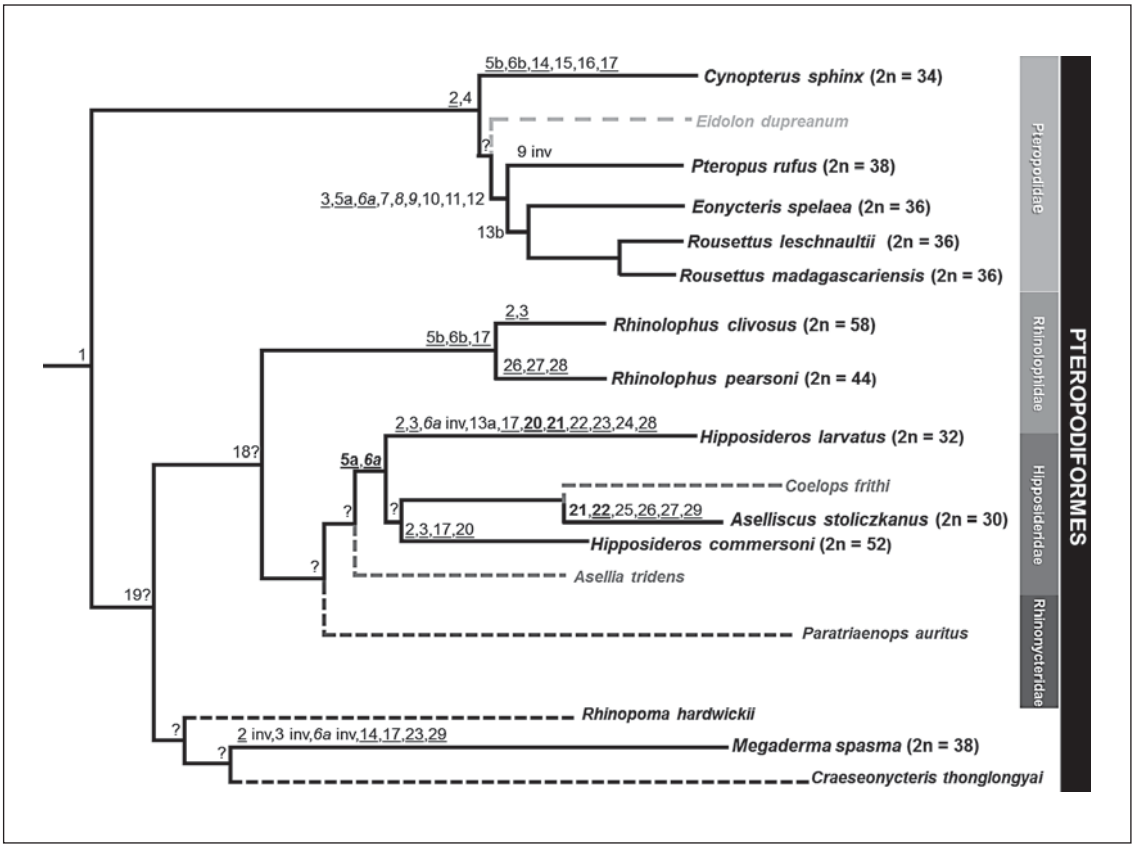

Fig. 5. Molecular DNA sequence-based phylogenetic tree [modified from Eick et al., 2005; Almeida et al., 2011; Foley et al., 2015] with mapped chromosomal characters. Dotted lines indicate the phylogenetic position of Pteropodiformes taxa not listed in table 2 for which chromosome painting data are not yet available. Possible homoplastic characters are underlined. Previously described key features of the hipposiderid ancestral karyotype are indicated in bold. Ancestral chiropteran karyotypic features are in italics. Inv = Inversion/rearranged; ? = possible synapomorphy/synapomorphy not known.

R. clivosus was included in this study as it bears a karyotype similar to *H. commersoni* s.s. The karyotype of *R. clivosus* has a diploid number of 2n = 58 (FN = 60, fig. 3c) and is dominated by acrocentric chromosomes. Two small metacentric pairs (1–2) were present.

