Cryptic Diversity in Common Mustached Bats Pteronotus cf. parnellii (Mormoopidae) in French Guiana and Brazilian Amapa

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Keywords: cryptic species – bioacoustics – mitochondrial DNA – Guianan Shield

Short running title:
Common Mustached Bats in the Guianan Shield

Abstract

The Common Mustached Bat (*Pteronotus parnellii*) is a mormoopid bat living in caves in lowland rainforests throughout the north and eastern Neotropics, including several Caribbean islands. Recent studies have shown that this taxon is certainly a composite of several cryptic species, especially in the western part of the Guiana Shield, where molecular reconstructions and bioacoustics point to the presence of at least two cryptic species that may not be related to genuine *P. parnellii*, native of Jamaica. The current taxonomy of this species complex is therefore confused. We examined here over 200 bioacoustically identified individuals to show that two phonic types live in sympatry in French Guiana with no overlap in frequencies of echolocation calls. Morphologic variation showed consistent and significant differences between the two phonic types, but external measurements were unable to discriminate all bats. Two mitochondrial markers analyzed in a selection of each of these phonic types were further used to evidence that they represent two genetically discrete groups, and to assign them to the existing molecular clades described elsewhere. Molecular comparisons with reference specimens sampled near the type localities of *P. parnellii* and *P. rubiginosus* further suggest that the 53 kHz phonic type found in French Guiana and Amapa (Brazil) should be assigned to the later species, while the 59 kHz phonic type represents an undescribed species.

Keywords: cryptic species – phonic type – mitochondrial – Guianan Shield

Introduction

Mormoopidae is a widely distributed family of Neotropical insectivorous bats ranging from the southwestern United States to southern Brazil, including many islands in the Antilles. The Mormoopidae is composed of two genera, *Mormoops* and *Pteronotus*, and 10 species (Simmons, 2005). One species, the Common Mustached Bat (*Pteronotus parnellii*) is the only high-duty cycle echolocating bat of the Neotropics, i.e. one that

uses harmonics of Constant-Frequency (CF) calls to orientate themselves (Schnitzler and Kalko, 1998). This species was described by Gray (1843) from animals originating in Jamaica, but it is traditionally viewed as a widespread species living across most of the Antilles, Central and tropical South America (e.g. Simmons, 2005). Eight subspecies have been described throughout this wide range, including some former continental species that are now considered as synonyms of *P. parnellii* (reviewed in Herd, 1983, and Simmons, 2005).

However, recent phylogenetic studies revealed that populations of the Common Mustached Bat (CMB) are not homogeneous and bear much hidden biodiversity across their geographic range. Using sequences of the Cytochrome b gene (Cyt b), Lewis-Oritt *et al.* (2001) showed that animals from the type locality in Jamaica are genetically very distinct (11 % sequence divergence) from specimens in Suriname or Guyana, while similar levels of divergence separate some bats from Suriname and those from Guyana or Mexico. By using an improved geographic sampling and combining mitochondrial and nuclear genes, Davalos (2006) confirmed the large molecular divergence between *P. parnellii* sampled in the Antilles (Jamaica, Puerto Rico and Hispaniola), and mainland lineages. One of these divergent lineages was located in French Guiana and Suriname, and the other in Mexico, Honduras and Guyana. Based on genetic, morphological and distributional data, Davalos (2006) and Van Den Bussche and Weyandt (2003) proposed that the mainland CMB might represent several cryptic species that are not conspecific with the true Jamaican *P. parnellii*. Davalos (2006) further suggested that the name *P. rubiginosus* (Wagner, 1843) would be appropriate to name the continental lineage represented by CMB from French Guiana, Guyana, Suriname, Mexico and Honduras. More recently, Clare *et al.* (2013) analyzed mitochondrial and nuclear gene variation together with morphological and some acoustic data to show that the CMB found in Central and South America represent at least three or four independent, biological species, and confirmed that the name *P. parnellii* should be restricted to bats from Jamaica and perhaps other Greater Antilles islands. These authors further used DNA barcodes of the Cytochrome oxydase 1 gene (CO1) to circumscribe four major clades of continental CMB that may represent each a distinct species. The correlation of these molecular clades with morphological characters was good, but none of the individual

measurements were able to discriminate all clades. Using this integrated approach, Clare *et al.* (2013) reached the following taxonomic conclusions for continental taxa: (i) their clade 1 (or Group 1) is comprised of all CMB sampled in Central America (from Panama to southern Mexico), are relatively small (mean forearm length 59.9 mm), emit CF echolocation calls at about 62 kHz and could be named *P. mesoamericanus*. (ii) their clade 2 (Group 2) is comprised of CMB sampled in Venezuela, Trinidad and western Guyana, are medium-sized (mean FA 62.4 mm), and emit CF calls at about 59 kHz (although no calls from continental animals could be obtained), and would be an unnamed species (*P. sp2*). (iii) their clade 3 (Group 3) includes specimens from Guyana and Suriname, are medium-sized (mean FA 63.2 mm), and represent most likely another unnamed species (*P. sp3*);. (iv) their clade 4 (Group 4) includes specimens from the same area as those of clade 3 (Guyana and Suriname), but are significantly larger (mean FA 65.2 mm), and represent a forth unnamed species (*P. sp4*). The echolocation calls recorded from free-flying CMB from Guyana had CF at about 53-54 kHz, but as no match with genetically identified animals could be obtained, Clare *et al.* (2013) were unable to associate these call characteristics to either clade 3 or clade 4.

In this study, we describe the bioacoustic variation of over 200 CMB sampled in French Guiana and nearby Amapa State in Brazil, to show that two distinct phonic types live in sympatry in extended parts of this geographic range. We also characterize the molecular identity of these two phonic types and link them to published samples or clades to reach informed taxonomic recommendations. Morphological characters, although not completely discriminant between the two phonic groups, are also described to provide an aid for their identification in the field or for museum specimens.

Materials and methods

Sampling

Mormoopid bats were sampled in 14 localities of French Guiana and Amapa (Fig. 1), from which we collected data of either or all of bioacoustics, external and cranial morphometry, and molecular systematics.

