# Geographical variation in the high-duty cycle echolocation of the cryptic common mustached bat *Pteronotus* cf. *rubiginosus* (Mormoopidae)

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## Abstract

The use of bioacoustics as a tool for bat research is rapidly increasing worldwide. There is substantial evidence that environmental factors such as weather conditions or habitat structure can affect echolocation call structure in bats and thus compromise proper species identification. However, intraspecific differences in echolocation due to geographical variation are poorly understood, which poses a number of issues in terms of method standardization. We examined acoustic data for *Pteronotus* cf. *rubiginosus* from the Central Amazon and the Guiana Shield. We provide the first evidence of intraspecific geographic variation in bat echolocation in the Neotropics, with calls significantly differing in almost all standard acoustic parameters for the two lineages of this clade. We complement our bioacoustic data with molecular and morphological data for both species. Considerable overlap in trait values prevents reliable discrimination between the two sympatric *Pteronotus* based on morphological characters. On the other hand, significant divergence in the frequency of maximum energy suggests that bioacoustics can be used to readily separate both taxa despite extensive intraspecific variability in their echolocation across the Amazon. Given the relative lack of barriers preventing contact between bat populations from the Central Amazon and French Guiana, the documented acoustic variation needs to be further studied in geographically intermediate locations to understand the potential isolation processes that could be causing the described divergence in echolocation and to determine whether this variation is either discrete or continuous.



**Keywords**: Amazon; bioacoustics; cryptic species; echolocation; Mormoopidae; speciation

## Introduction

Acoustic divergence is one of the key factors driving speciation processes and is commonly found in cryptic vertebrate species (Wilkins et al. 2013). However, it is still unclear whether it is the cause or the consequence of a reduction in levels of gene flow within the species (Jiang et al. 2013). Although it has been shown that at different geographical scales this sensory divergence may emerge as a result of either direct ecological selection, genetic drift, cultural drift or indirect ecological selection (Jiang et al. 2013, Keighley et al. 2017, Lin et al. 2014), our understanding of it is still far from complete (Jiang et al. 2013).

As a result of continuous technological advances many cryptic species are discovered every year (Caminer and Ron 2014, Csorba et al. 2011, Koubínová et al. 2013, Lin and Li 2013). Because they are morphologically and ecologically similar, cryptic species are usually difficult to identify in the field (Jörger and Schrödl 2013). Especially bats, due to their elusiveness and nocturnal habits, are a challenging group to study in the wild and therefore constitute an excellent target for the discovery of new species (Jones 1997). Their description and identification have been mostly based on the examination of external and cranial morphology (Eisenberg and Redford 1999, Vuilleumier et al. 1992, Wilson and Reeder 2005), but nowadays molecular techniques combined with behavioural and ecological information are rapidly unveiling new bat species.

Until the beginning of the 21st century, species identification based on the analysis of echolocation calls was rarely applied due to the lack of knowledge about bat bioacoustics. However, echolocation research is a rapidly evolving field and several studies have allowed scientists to unravel new species worldwide. For instance, based on differences in social calls Barlow and Jones (1997) were able to infer that bats previously identified as *Pipistrellus pipistrellus*, corresponded in fact to two different species (*P. pipistrellus* and *P. pygmaeus*). Similarly, Ramasindrazana et al. (2011) identified two species of *Miniopterus* bats from

Madagascar and the Comoros using bioacoustic parameters combined with genetic information and morphological characters.

In the Neotropics, the family Mormoopidae comprises two genera (*Mormoops* and *Pteronotus*) of insectivorous bats (Simmons et al. 2005, Smith 1972), occurring from the southern United States to Central and Northeastern Brazil, including some Caribbean islands (Patton and Gardner 2007, Pavan and Marroig 2016). *Pteronotus* contains the only New World bat species that uses high-duty cycle echolocation, a trait otherwise restricted to ~ 120 species in the Old World families Rhinolophidae and Hipposideridae (Kober and Schnitzler 1990). Barataud et al. (2013) published a reference call library for Neotropical bats (based on data mainly from French Guiana) in which two phonic groups were described for *Pteronotus parnellii* in French Guiana; one group displaying frequencies of maximum energy around 52 kHz, and the other around 58 kHz.