Myotis autosomal probes detected 28 regions of homology in the genome of *H. commersoni* s.s. and delimited 29 homologous chromosomal segments in *R. clivosus* (fig. 3a, c). Twenty-three homologous chromosomes/chromosomal segments were shared by the 2 taxa. Thirteen MMY chromosomes, including the X, were conserved in toto in *H. commersoni* s.s., whereas 14 MMY chromosomes were conserved in toto in *R. clivosus*. MMY10 was conserved as a single metacentric autosome in *H. commersoni* s.s., whereas it was separated into 2 separate chromosomes (RCL20 and 23, fig. 2c) in *R. clivosus*. A further 2 fusions involving MMY3 + 11 and MMY13 + 23 differentiated the karyotype of *H. commersoni* s.s. from *R. clivosus* (figs. 2d, e, 3). MMY8 and 12 each corresponded to 2 chromosomes in the genomes of *H. commersoni* s.s. and *R. clivosus*.

Comparative Analyses Based on G- and C-band Homology

Six autosomal pairs corresponding to MMY2, 5, 7i (see Volleth et al. [2011] for a detailed description of MMY7 partial chromosomal arms), 20, 22 and 25 were shared as one entity amongst the species investigated in this study (fig. 4). The banding patterns of 3 homologous chromosomes (MMY20, 22, 25) were unaltered, suggesting that they may represent ancestral elements of the suborder Pteropodiformes. Our analyses also revealed differences in the structure and/or banding of patterns of MMY7i homologues in the Afro-Malagasy species. In *Rousettus*,

the MMY7i homologue is bi-armed, whereas this element is acrocentric in the remaining species. Differences in the position of the centromere in chromosomes corresponding to MMY2 were also apparent. The banding pattern of PRU14 differed slightly from the hipposiderid and rhinolophid bats, suggestive of a paracentric inversion. G- and C-banding analyses revealed possible paracentric inversions on both the short and long arms of the X chromosomes. Two autosomal pairs homologous to MMY10 and the fusion chromosome of MMY13 + 23 were retained within the genomes of the pteropodid and hipposiderid taxa, but not *Rhinolophus*. Banding patterns within the p arm of the chromosomes homologous to MMY13 + 23 were conserved between *H. commersoni* s.s. and both pteropodid species (fig. 4). Two elements of MMY8 and 12 were present within the hipposiderid and rhinolophid species, as was a secondary constriction within the pericentromeric region of MMY21. Marker chromosomes bearing a secondary constriction and corresponding to MMY10 were also identified within the karyotypes of the Malagasy pteropodids.

Phylogenomic Relationships Based on Chromosomal Characters

Our chromosome painting data of *R. madagascariensis*, *P. rufus*, *H. commersoni* s.s. and *R. clivosus* were interpreted in the context of 7 additional taxa that have been analyzed using chromosome painting techniques. We identified 32 chromosomal characters, summarized in table 2 and overlaid on a DNA sequence-based phylogeny (fig. 5), that were used to describe phylogenomic relationships amongst Pteropodiformes taxa previously studied using chromosome painting. Dotted lines indicate the position of taxa not included in previous chromosome painting analyses. Rb rearrangements involving different arm combinations resulted in few shared chromosomal rearrangements across all species, apart from the presence of 2 elements homologous to MMY7, which was recorded in all species.

Possible plesiomorphic characters included the bi-armed state of MMY20 and 22 and the fusion product of MMY13 + 23. The synteny of MMY13 + 23 was conserved amongst 3 pteropodid genera, all 3 hipposiderid species, and the single species belonging to the family Megadermatidae, albeit in slightly different combinations. Chromosomes homologous to MMY10 were conserved as a single element within the karyotypes of *R. madagascariensis*, *R. leschenaulti*, *P. rufus*, *Eonycteris spelaea*, *Aselliscus stoliczkanus*, *H. larvatus*, and *H. commersoni* s.s. Very few chromosomal characters were common across all analyzed pteropodid taxa. The secondary constriction present on chromosomes or chromosomal segments homologous to MMY10 was the only character shared exclusively among all pteropodids, including the Malagasy representatives. Six chromosomal characters, each representing a centric fusion, were common to the genera *Rousettus*, *Pteropus*, and *Eonycteris*: MMY1 + 14, 3 + 9, 4 + 19, 6 +11, 7 + 15, and 18 + 21. Homologues to MMY16/17 + 24 were present in different combinations within the genomes of the genera *Eonycteris*, *Rousettus,* and in *H. larvatus*. This fusion product was not present in genomes of *P. rufus*, *Cynopterus sphinx*, *H. commersoni* s.s., *A. stoliczkanus*, *Megaderma spasma,* and the 2 *Rhinolophus* spp. (table 2). The fission state of the MMY8 homologue and the secondary constriction on chromosomes homologous to MMY21 were present in Hipposideridae and Rhinolophidae. Our comparative analyses failed to identify synapomorphic characters for Hipposideridae. The fusion product of MMY3 +11 represented the only chromosome limited to *Hipposideros* spp. Similarly, the fission of MMY12 was a feature common to only the *Hipposideros* spp., and not *Aselliscus*. MMY20 and 22 homologues and the fission of MMY12 were also present in the karyotype of *R. clivosus*.