Capture methods included essentially mist nets set either at the entrance of caves or across nearby corridors such as trails in the forest. Mist nets of 2.6 × 6 m and 2.6 x 10 m were employed at ground level. In addition, we used at Cacao (locality 3 in Fig. 1) a three-frame harp trap (AUSTBAT Research Equipment, Victoria, Australia), with a catching surface of 1.0 m2 erected across a trail acting as a corridor for bats flying out of a nearby cave system.

External measurements and ultrasonic calls were recorded for all captured bats after which they were released in the same spot. A small biopsy punch (Worthington and Barratt, 1996) used for DNA analyses was also taken from some individuals prior to release, and a small selection of specimens were preserved as scientific vouchers for further cranial measurements. These vouchered specimens were euthanized, fixed in 10% buffered formalin and finally stored in 70% ethanol. Appendix 1 lists the origin and institutions housing these vouchers.

Morphology

Gender and reproductive status were acquired from external characteristics (e.g. enlarged nipples or testis) or from gross examination of dissected specimens (Racey, 2009). All specimens reported here were adults as indicated by completely fused phalangeal epiphyses. The following external measurements were taken with a dial caliper accurate to 0.1 mm: forearm length (FA), metacarp of third digit (MC3), metacarp of 4 digit (MC4), total length of fourth digit (D4), hindfoot length excluding claw (HF), tibia length (TI), and ear length from basal-ventral notch of the pinna to its tip (Ea). Digit measurements were taken from the wing held flat on a solid surface and excluding wrist.

Skull and dental measurements were taken with a dial caliper (accurate to 0.05 mm) following the methods detailed in Gutierrez and Molinari (2008) and Simmons and Voss (1998) except when noted: greatest length of skull (bone-to-bone: GLS) and greatest length of skull including the upper incisors (GLSI), braincase depth (BRD), maxillary toothrow length (MTL), condylo-incisive (CIL) and condylo-canine lengths (CCL), palatal length (distance between the posterior palatal notch and the external border of the incisives PL), length of upper molars (M1M3), zygomatic breadth (ZB), mastoid breadth

(MB), breadth across molars (BaM), mandibular toothrow length (MDT), mandibular condylo-canine length (MCC), coronoid height of mandible (CRH). For comparing the French Guianan material with topotypic material of *P. rubiginosus*, we examined a museum specimen (MZUSP-35152) caught at Cuiabá, Mato Grosso, Brazil. This city is located 180 km east-north-east from Cáceres, which is close to the type-locality of *P. rubiginosus* defined by Gardner (2007) as being at 16°04' S, 57°43' W.

To differentiate both phonic types, we performed with Statistica 6.0 (StatSoft Inc., USA) two discriminant function analyses (DFA), one based on three external measurements taken in all 201 recorded bats (FA, MC3 and MC4), and one based on 14 cranial (M1M3, GLS, GLSI, CIL, CCL, ZB, MB, MTL, BaM, CRH, PL, MDT, MCC, BRD) measurements of a subset of 23 vouchered specimens. Each bat was classified a priori according to its recorded phonic type (53 or 59 kHz), except for the Mato Grosso specimen which was left unclassified prior to analyses. To reduce the number of cranial variables entered into the discriminant function, we computed a stepwise discriminant function, where only those first variables contributing the most to the discrimination of the two groups are entered. Univariate statistical tests for comparing measurements or acoustic calls between groups included Mann–Whitney nonparametric test, as implemented by the software PAST (Hammer *et al.*, 2011).

Bioacoustics

Echolocation calls were recorded with Pettersson bat detectors (models D240X, D500X, D1000X: Pettersson Elektronik AB, Uppsala, Sweden) and stored using a Roland - R-05 recorder. Calls were analyzed with the program BatSound Pro 3.31 (Pettersson Elektronik AB, Uppsala, Sweden) based on spectrograms with Hanning window at a sampling rate of 44,100 Hz and Fast Fourier Transformation (FFT) size of 512. Part of the sonograms were also created with R 2.14.1 (R Development Core Team, 2011) using the package Seewave (Sueur *et al.*, 2008). For each recorded call the [second] harmonic containing most energy was identified from the power spectrum and measurements taken from the Constant Frequency (CF) component of the call, as detailed in previous studies (Schnitzler and Kalko, 1998).

Molecular

For molecular investigations, DNA was extracted from 95% ethanol-preserved tissues with the EasyMag © Biomérieux robot. Two mitochondrial genes were targeted: the Cytochrome b (Cyt b), amplified using the pairs MVZ05/NEW12 and UMMZ04/UMMZ13 primers (Jansa *et al.* 1999, Dávalos and Jansa 2004), and the Cytochrome oxydase 1 (CO1), amplified with the methods commonly used in barcoding screening (Borisenko et al 2008; Clare *et al*., 2013). After amplification, PCR products were sent for purification and sequencing at Cogenics (Takeley, UK). Sequences were aligned and checked manually with MEGA 5.1 (Tamura *et al.* 2011) for absence of gaps or stop codons, to ensure that these were not pseudogenes. Besides the French Guianan, Amapa and the *rubiginosus* specimen from Mato Grosso (MZUSP-35152), the Cyt b and CO1 sequences were acquired from three additional specimens caught by B. Lim in Jamaica, for the sake of having comparative materials from near the type-locality of *P. parnellii* as well. All sequences obtained in this study have been deposited in GenBank and are listed by their accession number in Appendix 1.