In the last years, several lines of evidence, among them differences in echolocation, have revealed that *Pteronotus parnellii* represents a complex of several cryptic species (Clare et al. 2013, López-Wilchis et al. 2016, Pavan and Marroig 2016, Thoisy et al. 2014). Using genetic, morphological and acoustic evidence, Clare et al. (2013) recognized the existence of four distinct taxa in Central and northern South America; one single species in Central America (*P. mesoamericanus*) and three additional species in northern South America. Thoisy et al. (2014) provided evidence of segregation between two sympatric groups in French Guiana and the state of Amapá in Brazil at the level of mitochondrial DNA complemented with acoustic and morphological data (lineages named as *P*. sp 3 -also named P. sp 1 by Pavan & Marroig 2016- and *P*. sp 4 *sensu* Clare et al. 2013). Recently, Pavan and Marroig (2016) proposed a new phylogenetic hypothesis for the genus *Pteronotus*, recognizing the existence of eight species within the *P. parnellii* complex (subgenus *Phyllodia*). This study corroborates the presence of two syntopic lineages in the Guiana Shield and the Brazilian Amazon: one of them already described in the group taxonomy as *Pteronotus rubiginosus* and referred to as *P*. sp 4 by previous studies (Clare et al. 2013, Thoisy et al. 2014)

and the other lineage (referred to as *P*. sp 3 by the same authors, representing an undescribed species in the group. In this study, we explore the potential for separating these cryptic species in the Central Amazon based on acoustic information, and describe two examples of intraspecific geographic divergence in bat echolocation. We provide bioacoustic and genetic evidence that individuals of *P*. cf. *rubiginosus* captured in the Central Brazilian Amazon correspond to the same two distinct cryptic species found in sympatry in French Guiana (Thoisy et al. 2014), but also show clear geographic variation within their calls.

## Materials and methods

*Study site*

Fieldwork was carried out at the Biological Dynamics of Forest Fragments Project (BDFFP), a large-scale fragmentation experiment located in the Central Amazon, 80 km north of Manaus, Brazil (2°20'S, 60°6'W, altitude of 30-125 m a.s.l). The area is characterized by a mosaic of *terra firme* rainforest (30-37 m of mean canopy height, with emergent trees up to 55 m) with secondary forest mainly composed by *Vismia* spp. and *Cecropia* spp. (Mesquita et al. 1999). Annual rainfall across the region ranges from 1900 to 3500 mm, with a rainy season between October and May (Laurance et al. 2011). Average temperature is around 26 ºC (de Oliveira and Mori 1999). There are no large gradients of altitude, with elevations ranging from 80 to 160 m.

*Mist-netting*

As part of a 3-year project (2011-2014) at the BDFFP we captured bats using both ground- and canopy-level mist-netting during the dry and wet season. Bats were sampled in a variety of habitats, ranging from primary *terra firme* rainforest, secondary forest, and forest fragments to temporary lakes and small ponds, rivers and streams, campsites, roads, trails and pastures. Captured bats were identified using different taxonomic keys (Lim and Engstrom 2001, López-Baucells et al. 2016). Throughout this paper taxonomic nomenclature follows Clare et al. (2013). A total of 87 individuals of *Pteronotus* cf. *rubiginosus* were captured, of which fifteen individuals were collected as voucher specimens: 2 males and 5 females for the 55 kHz phonic group and 3 males and 5 females for the 60 kHz phonic group. These specimens were deposited at the Mammal Collections of the *Instituto Nacional de Pesquisas da Amazônia* (accession numbers are provided in the Supplementary Material, Table 1) under ICMBio permit (no. 26877-2). For all the other specimens, biopsy punches (2 mm, Stiefel Laboratories, Inc., Germany) were taken from the wings for barcoding analyses. We followed the guidelines approved by the American Society of Mammalogists in our procedures (Sikes and Gannon 2011).