Discussion

Karyotypic Evolution among Malagasy Pteropodids

We present the first painting analysis of a member of the Pteropodinae, *P. rufus*. To date, karyotypic data for *Pteropus* spp. are largely derived from conventionally stained karyotypes [Harada and Tsuneaki 1980; Hood et al., 1988; Rickart et al., 1989]. Due to the inadequacy of conventional cytogenetic studies in delimiting chromosomal rearrangements, karyotypic comparisons between *Pteropus* and other pteropodid genera have remained incomplete. Despite an overall similarity in diploid numbers of *P. rufus* (2n = 38) and *R. madagascariensis* (2n = 36), our chromosome painting analyses with *M. myotis* revealed several karyotypic differences between the Malagasy species. Chromosomal rearrangements responsible for differences in diploid number and fundamental number between Malagasy pteropodids included a single chromosomal fusion, 2 pericentric inversions, and heterochromatic polymorphisms on 4 homologous chromosomal pairs. Such rearrangements as mentioned before have been implicated in the genome evolution of African pteropodids [Haiduk et al., 1981].

Table 2. Chromosomal characters represented among 11 Pteropodiformes taxa from 4 families

Character	Character description	Pteropodidae					Hipposideridae			Rhinolophidae		Megadermatidae
		CSP[a] 2n = 34	ESP[b] 2n = 36	RLE[c] 2n = 36	RMA[d] 2n = 36	PRU[d] 2n = 38	AST[a] 2n = 30	HLA[b] 2n = 32	HCO[d] 2n = 52	RPE[c] 2n = 44	RCL[d] 2n = 58	MSP[e] 2n = 38
1	Fi 7	+	+	+	+	+	+	+	+	+	+	+
2	Fu 22	+	+	+	+	+	-	+	+	-	+	+
3	Fu 20	–	+	+	+	+	–	+	+	–	+	+ inv
4	SC 10	+	+	+	+	+	–	–	–	–	–	–
5a	Fu 10	–	+	+	+	+	+	+	+	–	–	–
5b	Fi 10	+	–	–	–	–	–	–	–	+	+	+
6a	RF 13 + 23	–	+	+	+	+	+	+ inv	+	–	–	+ inv
6b	Fi 13 + 23	+	–	–	–	–	–	–	–	+	+	–
7	RF 1 + 14	–	+	+	+	+	–	–	–	–	–	–
8	RF 3 + 9	–	+	+	+	+	–	–	–	–	–	–
9	RF 4 + 19	–	+	+	+	+ inv	–	–	–	–	–	–
10	RF 6 + 11	–	+	+	+	+	–	–	–	–	–	–
11	RF 7 + 15	–	+	+	+	+	–	–	–	–	–	–
12	RF 18 + 21	–	+	+	+	+	–	–	–	–	–	–
13a	RF 16/17 + 24	–	–	–	–	–	–	+	–	–	–	–
13b	Fu 16/17 + 24	–	+	+	+	–	–	–	–	–	–	–
14	RF 3 + 7ii	+	–	–	–	–	–	–	–	–	–	+
15	RF 4 + 6	+	–	–	–	–	–	–	–	–	–	–
16	RF 8i + 15	+	–	–	–	–	–	–	–	–	–	–
17	Fi 12	+	–	–	–	–	–	+	+	+	+	+
18	SC 21	–	–	–	–	–	+	+	+	+	+	–
19	Fi 8	–	–	–	–	–	+	+	+	+	+	+
20	RF 3 + 11	–	–	–	–	–	–	+	+	–	–	–
21	RF 7i + 19	–	–	–	–	–	+	+	–	–	–	–
22	RF 8ii + 14	–	–	–	–	–	+	+	–	–	–	–
23	RF 4 + 18	–	–	–	–	–	+	–	–	–	–	+
24	RF 7ii + 21	–	–	–	–	–	–	+	–	–	–	–
25	RF 3 + 16	–	–	–	–	–	+	–	–	–	–	–
26	RF 1 + 16/17	–	–	–	–	–	+	–	–	+	–	–
27	RF 3 + 15	–	–	–	–	–	+	–	–	+	–	–
28	RF 4 + 5	–	–	–	–	–	–	+	–	+	–	–
29	RF 4 +18	–	–	–	–	–	+	–	–	–	–	+