Genetic relationships of CO1 haplotypes were visualized on statistical parsimony networks with the minimum connection probability set to 95% (Clement *et al.*, 2000). The phylogenetic relationships between samples were evaluated using maximum likelihood (ML), Neighbor-Joining (NJ), and Bayesian procedures. The GTR model (Tavaré, 1986), with gamma (G) rate parameter and a proportion of invariant sites (I) was identified with MEGA 5.0 (Tamura *et al.* 2011) as the best fitting nucleotide substitution model for both genes. Ten thousand replicates were used for maximum likelihood analyses in Mega 5.0, yielding support values of Bootstrap Percentage (BP). The Bayesian approach was carried out with MrBAYES 3.2 (Ronquist *et al.* 2012). Markov Chain Monte Carlo (MCMC) simulations were run twice independently for 10 million generations with four simultaneous chains, using a sample frequency of 1,000 and a burn-in of 2,500 trees; support values are indicated by Posterior Probabilities (PP), and were calculated from the remaining trees. Intra and inter-clade haplotypic and nucleotidic variabilities were calculated with ARLEQUIN 3.5 (Excoffier and Lischer 2010). Pairwise genetic distances were calculated with MEGA using the K2P model in order to be easily comparable with other similar studies.

For the sake of clarity and for facilitating comparisons with previous works, we indicate in Appendix 2 the names we have used for the sequences retrieved from GenBank, their approximate localities, and the voucher numbers (when known).

Results

1) Bioacoustics

Grotte Mathilde (locality 5; Fig. 1) is a cave surrounded by primary rainforest located ca. 40 km south-east from Cayenne. At this locality, we verified that CMB held individually in cotton-bags emitted ultrasonic calls that had CF characteristics indiscernible from those emitted when flying (upon release). Differences measured for each bat recorded in the bag or upon release were negligible (standard deviation less than 0.2 kHz), which validates the assignation of all handled bats into their respective phonic types.Based on these coustic assignations, 63 CMB caught at Grotte Mathilde emitted ultrasounds with a CF component around 53 kHz (called hereafter the 53 kHz phonic type), and 57 bats had CF components at around 59 kHz (i.e. the 59 kHz phonic type).

We then addressed the population variability of the CF component of both phonic types in samples from Grotte Mathilde (the same 120 individuals), Cacao (87), and Haute-Camopi (38), where these phonic types were recorded in sympatry. At those three sites the two distinct phonic types were clearly discernible with no intermediate CF value (Table 1). At any site, the difference between the highest CF value in the 53 kHz phonic type and the lowest in the 59 kHz phonic type was at least 2.3 kHz (Grotte Mathilde), whereas the difference in mean CF values between each phonic type varied between 5.45 kHz (Cacao) and 6.13 kHz (Haute Camopi). Part of the slight differences observed between localities among each phonic type could be due to technical aspects related to the different models of bat-detectors used to record the CF calls, but again these differences were much smaller than those characterizing each phonic type (Table 1).At the regional scale, all individuals from 6 different localities in French Guiana were

assigned to their respective phonic type (53 or 59 kHz), and again CF calls showed minimal variability within each group (53.4 ± 0.62 kHz, N = 130; 59.2 ± 0.68, N = 127; respectively). No overlap was observed between phonic types (Fig. 3). The classification of all handled or free-flying CMB through their ultrasounds thus provides an easy and reliable character for recognizing the two phonic types in French Guiana.

2) Molecular systematics and taxonomy

The CO1 barcode fragment (657 bp) was sequenced in 14 vouchered specimens from the area of sympatry of Grotte Mathilde and whose ultrasonic calls were also recorded. The alignment of sequences resulted in five distinct haplotypes. Two were unique to the six 53 kHz phonic type CMB, and three unique to the eight 59 kHz animals. TCS networks confirm that haplotypes from each phonic type form statistically separate networks (at the 95% threshold), and differ by a mean K2P distance of 4.9 % (Fig. 4). Haplotypes differed by one mutation (0.06 % net distance) and by up to 9 mutations (0.56% net distance) within the 53 and 59 kHz phonic types, respectively

The more global phylogenetic analyses of DNA barcodes (CO1 fragments) included 5 distinct sequences from French Guiana, 2 from Brazilian Amapa, one from Brazilian Mato Grosso, and the unique haplotype found in 3 CMB from Jamaica, as well as 17 published CO1 sequences from various localities in Suriname, Guyana, Venezuela, El Salvador and Mexico (Clare *et al.*, 2011; 2013). Phylogenetic analyses indicate that the French Guianan and Amapa bats of each phonic type also segregate in two monophyletic clusters (Fig. 5). Noteworthy, the CO1 sequence of specimen MZUSP-35152 is nested within the cluster containing all CMB identified as *Pteronotus sp4* by Clare *et al.*(2013), with less than 0.1% K2P distance from other sequences in this clade. Each major clade (denoted as *P. sp3* and *P. sp4*) contains animals from various localities of Suriname and Guyana – see Appendix 2 - and both are well supported by high bootstrap and PP values (Fig. 5). These two clades show low intraspecific variability (less than 1.4% K2P) and form closely related, sister taxa differing by a mean of 5.1% K2P distance.They are more distantly related to any other named species of *Pteronotus* sampled in this analysis.

Also apparent on the phylogenetic tree is a cluster of four CO1 sequences containing CMB from Mexico (ROM-95741), El Salvador (101476), Venezuela (107924), and western Guyana (101046) and corresponding to *P. sp1* and *P. sp2* of Clare *et al.* (2013). This second group is sister to the other species (*P. sp3* and *P. sp4*) from the Guiana Shield. The CO1 haplotype from Jamaica (ROM-120826) representing *P. parnellii* is external to the clade containing *P. sp1* to *P. sp4* (Fig. 5) and differs from continental CMB by a mean K2P of 11.4 %.

The Cyt b gene was sequenced from 4 vouchered specimens whose ultrasonic calls were previously recorded at Grotte Mathilde, from one specimen in Cacao, two specimens in Amapa, and one specimen in Mato Grosso (Appendix 1). Additional Cyt b sequences of CMB and other species of *Pteronotus* (Lewis-Oritt *et al.*,2001; Davalos 2006) retrieved from Genbank and included in phylogenetic analyses for a total of 32 mormoopid and 4 outgroup taxa (Fig. 6). Details on origins of these sequences are given in Appendix 2.

All sequences of the 59 kHz phonic type bats appear among the putative species *P. sp3*, while those of the 53 kHz phonic type group within *P. sp4*, both with strong support (BP of 91 and 99, respectively; 1.0 PP: Fig. 6). The latter putative species also includes the single specimen from near the type-locality of *rubiginosus*. Average divergence within these two clades was 1.0 and 1.6 % nucleotides, respectively. Each clade included animals caught in sympatry in French Guiana or Amapa, and appear as each other's closest relative (at 6.6 % K2P) in all reconstructions (Fig. 6).