*Bioacoustics*

Vocalizations from a total of 87 individuals of both phonic groups were recorded. Echolocation recordings were obtained from captured individuals using a Pettersson D1000X detector (Pettersson Elektronik AB, Uppsala, Sweden) just after the bats were released in open areas and forest clearings. Recordings were made with the detector placed 15 m from the point of release. We used a sampling frequency of 250 kHz, with 16 bits/sample. For both spectrograms and power spectra, a customized 512 point fast Fourier transform (FFT) with a Hanning window of 44.1 kHz was used. The following seven standard echolocation call parameters were measured from the main harmonic of each pulse using BatSound version 1.3 (Pettersson Elektronik AB, Uppsala, Sweden) and following (López-Baucells et al. 2016): Frequency of maximum energy (FME): the frequency containing most energy; Bandwidth (BW): the difference between minimum and maximum frequency; Start frequency (Startfreq); End frequency (Endfreq); Maximum frequency (Maxfreq); Minimum frequency (Minfreq) and Pulse duration (Duration). To minimize measurement errors and biases, we only measured those pulses from the recorded echolocation call sequences whose intensity was around 20 dB higher than background noise. When possible, ten pulses were measured for each individual.

*Molecular data*

Voucher specimens from both phonic groups were selected for molecular analyses (Supplementary material, Table 1). Total genomic DNA was extracted from muscle tissue or wing punches using a Qiagen DNeasy Blood & Tissue Kit (Qiagen, Inc.) and following the manufacturer's protocol. Two molecular markers from the mitochondrial DNA were selected - the entire 1140 bp cytochrome *b* gene (*CytB*) and a 651 bp fragment of the COI gene (COI). The COI fragment was sequenced for ten specimens, five from each phonic group; the complete *CytB* gene was sequenced for four specimens, two from each phonic group. The sequences were included in the COI and *CytB* datasets with other *P.* cf. *rubiginosus* sequences generated by Thoisy et al.

(2014) and (Pavan and Marroig 2016). The COI and *CytB* fragments were amplified via Polymerase Chain Reaction (PCR) with primers and protocols described previously (Borisenko et al. 2008, Pavan et al. 2013). The primers used for *CytB* sequencing were designed specifically for *Pteronotus* species and are provided in the Supplementary Material, Table 2. All sequences were assembled and checked for quality using the program Geneious v.7.1 (Biomatters) and aligned by eye. The generated sequences were stored at GenBank (accession numbers in the Supplementary material, Table 2).

Phylogenetic relationships among specimens were inferred through Bayesian and Maximum Likelihood approaches for COI and *CytB* datasets. Bayesian Inference (BI) was performed in MrBayes 3.2.6 (Ronquist et al. 2012). We conducted two independent runs consisting of four Markov chain Monte Carlo (MCMC) chains each, which were run for 3 million generations. Chains were sampled every 1000 generations and the first 25% of the sampled trees and estimated parameters were discarded as burn-in. Stationarity of runs was checked in Tracer v.1.6 (Rambaut et al. 2014) by examining the average standard deviation of split frequencies (Ronquist and Deans 2010). Maximum likelihood (ML) analysis was implemented in GARLI 2.0 (Zwickl 2006), with 5 independent searches of 5 million generations each. The best topology, i.e., the tree with the smallest likelihood (Ln) value, was used to plot the result of 100 bootstrap replicates. Nucleotide substitution models best explaining the variation observed in the datasets were estimated with MEGA 6 (Tamura et al. 2013) and applied for both BI and ML approaches. We rooted the analyses using sequences of *Pteronotus psilotis* and the remaining species in the subgenus *Phyllodia*.

*External morphometry and craniodental characters*

A suite of ten external morphological and 21 craniodental characters were measured based on Eger (1977) and Freeman (1981), in millimetres (mm), using digital callipers accurate to 0.01 mm. External characters included: Forearm (Forearm); Total length (Total length); Tail length (Tail);

Thumb length (Thumb); Calcar length (Calcar); Tragus width (TragusW); Tragus height (TragusH); Hind foot length (Foot); Ear length (Ear); Tibia length (Tibia) and Nail length (Nail). Craniodental characters were: Occipitonasal length with incisor (ONLI); Occipitonasal length without incisor (ONL); Condylobasal length with incisor (CBLI); Condylobasal length without incisor (CBL); Zygorostral length (ZRL); Braincase depth (BD); Braincase width (BW); Maxillary toothrow length (MTL); Upper canine height (UCH); Rostral width (RW); Interorbital width (IOW); Zygomatic width (ZW); Palatal width (PW); Palatal length (PL); Canine-canine width (CCW); Molar-molar width (MMW); Mastoid width (MW); Ectotympanic bulla length (ETBL); Mandibular toothrow length (MDL); Mandibular condylocanine length (MCCL) and Mandibular intercondylar width (MICW) (see Supplementary material, Fig. 1).