Characters are described based on *M. myotis* homologies. MMY chromosomal segments as according to Volleth et al. [2011]: 7i = HSA 19/8/4 homologous segments; 7ii = HSA 5 homologous segment; 8ii = HSA 7/5 homologous segments.

CSP = *C. sphinx*; ESP = *E. spelaea*; RLE = *R. leschenaulti*; RMA = *R. madagascariensis*; PRU = *P. rufus*; AST = *A. stoliczkanus*; HLA = *H. larvatus*; HCO = *H. commersoni* s.s.; RPE = *R. pearsoni*; RCL = *R. clivosus*; MSP = *M. spasma*; Fi = fission; Fu = fusion; inv = inversion/rearranged state; RF = Robertsonian fusion; SC = secondary constriction.

[a] Ao et al. [2007]. [b] Volleth et al. [2002]. [c] Mao et al. [2007]. [d] This study. [e] Mao et al. [2008].

Phylogenomic Relationships amongst Pteropodidae

Chromosomal characters based on G-banded comparisons and chromosome painting analyses were used to assess the phylogenomic relationships amongst 5 pteropodid species including the Malagasy representatives studied herein. The character common to all pteropodid species, to the exclusion of other Pteropodiformes taxa, was the secondary constriction present within chromosomes/chromosomal segments homologous to MMY10 (table 2). This chromosome was conserved as a single element within the karyotypes of *R. madagascariensis*, *R. leschenaultii*, *P. rufus*, and *E. spelaea*. Homologues to MMY10 appear as 2 elements on separate bi-armed chromosomes in *C. sphinx*, one of which bears a secondary

constriction adjacent to the pericentromeric region (CSP4) [Ao et al., 2007]. Such marker chromosomes displaying a secondary constriction have been reported from most karyotype studies of pteropodids, with the exception of *Scotonyteris ophiodon* [Haiduk et al., 1981], now generally placed in the genus *Casinycteris* [Hassanin et al., 2015]. A study of 10 Philippine pteropodids revealed the secondary constriction to correspond to nucleolar organizer regions [Rickart et al., 1989]. Additional investigations using silver-staining and/or hybridization experiments with rDNA probes are needed to determine whether this is the case for Malagasy pteropodids.

Six chromosomal characters, each representing a centric fusion, were common to the genera *Rousettus*, *Pteropus*, and *Eonycteris*: MMY1 + 14, 3 + 9, 4 + 19, 6 + 11, 7 + 15, and 18 + 21. Three fusion products corresponding to MMY3 + 9 (HSA 6), 4 + 19 (HSA3 + 21), and 13 + 23 (HSA11) (HSA homology based on Volleth et al. [2002, 2011]), represent conserved elements within the Eutherian ancestral karyotype [Robinson and Ruiz-Herrera, 2008; Ruiz-Herrera et al., 2012]. Within Chiroptera, these 3 chromosomal features have only been reported in synteny from pteropodids [see Volleth et al., 2011] with the exception of *C. sphinx* [Ao et al., 2007]. Comparisons between the G-banded karyotypes of *R. madagascariensis* and *P. rufus* (this study) and published karyotypes of *E. spelaea* [Volleth et al., 2002; Volleth, 2013] and *R. leschenaultii* [Mao et al., 2007] revealed that the banding patterns of chromosomes homologous to MMY3 + 9 and 13 + 23 were conserved across all taxa. Previous painting studies have demonstrated MMY19 homologous sequences in the p arm and MMY4 homology in the q arm of pteropodids [Volleth et al., 2002]. However, a more recent study has revealed that a small segment homologous to MMY4 extends into the proximal portion of the p arm of *E. spelaea* [Volleth et al., 2011]. Our painting data revealed the MMY4 + 19 homology of *P. rufus* chromosomal pair 4 as a distinct and derived state, with homologous elements of MMY4 + 19 located in both the p and q arms of PRU4. Experiments with human painting are needed to confirm the chromosomal segmental order within PRU4.