The putative species *P. sp2* (from western Guyana) and *P. sp1* (Mexico; Honduras) form another pair of closely related taxa and appear in a clade (99 BP, 1.0 PP) which is sister to a well supported group (97 BP, 1.0 PP) containing the Antillan species *P. parnellii* (Jamaica), *P. pusillus* (Dominican Republic), and *P. portoricensis* (Puerto Rico).

All four putative species and the Antillean CMB cluster in a large, well supported clade (99 BP, 1.0 PP) which forms a polytomy together with the seven remaining species of *Pteronotus* (*macleayii*, *quadridens*, *fulvus*, *davyi*, *gymnonotus*, *psilotus* and *personatus*).

3) External and cranial morphology

Forearm and third metacarpal measurements tended to be larger in females than in males in both phonic types, but differences were mostly insignificant (Table 2) and hence we pooled genders for other comparisons. Differences among phonic types sampled across French Guiana were in turn all highly significant (Table 3) with individuals of the 53 kHz phonic type being on average larger than those of the 59 kHz phonic type. Although mean values differ, the ranges of the external measurements overlapped broadly, as illustrated with FA length (Fig. 7).

Similarly, the three external variables (FA, MC3, MC4) retained to best discriminate the two phonic types are insufficient to identify each phonic type with confidence, although the discriminant function analysis (DFA) is highly significant [$F(df\ 3,196) = 67.168$, $p<0.0001$]. Indeed 19 out of 110 individuals identified by their ultrasounds as 59 kHz phonic type and 12 out of 89 identified as 53 kHz phonic type were misclassified by this discriminant function (results not shown). Interestingly, the male individual from Mato Grosso (MZUSP-35132) is classified as a 53 kHz phonic type (posterior probability 0.76) with this simple function. Results were similar if sex were separated or pooled in the discriminant analyses. Clearly none of these external characters taken alone or in combination can be used for a reliable phonic type discrimination.

Cranial and dental measurements also indicate that 53 kHz CMB are larger than 59 kHz bats and, despite small sample sizes, differ significantly from each other (Table 4). Most cranial measurements do not overlap between individuals of the two phonic types (Table 4), but these results should be considered preliminary due to the small number of skulls examined.

A discriminant function analysis including all 14 variables is highly significant [$F(df\ 14, 7) = 20.23$, $p<0.0003$] and classified all 22 skulls in their correct phonic type (results not shown). We present here only results of the stepwise discriminant function [$F\ (df\ 3, 18) = 64.42$, $p < 0.0001$; Fig. 8] that includes the three most discriminant cranial variables (MDT, BRD and CCL) and which classifies all individuals in their correct (a priori) phonic type. Again the skull of the Mato Grosso *Pteronotus* (specimen MZUSP-35152) is classified a posteriori as a 53 kHz phonic type in these discriminant analyses. This male individual was thus classified as a 53 kHz phonic type with high posterior

probability (>0.76) in all preformed DFA, whether based on external or on cranial measurements.

Discussion

Our results clearly support the existence of two phonic types among CMB living in French Guyana and Brazilian Amapa. Each phonic type emits constant frequency calls at non-overlapping ranges (Fig. 3). Indeed both sexes of the 53 kHz phonic type emit around 53 kHz with very little variation across the region (extremes 51.5-54.5 kHz; Table 1). Likewise, CMB of the 59 kHz phonic type emits at around 59 kHz (range 56.8-60.1 kHz; Table 1) in all recorded localities. These two phonic types are therefore easy to identify throughout their echolocation calls, including when they are recorded when held captive in cotton bags.

The two phonic types are found throughout French Guiana and Brazilian Amapa (Fig. 1), but further bioacoustic studies are needed to understand their exact distribution in this region. Where they are found in sympatry, both phonic types occur in comparable numbers (Table 5), suggesting that they can coexist in similar habitats without major ecological competition. Indeed, they may occupy the few cave roosts existing in this area, while exploiting a different trophic niche of their habitat, as was evidenced in other sympatric, sibling species of bats sharing roosts (e.g. Arlettaz *et al.* 1997).

Molecular analyses of two mitochondrial genes (CO1 and Cyt b; Figs 5 and 6) consistently identify these two phonic types in the clades defined by Clare *et al.* (2013). The sequences of 59 kHz phonic type bats correspond to clade 3, which is considered as a putative species *P. sp 3*, while the 53 kHz phonic type sequences cluster within clade 4 (*P. sp 4*). Both phonic types are closely related (mean 6.6% K2P distance for the Cyt b and 5.1% for CO1), sister clades in all phylogenetic reconstructions and present little genetic variation within clades (Figs 5 and 6). Although sample sizes of CMB characterized by both bioacoustics and genetic data are still limited (14 individuals), there is complete concordance between phonic types and molecular clades (Fig. 4), indicating that no gene flow occurs between animals of both phonic types, even when they share roosts. Our results therefore fully corroborate the conclusions of Clare *et al.*

(2013) who consider that clades 3 and 4 represent independent, biological species. Interestingly Clare *et al.* (2011) identified molecularly nearly the same number of *P. sp3* (n= 143) and *P. sp4* (n= 151) individuals in a large survey (355 barcoded bats) of CMB sampled in Suriname and Guyana, a pattern of relative abundance similar to that found in French Guiana.

Morphologically, these two species differ by size, the 53 kHz phonic type being significantly larger than the 59 kHz type in all external (Table 3) and cranial (Table 4) variables measured. These differences in body size and in echolocation calls parameters are consistent with expectations from the scaling effect of size (Jones, 1999), where larger species tend to emit calls at lower frequencies when compared to smaller ones. Unfortunately, these morphological differences, although highly significant, do not allow for a unambiguous separation of both phonic types, as most external characters show large measurement overlaps (e.g. forearm length, Fig. 7). Clearly, ultrasound recording is a much more reliable method to identify living CMB of both phonic types in French Guiana and Amapa. Individual measurements of the skull are much less overlapping (Table 4) and allow for a complete discrimination of all bats in a simple discriminant analyses (Fig. 8). Skull dimensions are therefore also appropriate to identify dead or museum specimens, e.g. for proper taxonomic comparisons.