*Statistical analysis*

Discriminant Function Analysis (DFA) with Jackknife cross-validation was performed separately for the acoustic, external morphological and craniodental datasets, and visualized using Principal Component Analysis (PCA). Groups were defined according to the different phonic types. In order to reduce multicollinear variables in the analysis, a multiple correlation test was performed with the R package "corrplot" (Wei 2013). All variables with correlations > 0.7 were discarded. The following parameters were kept for the analyses: ONLI, BD, BW, RW, ZW, PL, MMW and MW for craniodental measurements; Forearm, Thumb, Nail, Ear, TragusW, TragusH, Foot, Tail, Calcar and Total length for external morphology; and lastly, Start freq, End freq, FME and Duration for echolocation. In order to compare echolocation, external morphological and craniodental measurements between phonic groups, the non-parametric univariate Mann-Whitney U-test was used for those variables that did not follow normal distributions (Shapiro test $p>0.05$), while for normally distributed variables we chose the univariate parametric Student's t-test. Geographical variation in FME was also visualized with a kernel density plot. All plots were built with the "ggplot2" (Wickham 2009), "ggfortify" (Horikoshi 2009) and "gridExtra" (Auguie 2012)

R packages. All analyses were conducted using R software, version 3.2.4. (R Foundation for Statistical Computing, 2016).

## Results

*Intraspecific geographical variation (French Guiana vs Brazil)*

Comparison of the echolocation call characteristics of *P. rubiginosus* and *Pteronotus* sp. 3 from French Guiana and the Central Amazon revealed significant intraspecific differences for both species between the two localities in FME ($p<0.05$, Mann-Whitney U-test) (Table 1, Fig. 1 & 2). Although all individuals had the same pulse structure - mainly a long constant frequency pulse with short modulated tails at the start and end - *Pteronotus rubiginosus* from the Central Amazon had FME between 55-56 kHz, while in French Guiana it was mainly 52-54 kHz. In fact, the maximum FME found by Thoisy et al. (2014) among all 257 samples was 54.5 kHz, while 93.4% of our recordings were above 54 kHz. Similarly, *Pteronotus* sp. 3, with constant frequency calls around 60 kHz in the Central Amazon, had FME of 59 kHz in French Guiana. Although duration, start and end frequencies were also significantly different within each species across localities ($p<0.05$, Mann-Whitney U-test), all of these parameters showed greater overlap than FME. In all cases, individuals from French Guiana had lower start and end frequencies, but longer durations. DFA based on echolocation data supports a clear separation of 2 clusters within each species with high levels of accuracy (96% and 86% respectively, Fig. 2).

Comparing our measurements with those reported in the literature, also reveals a substantial intraspecific overlap in external morphological measurements (forearm, weight and tibia) between the populations from the Central Amazon and French Guiana (Table 1). In contrast, some craniodental measurements did not overlap (CBL, MTL, PL, MMW, MDL, MCCL for *Pteronotus* sp. 3, and MTL and MMW for *P. rubiginosus*) (Table 1), with both *Pteronotus* sp. 3 and *P. rubiginosus* from the Central Amazon being slightly smaller than those from French Guiana.

229 However, in terms of intraspecific molecular divergence in *CytB* and COI, haplotypes from the
230 Central Amazon are similar or identical to haplotypes from French Guiana (Fig. 4).

231

*Interspecific variation*

DFA based on echolocation data from a total of 87 *Pteronotus* cf. *rubiginosus* (*P. rubiginosus* and *Pteronotus* sp. 3) from the Central Amazon provided substantial evidence regarding the existence of two very distinct groups with high levels of accuracy (99.9%, Fig. 2). The two phonic groups could be easily discerned with no intermediate frequency FME values (Table 1). Significant differences between species in the following variables were found: FME, Startfreq, Endfreq, Maxfreq and Minfreq ($p<0.05$, Student t-test and Mann-Whitney U-test, Fig. 3). However, pulses were similar in duration. FME was the only acoustic parameter that did not show any overlap between species. The first phonic type had a lower FME around 55 kHz while the second group had higher FME values around 60 kHz (Table 1).