The non-centric fusion of homologues to MMY16/17 + 24, or Rb fusion coupled with a centromere shift, appears to be characteristic of the *Eonycteris* and *Rousettus* genera and may possibly represent a shared feature of this rousettine clade. This study and other published chromosomal data [Volleth et al., 2002; Mao et al., 2007] thus support the molecular hypothesis of a close association between *Eonycteris* and *Rousettus* (Rousettinae).

Phylogenomic Relationships between H. commersoni s.s. and Other Hipposiderids

Karyotype analyses of hipposiderids revealed diploid numbers varying between 2n = 30–52, with most species exhibiting a bi-armed karyotype of 2n = 32 [see reviews of sreepada et al., 1993; Bogdanowicz and Owen, 1998]. *H. commersoni* s.s., endemic to Madagascar and forming a species complex with at least 2 other large-bodied African forms, *H. gigas* and *H. vittatus*, exhibits an atypical diploid number of 2n = 52 (*H. commersoni* s.s., Volleth et al. [2011]; *H. gigas* from Central and Western Africa, Koubínová et al. [2010], Porter et al. [2010]; *H. vittatus* from southern Africa, Rautenbach et al. [1993]). Our understanding of karyotypic evolution within the family remained limited as only species with 2n = 30 *(A. stoliczkanus)* [Ao et al., 2007] and 2n = 32 *(H. armiger, H. larvatus, H. pomona, H. pratti)* [Mao et al., 2010] were studied using chromosome painting techniques.

Despite limited taxon sampling, several synapomorphies have been proposed for Hipposideridae based on findings of chromosome painting analyses using human, *Myotis,* and *Aselliscus* probes [see Volleth et al., 2002; Ao et al., 2007; Mao et al., 2010]. The syntenic associations of MMY8ii + 14 (homologous to HSA5 + 7 + 9) and MMY7i + 19 (HSA3 + 19 + 8 + 4) proposed synapomorphies of Hipposideridae [Volleth, 2013], were not present in the genome of *H. commersoni* s.s. However, 2 chromosomes corresponding to the homologues of MMY10 and 13 + 23, considered key features of the ancestral karyotype of Hipposideridae, were conserved as bi-armed elements in *H. commersoni* s.s. Importantly, MMY13 + 23, equivalent to HSA11 and a postulated symplesiomorphy of Eutheria [Robinson and Ruiz-Herrera, 2008; Ruiz-Herrera et al., 2012], was also present in 4 of the 5 pteropodid species analyzed thus far, including the Malagasy taxa.

Ao et al. [2007] proposed *A. stoliczkanus* (AST) as the likely basal taxon within Hipposideridae as this species shared plesiomorphic chromosomal characters with pteropodids, including the retention of MMY10 and 12 as bi-armed elements and the arrangement of MMY23 on the p arm of AST11. *Hipposideros* spp. studied previously using chromosome painting displayed an altered G-banding pattern based on one or more paracentric inversions in the p arm of chromosomes homologous to AST11 [Mao et al., 2010]. Our results, however, show that the G-banding pattern in the p arm of HCO2, unlike other *Hipposideros* spp. studied so far, was the same as that of AST11 and pteropodids considered to display the ancestral condition. The fusion product of MMY3 + 11 repre-

sented the only chromosome limited to *Hipposideros* spp. Similarly, the retention of 2 chromosomal elements corresponding to MMY12 was a feature common to only the *Hipposideros* spp. and not to *Aselliscus*. However, the fusion of MMY16/17 + 24 within *Hipposideros* spp., to the exclusion of *H. commersoni* s.s., bears a different banding pattern to that seen in *Eonycteris* and *Rousettus* and is suspected to have arisen via a pericentric inversion [see Volleth et al., 2002]. This fusion product was also not present in the genome of *A. stoliczkanus* [Ao et al., 2007]. The retention of MMY16/17 + 24, MMY8ii + 14, and MMY7i + 19 on separate chromosomes in the genome of *H. commersoni* s.s., coupled with the G-banding pattern on the p arm of HCO2, lends support to the hypothesis that the genus *Hipposideros* is paraphyletic and that the *H. commersoni* s.s. species group would be better placed in a different genus [Foley et al., 2015].