In an effort to assign an appropriate taxonomic name to the two cryptic phonic types, we included one CMB (MZUSP35152, see Appendix 1) sampled near the type locality of *rubiginosus* (Wagner, 1843). This taxon name was suggested by Davalos (2006) for CMB of eastern South America, but because there are clearly more than one species of CMB living in this region, it was unclear if this name would be applicable. In all comparisons the specimen MZUSP35152 unambiguously is part of the 53 kHz phonic type (= *P. sp4*). Indeed, despite extensive geographic distance separating samples of *P. sp4* from French Guiana and the Mato Grosso (over 2’000 km), both mitochondrial markers show little genetic differentiation (Fig. 6). Morphologically the relatively large external measurements of this Mato Grosso CMB also fits the range of variation displayed by CMB of the 53 kHz phonic type in French Guiana (Table 3) and cranially it matches skull dimensions typical of specimens from this phonic type (Fig. 8, Table 4). If the Mato Grosso individual truly represents *rubiginosus*, then this name would apply to

the 53 kHz phonic type (*P. sp 4*) and would considerably extend its geographic range beyond the Guianan Shield. These characteristics also fit with the brief morphological description of clade 4 given in Clare *et al.* (2013).

The smaller CMB of the 59 kHz phonic type and representing *P. sp3* are more difficult to classify. So far, these animals are found only on the Guianan Shield (French Guiana, Brazilian Amapa, Guyana and Suriname, Clare *et al.*, 2013 and Fig. 1) where no endemic taxon have been named. This phonic type most probably represents a new cryptic species in the CMB complex, as suggested earlier. At a broader geographical scale, a third taxon of CMB denoted *P. sp2* and also characterized by constant frequency calls around 59 kHz is found in the north-western part of the Guianan Region (Guyana and the Venezuelan states of Amazonas and Bolivar; Clare *et al.* 2013). Representatives of this third cryptic species are genetically very distinct from the 53 and 59 kHz phonic types from French Guiana in all reconstructions (Figs 5 and 6) and certainly represents another cryptic species, related to the Central American *P. sp1* (= *P. mesomaricanus* in Clare *et al.*, 2013). We did not sample *P. sp2* in French Guiana or Brazil, and as no precise morphological or bioacoustics clues are provided for its identification, it is also premature to name it properly. Clearly reference material from other type localities of CMB taxa and more specimens characterized with multiple methods are needed before all cryptic species within the CMB complex can be critically reviewed for an adequate systematic arrangement.

The existence of at least three cryptic species of CMB living in the Guianan Shield, some of which may live in strict sympatry and share roosts (Fig. 1) has important bearings for their conservation, as their exact distribution, ecological needs and abundance are largely unknown.

Much remains to be done for getting a complete picture of *Pteronotus* systematics and biodiversity in Central and South America. Studies should investigate molecular and ultrasonic call characters in Venezuela, where Gutierrez and Molinari (2008) have shown through comparative morphometrics that two taxa live in parapatry, namely *P.* (*parnellii*) *fuscus* north from the Rio Orinocco and *P.* (*parnellii*) *rubiginosus* south from the Rio Orinocco, i.e. in the north-western part of the Guianan Region. An appealing

hypothesis is that *P.* (*parnellii*) *rubiginosus* of Guttierez and Molinari (2008) corresponds to Group 2 (*P. sp*ecies 2) of Clare *et al.* (2013), i.e. animals from the Guianan Region in western Guyana and in Venezuela (states of Amazonas and Bolivar), for which we here use the taxon name *Pteronotus sp2*. Gutierrez and Molinari (2008) indicate that the Venezuelan CMB north of the Orinoco (which they call *P. parnellii fuscus*) have a Constant Frequency echolocation call at around 62 kHz, a value similar to the one reported by Clare *et al.* (2013) for what those latter authors call *Pteronotus sp1* (or *P. mesoamericanus*). Clearly, more work combining different kinds of characters is needed for understanding those conflicting interpretations, as Clare *et al.* (2013) circumscribe their *P. mesoamericanus* CMB to Central America (from Panama to Southern Mexico) whereas Gutierrez and Molinari (2008) have their *P. fuscus* CMB limited to Caribbean regions of Colombia and Venezuela.

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Table 1: Variability of the constant frequency (CF) component of echolocation calls for two phonic type CMB caught in sympatry in French Guiana. Values are expressed in kHz and correspond to the frequency containing maximum energy (FME), with mean, standard deviation (SD), minimum (min) and maximum (max) values; N is the number of bats recorded.

Locality	**Phonic type**	**N**	**mean**	**SD**	**min**	**max**
Cacao	*53 kHz*	47	53.79	0.31	53.1	54.3
Cacao	*59 kHz*	40	59.24	0.53	57.6	60.1
Grotte Mathilde	*53 kHz*	63	53.10	0.65	51.6	54.5
Grotte Mathilde	*59 kHz*	57	59.10	0.72	56.8	59.9
Haute-Camopi	*53 kHz*	21	52.85	0.55	51.5	53.9
Haute-Camopi	*59 kHz*	17	58.98	0.59	58.3	60.0

Table 2: Sexual dimorphism in two external measurements for the 53 kHz and 59 kHz phonic types recorded at various localities in French Guiana. Measurements are given in millimetres. Abbreviations: FA = forearm length; MC3 = third metacarpal length; N = sample size; SD = standard deviation; p-value = significance of the Mann-Whitney test between genders.

Phonic type		**Males**			**Females**			**p-value**
		N	**mean**	**SD**	**N**	**mean**	**SD**	
53 kHz	FA	65	64.0	0.14	24	64.7	0.26	0.055
59 kHz	FA	69	61.6	0.15	41	62.1	0.20	0.067
53 kHz	MC3	65	52.9	0.20	24	52.7	0.27	0.537
59 kHz	MC3	70	50.2	0.17	41	50.9	1.18	0.004

Table 3: External measurements (in millimetres) for Common Mustached Bats of the 53 kHz and 59 kHz phonic types from French Guiana. The last column correspond to measurements of the specimen MZUSP-35152 from Cuiabá, Mato Grosso, Brazil. Abbreviations: n.a. = not available; other abbreviations are as defined in table 1.

	53 kHz			**59 kHz**				**MZUSP-**
	N	**mean**	**SD**	**N**	**mean**	**SDd**	**p-value**	**35152**
Forearm	89	64.2	0.13	111	61.8	0.12	< 0.001	63.6
Metacarp-3	89	52.8	0.16	111	50.5	0.13	< 0.001	52.6
Metacarp-4	89	50.9	0.19	111	49.1	0.15	< 0.001	50.9
Digit-4	89	77.0	0.36	111	75.3	0.32	0.002	n.a.
Tibia	44	25.6	0.18	76	24.0	0.11	< 0.001	25.6
Weight (g)	43	23.9	0.19	65	21.7	0.16	< 0.001	n.a.
Wingspan	9	418	7.6	14	398	8.9	< 0.001	n.a.

Table 4: Cranial and dental measurements (in mm) in French Guianan Common Mustached Bats: 8 individuals of the 53 kHz phonic type and 14 individuals of the 59 kHz phonic type. Mann-Whitney tests of differences between the phonic types are all significant at $p < 0,001$. The far right column is for specimen MZUSP-35152, from Cuiabá, Mato Grosso, Brazil (see text for further details). Abbreviations: GLS = greatest length of skull , GLSI = greatest length of skull including upper incisors, CIL =condyle-incisive length, CCL = condylo-canine length, PL = palatal length, ZB = zygomatic breadth, BaM = breadth across molars, MCC = mandibular condyle-canine length, MTL = maxillary toothrow length, M1M3 = length of upper molar teeth, MDT = mandibular toothrow length.

	53 kHz			**59 kHz**			**MZUSP**
	mean	**min**	**max**	**mean**	**min**	**max**	**35152**
GLS	22.85	22.25	23.30	21.80	21.46	22.20	23.04
GLSI	23.54	22.94	24.30	22.38	21.74	22.83	23.65
CIL	22.55	22.22	22.90	21.48	21.03	22.11	22.15
CCL	21.48	20.96	21.90	20.27	19.90	20.70	21.50
PL	11.47	11.15	11.72	10.82	10.58	11.08	11.24
ZB	13.56	13.30	13.85	12.98	12.50	13.40	13.00
BaM	8.76	8.55	8.94	8.35	8.14	8.80	8.62
MCC	16.71	16.45	17.07	15.59	15.20	15.91	16.44
MTL	10.21	10.00	10.44	9.54	9.22	9.83	9.75
M1M3	5.95	5.72	6.09	5.59	5.41	5.76	5.60
MDT	11.56	11.35	11.77	10.87	10.57	11.11	11.08

Table 5: Sample size of CMB recorded in six localities of French Guiana. Individual bats were assigned to their respective phonic types according to their calls after being caught, except in localities of Saut-Pararé and Haute Camopi where they were recorded when flying above the observer. Numbers in parenthesis correspond to the localities mapped in Fig. 1.

Locality	**59 kHz**	**53 kHz**	**ratio**
Cacao (loc. 3)	38	47	0.8 : 1
RN Trésor (loc. 4)	9	11	0.8 : 1
Grotte Mathilde (loc. 5)	49	39	1.3 : 1
Montagne des Gouffres (loc. 6)	32	9	3.6 : 1
Saut-Pararé (loc. 7)	36	45	0.8 : 1
Haute Camopi (loc. 8)	12	15	0.8 : 1
French Guiana	176	166	1.1 : 1

Appendix 1

Specimens examined are housed in the following institutions: Muséum d'Histoire Naturelle de Genève (MHNG), collection of Pierre-Charles Dominique housed at the Laboratoire d'Ecologie du Muséum at Brunoy, France (MNHN-PCD), Instituto de Pesquisas Cientificas e Technologicas do Estado do Amapa, at Macapa (IEPA); Museum of Zoology of the University at Sao Paulo (MZUSP) ; Royal Ontario Museum at Toronto (ROM). For each taxon, specimens are listed according to country, political division, locality, coordinates, and museum catalog number (except for R266, which was released after recording). When available, Genbank accession number of both sequenced genes (CO1 = Cytochrome oxidase-1; Cyt b = Cytochrome-b) are given in parenthesis after the catalog number.

Pteronotus rubiginosus (P. sp4). – Brazil: Amapa: Parque Nacional Montanhas do Tumucumaque, Rio Mutum, Calçoene: IEPA-554 (CO1 KF636800, Cyt b KF636804); Mato Grosso: Cuiabá MZUSP-35152 (CO1 KF636799, Cyt b KF636801). French Guiana: municipality of Roura: Cacao MHNG-1983.064 and 65; 1979.073; municipality of Regina: Grotte Mathilde MHNG-1978.076 (CO1 KF636795; Cyt b KF636803), 1978.079 (CO1 KF636797; Cyt b KF636802), 1978.080 (CO1 KF636798), 1978.083 (CO1 KF636796), 1978.084 (CO1 KF636794), 1978.088 (CO1 KF636793).

Pteronotus sp3. - Brazil: Amapa: Iratapuru village, near to Laranjal do Jari, Jari river: IEPA-1843 (CO1 KF636815, Cyt b KF636817). French Guiana: municipality of Roura: Cacao MHNG-1980.091 and 92 (CO1 KF636813, Cyt b KF636816), 1983.043, 58 and 69; Trésor Natural Preservation R266 (CO1 KF636814); municipality of Regina: Grotte Mathilde MHNG-1980.094, 1978.077 (CO1 KF636809), 1978.078 (CO1 KF636811, Cyt b KF636818), 1978.081 (CO1 KF636810), 1978.082 (CO1 KF636805), 1978.085 (CO1 KF636807), 1978.086 (CO1 KF636806, Cyt b KF636819), 1978.087 (CO1 KF636812), 1978.089 (CO1 KF636808); municipality of Regina: camp inselberg des Nouragues (04°02' N; 52°42' W) MNHN-PCD-433, 485, 1088, 1095, 1228, 1229 and 1230.