Based on mtDNA molecular data, Benda and Vallo [2009] suggest that *A. stoliczkanu*s occupies a terminal branch in a clade containing the genera *Asellia* and *Coelops*, representing the successive lineage to *Hipposideros*. However, the nuclear DNA study of Foley et al. [2015] provided evidence for the recognition of *Asellia* as the most basal taxon in Hipposideridae s.s., followed by a clade comprising *Aselliscus*, *Coelops*, and the large Malagasy *H. commersoni* and African *H. vittatus* characterized by 2n = 52. Other molecular phylogenies also place the 2n = 52 *Hipposideros* spp. basal to other Afro-Malagasy *Hipposideros* spp. displaying 2n = 32 karyotypes [Eick et al., 2005; Vallo et al., 2008; Monadjem et al., 2013]. A largely acrocentric chromosomal complement has been postulated as ancestral for both the Hipposideridae [Bogdanowicz and Owen, 1998] and the Rhinolophidae [Mao et al., 2007]. Our data indicate that *H. commersoni* s.s. shares several chromosomal features with both the Pteropodidae (e.g. bi-armed state of MMY10 and MMY13 + 23) and the Rhinolophidae and Megadermatidae (bi-armed state of MMY20 and 22 and the disruption of MMY8 and 12). The disrupted synteny of MMY12, retention of MMY20 and 22 homologues as whole chromosomes, and a secondary constriction on the MMY21 homologue were features present in the karyotype of *H. commersoni* s.s., other hipposiderids, and *R. clivosus*. Hence, the karyotype of *H. commersoni* s.s. appears to be more representative of the ancestral hipposiderid chromosomal complement. The above-mentioned data bring into question the supposition that *A. stoliczkanus* possesses the most primitive hipposiderid chromosomal complement [Ao et al., 2007].

The inclusion of other 2n = 52 species, such as *H. gigas* and *H. vittatus*, in future painting studies of Hipposideridae may provide further evidence that corroborate our findings and those of recent molecular investigations [Foley et al., 2015]. Comprehensive painting studies of true hipposiderid genera are needed to provide conclusive resolution of intergeneric phylogenomic relationships within the hipposiderid family at large. In addition, detailed comparative maps of members of the closely related Rhinonycteridae may allow for further inferences regarding the evolutionary history of the Hipposideridae.

Conclusion

By expanding chromosome painting studies of Pteropodidae and Hipposideridae to include Malagasy endemic species, we have refined our knowledge of the phylogenomic relationships among the 2 families and the chromosomal characters that have played an important role in their karyotypic evolution. Our results confirm Rb rearrangements as an important mode of karyotype evolution in Chiroptera. Despite the limitations of these rearrangements in resolving interfamilial relationships amongst bats due to widespread convergent events [Mao et al., 2008], we found these characters (chromosomal fusion and fission events) to be useful in inferring phylogenetic relationships at the generic level. Our study also highlights the utility of inversions in phylogenomic studies of Pteropodiformes taxa. Further Zoo-FISH with HSA paints and probes derived from species with fragmented karyotypes are necessary to resolve the segmental associations of certain chromosomal elements within the karyotypes of the species studied herein. These include clarifying the structural composition of PRU4 and determining whether the paracentric inversion within the MMY2 homologous segment, a suggested synapomorphy for Pteropodiformes, is present within the Malagasy taxa.

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Statement of Ethics

The experiments comply with the current laws of the country in which they were performed. All institutional and national guidelines for the care and use of laboratory animals were followed. Bat capture and euthanasia were conducted according to ethical guidelines of the American Society of Mammalogists [Sikes et al., 2011] and with the approval of the Animal Ethics Committee of the University of KwaZulu-Natal. Voucher specimens were deposited in the Durban Natural Science Museum, South Africa; the Field Museum of Natural History, Chicago, USA; or Université d'Antananarivo, Département de Biologie Animale, Antananarivo, Madagascar. Research permits were obtained from the Direction des Eaux et Forêts and Madagascar National Parks in Madagascar and eZemvelo KZN Wildlife in South Africa.

Disclosure Statement

The authors declare no conflicts of interest.

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