Pteronotus parnellii – Jamaica : Saint Elisabeth, Oxford Cave (18°09’N ; 77°05’ W) ROM-120788 (Cyt b KF636822); Saint Andrew, 10 km N of Kingston (18°05’ N; 76° 43’W) ROM-120826 (CO1 KF636820, Cyt b KF636821).

Appendix 2: Reference list of the *Pteronotus* spp. sequences used in Figures 5 and 6. The first column lists the taxon name used in this paper, followed by Genbank and voucher numbers, and the approximate location of origin of the specimen. Sp1 to sp4 are taxon names given by Clare *et al.* (2013) to designate genetically and morphologically distinct continental Common Mustached Bats. The acronyms TK (Museum of Texas Tech University), ROM (Royal Ontario Museum), AMNH (American Museum of Natural History), AMCC (Ambrose Monell Cryo Collection, at New York) and USNM (United States National Museum) designate the institutions housing the sequenced specimens.

Name used in this paper	**GenBank**	**Specimen / voucher**	**Country / locality**
Pteronotus davyi	AF338669	TK-15571	Dominica: St. Joseph Parish, mouth of Layou River
Pteronotus davyi	AF338671	TK-25127	Trinidad and Tobago: Trinidad, Nariva, Arena Reserve
Pteronotus fulvus	JF446541	ROM-101305	El Salvador: Ahuachapan, El Imposible, El Refugio
Pteronotus fulvus	AF338672.	TK-27642	Mexico: Jalisco, Chamela
Pteronotus gymnonotus	EF080590	ROM-115628	Guyana: Essequibo Islands-West Demerara
Pteronotus gymnonotus	EF080591	ROM-109253	Guyana: Potaro-Siparuni
Pteronotus gymnonotus	JF447432	ROM-104265	Panama
Pteronotus gymnonotus	AF338674	TK-22845	Peru: Huanuco Department, Leoncia Prado
Pteronotus macleayii	AF338683	TK-32162	Cuba: Guantanamo Province, Guantanamo Bay Naval Station
Pteronotus macleayii	AY604461	AMCC102719	Jamaica
Pteronotus mesoamericanus (sp1)	JF448266	ROM-101476	El Salvador: Santa Ana, Parque Nacional Montecristo, Los Planes
Pteronotus mesoamericanus (sp1)	AF338662	TK-40197	Honduras: Valle, 8.5 mi SSW San Lorenzo
Pteronotus mesoamericanus (sp1)	AF338664	TK-13108	Mexico: Vera Cruz, 14 km N 22 km E Cordoba
Pteronotus mesoamericanus (sp1)	JF448279	ROM-95741	Mexico:Campeche, 44 Km S of Constitucion
Pteronotus parnellii	AY604456	AMCC102714	Jamaica
Pteronotus parnellii	AF338661	TK-27704	Jamaica: St. Ann's Parish, 24 km W St. Ann's Bay
Pteronotus personatus	EF080596	ROM-115597	Guyana: Essequibo Islands-West Demerara
Pteronotus personatus	EF080597	ROM-109298	Guyana: Potaro-Siparuni

Pteronotus personatus	JF455427	ROM-97943	Guyana: Upper Takutu, Karanambo
Pteronotus personatus	AF338679	TK-10336	Suriname: Nickerie, Grassalco
Pteronotus personatus	AF338678	TK-19079	Venezuela: Bolivar, 0.5 km E El Manteco
Pteronotus portoricensis	AF338665	TK-21800	Puerto Rico: Naguabo, Caribbean National Forest
Pteronotus portoricensis	AF338666	TK-21806	Puerto Rico: Naguabo, Caribbean National Forest
Pteronotus psilotus	AF338680	TK-12043	Mexico: Oaxaca, Tehuantepec
Pteronotus pusillus	AY604455	AMCC103048	Dominican Republic
Pteronotus pusillus	AY604454	AMCC103050	Dominican Republic
Pteronotus quadridens	AF338681	TK-32171	Cuba: Guantanamo Province, Guantanamo Bay Naval Station
Pteronotus quadridens	AF338682	TK-9487	Jamaica: St. Catherine Parish, St. Clair Cave
Pteronotus rubiginosus (sp4)	JF448246	ROM-100417	Guyana: East Berbice-Corentyne
Pteronotus rubiginosus (sp4)	EF080592	ROM-108916	Guyana: Potaro-Siparuni
Pteronotus rubiginosus (sp4)	EF080593	ROM-108934	Guyana: Potaro-Siparuni
Pteronotus rubiginosus (sp4)	JF448388	ROM-102896	Guyana: Upper Takutu-Upper Essequibo, Takutu
Pteronotus rubiginosus (sp4)	JF448455	ROM-113395	Guyana: Upper-Demerara-Berbice, Pibiri
Pteronotus rubiginosus (sp4)	AF330807	TK-17953	Suriname: Marowiejne, Oelemarie
Pteronotus rubiginosus (sp4)	EU096920	ROM-117591	Suriname: Sipaliwini
Pteronotus rubiginosus (sp4)	EU096918	ROM-117608	Suriname: Sipaliwini
Pteronotus rubiginosus (sp4)	EU096924	ROM-117654	Suriname: Sipaliwini
Pteronotus sp2	JF448375	ROM-101046	Guyana: Barima-Waini, Baramita, Old World
Pteronotus sp2	AF338668	USNM-582260	Guyana: NW District, Baramita
Pteronotus sp2	JF448283	ROM-107924	Venezuela:Bolivar, Hato La Florida, 35 Km Ese of Caicara
Pteronotus sp3	AY604457	AMNH-269115	French Guiana: Paracou (Sinnamary)
Pteronotus sp3	JF448247	ROM-100427	Guyana: East Berbice-Corentyne
Pteronotus sp3	JF448433	ROM-111664	Guyana: Potaro-Siparuni
Pteronotus sp3	JF448179	ROM-115735	Guyana: Potaro-Siparuni
Pteronotus sp3	JF448197	ROM-113511	Guyana: Upper Takutu-Upper Essequibo, Takutu
Pteronotus sp3	JF448451	ROM-113368	Guyana: Upper-Demerara-Berbice, Pibiri
Pteronotus sp3	JF448457	ROM-113407	Guyana: Upper-Demerara-Berbice, Pibiri
Pteronotus sp3	JQ601193	ROM-120589	Suriname: Kutari
Pteronotus sp3	EU096921	ROM-117176	Suriname: Sipaliwini
Pteronotus sp3	EU096925	ROM-117198	Suriname: Sipaliwini

Pteronotus sp3	EU096919	ROM-117576	Suriname: Sipaliwini
Pteronotus sp3	HQ919698	ROM-120339	Suriname: Sipaliwini
Pteronotus sp3	JQ601320	ROM-119488	Suriname: Tafelberg

FIGURE CAPTIONS

Figure 1: Map of French Guiana (localities 1 to 9) and Amapa, Brazil (10 to 14) locations where bats of the 53 kHz (white circles) and the 59 kHz phonic types (black circles) have been characterized through bioacoustics, skull morphometry and/or molecular genetics. Localities harbouring both phonic types in sympatry are indicated by a pie circle.

1 = Macouria River, municipality of Tonate, 05°02'N; 52°30' W

2 = Quartier Torcy, municipality of Roura, 04°50' N; 52°16' W

3 = Cacao, municipality of Roura, 04°34' N; 52°27' W

4 = Trésor Natural Preservation, municipality of Roura, 04°37' N; 52°17' W

5 = Grotte Mathilde, municipality of Regina, 04°31' N; 52°07'W

6 = Montagne des Gouffres, municipality of Regina, 04°20' N; 52°16' W

7 = Saut-Pararé, municipality of Regina, 04°02' N; 52°42' W

8 = Montagne Cacao by Haute Camopi, municipality of Camopi, 02°20' N; 53°12' W

9 = La Trinité, municipality of Saint Elie, 04°61' N ; 53°40' W

10 = Iratapuru village, near to Laranjal do Jari, Jari river, 00°37' N, 51°31' W

11 = Itapeuara village, near to Laranjal do Jari, Jari river, 00°29' N ; 52°41' W

12 = P.N.Tumucumaque, Rio Mutum, municipality of Calçoene, 01º23' N; 51º55' W

13 = P.N.Tumucumaque, Rio Anoteie, municipality of Oiapoque, 03º13' N; 52º01' W

14 = Aricari farm/BR156 road, km147, municipality of Tartarugalzinho, 00º56' N; 51º14’ W.

Figure 2: Sonogram of the second harmonic of the echolocation call emitted by Common Mustached Bats in French Guiana. The sonogram was produced using a 512-point FFT (Fast Fourier Transform) created with R 24 (R Development Core Team, 2011) using the package Seewave (Sueur *et al.*, 2008). The Frequency of Maximal Energy (FME) is measured at around 60 kHz (left) or 54 kHz (right) and corresponds to the 59 kHz and 53 kHz phonic types, respectively.

Figure 3: Histograms of constant frequency (CF) values emitted by 257 Common Mustached Bats caught in French Guiana. The median constant frequency value (CF) emitted by 130 individuals of the first phonic type (left) was 53.5 kHz (standard deviation 0.62 kHz), and that of the 127 individuals of the second phonic type (right) was 59.3 kHz (standard deviation 0.68 kHz).

Figure 4: Parsimony haplotype networks for 14 CO1 sequences of Common Mustached Bats corresponding to the 53 kHz (below) and 59 kHz (above) phonic types, respectively. All bats were caught in syntopy at Grotte Mathilde, French Guiana. Ovals indicate haplotypes found in only one specimen, whereas the rectangles represent haplotypes shared by 6 (59 kHz phonic type) and 5 (53 kHz phonic type) animals, respectively. Numbers in the symbols correspond to the last three digits of the vouchered specimens (MHNG-1978.xxx).

Figure 5: Maximum likelihood tree showing the phylogenetic relationships among 26 CO1 barcodes of Common Mustached Bats from Amapa, Mato Grosso, French Guiana, Guyana, Suriname, Venezuela, El Salvador, Mexico, and Jamaica. Bootstrap support from ML and posterior probabilities (PP) from a Bayesian analysis are shown above and below major nodes, respectively. Labels for continental CMB are composed of the same species names as in Clare *et al.* (2013) followed by specimen reference (see Appendix 1 and 2). The tree was rooted with sequences of *P. personatus* and *P. gymnonotus*. Specimens (with voucher numbers) were assigned to *Pteronotus sp1* to *sp4* according to Clare *et al.* (2013). The phonic type of the individually recorded bats is also given in the label.

Figure 6: Maximum likelihood tree showing the phylogenetic relationships among 31 Cyt b sequences of *Pteronotus* specimens from Central, South America and the Caribbean region. The legend is the same as for Fig. 5 except that the tree was rooted

with sequences of *Artibeus* and *Noctilio.* Branches poorly supported (BP less than 80 in ML or PP less than 0.8 in Bayesian analysis) were collapsed into a polytomy.

Figure 7: Distribution of forearm lengths (in mm) of the 53 kHz phonic type (white bars) and 59 kHz phonic type (black bars) Common Mustached Bats in French Guiana. Sample sizes are 111 and 89 individuals for each phonic type, respectively. Sexes and localities were not differentiated.

Figure 8: Results of the stepwise discriminant function analysis [F (df 3, 18) = 64.42, $p < 0.0001$] including the three most discriminant cranial variables (MDT = mandibular toothrow length, BRD = braincase depth, and CCL= condylo-canine length) in 8 specimens of the 53 kHz (white bars) and 14 of 59 kHz phonic type (black bars), as well as the specimen MZUSP-35152 from Mato Grosso (grey bar).