When comparing external morphological measurements between the two cryptic species, most of them showed significant differences, but with often broad overlap between measurements (Supplementary material Fig. 2). DFA showed an accuracy of 89.3% when separating both groups after Jackknife cross-validation (Supplementary material Fig. 3a). Several measurements (forearm, tragusW and tragusH, total length, ear, tibia, nail and weight of the individuals) showed significant differences indicating that *Pteronotus* sp. 3 is slightly smaller than *P. rubiginosus* ($p<0.05$; Student t-tests and Mann-Whitney U-tests, Fig. 3). No significant differences were found in either thumb or foot length. In terms of craniodental measurements, most of them also showed significant differences between both species ($p<0.05$, Supplementary material Fig. 4), also supporting the hypothesis that *Pteronotus* sp. 3 is slightly smaller than *P. rubiginosus*. However, this difference was not significant for BD, UCH, IOW, PW, CCW, ETBL and MICW. The DFA including craniodental variables showed significant differences between the two sympatric species slightly better than the one based on external measurements with 92.8% of accuracy (Supplementary material, Fig. 3b).

In total, the COI dataset included 133 specimens with 442 conserved and 209 variable sites, 124 of them being parsimony-informative (Fig. 4A). The *CytB* dataset presented a similar level of genetic variation, with 751 conserved and 389 (229 parsimony-informative) variable sites among 131 individuals (Fig. 4A). Phylogenetic analyses for each molecular marker were highly congruent, pointing to the existence of two partially sympatric sister clades in the Amazon region (Fig. 4A & 4B). Specimens were always assigned to the same phylogenetic position irrespective of the analysis approach (BI or ML) and the molecular marker (COI or *CytB*). These clades are, on average, 6% and 5% divergent from each other in *CytB* and COI haplotypes, respectively.

One of the clades matches the mitochondrial lineage of *P. rubiginosus*, corresponding to *P.* sp. 4 *sensu* Clare et al. (2013), including all samples of the 53 kHz phonic type described by Thoisy et al. (2014) as well as our specimens classified under the 55 kHz category (see Fig. 4A). The second clade is the mitochondrial lineage of *Pteronotus* sp. 3 identified as the 59 kHz phonic type by Thoisy et al. (2014). This clade, correspondingly, encompasses all the samples from our 60 kHz category (see Fig. 4B).

## Discussion

We provide the first evidence of the occurrence of two sympatric cryptic species from the *Pteronotus* cf. *rubiginosus* complex in the Central Amazon, and further demonstrate the existence of significant geographical variation in echolocation call parameters of both species between localities in the Central Amazon and French Guiana. These species correspond to *P. rubiginosus* and *Pteronotus* sp. 3, which are known to occur in sympatry in Suriname, Guyana, French Guiana and the states of Amapá and Pará in the north-eastern Brazilian Amazon (Thoisy et al. 2014). Although in our study area both species were found to coexist in the same habitats - a finding that mirrors the patterns observed for French Guiana (Thoisy et al. 2014) - in some other Central Amazonian localities sympatry between *P. rubiginosus* and *Pteronotus* sp. 3 has not been found (e.g. Appel et al. 2017, de Oliveira et al. 2015). This suggests that although sympatry is found between the species, microhabitat segregation or specific requirements might also occur among them. This supports the hypothesis that both species may coexist in similar habitats without major ecological competition, as described for *Rhinolophus mehelyi* and *Rhinolophus euryale* by Russo et al. (2005). Contrary to what was suggested by López-Wilchis et al. (2016), our results indicate that the distributions of these lineages in South America do not follow an arch-like biogeographical shape. Our reports increase the extent of the known ranges for both species in the Brazilian Amazon, filling major knowledge gaps in their distribution in central South America (López-Wilchis et al. 2016, Pavan and Marroig 2017). In addition, the intraspecific geographic variation in echolocation call parameters found in both species opens new research questions regarding the origin and persistence of such variation between populations.

*Interspecific variation (Pteronotus rubiginosus vs Pteronotus sp. 3)*

As previously reported for French Guiana (Barataud et al. 2013, Thoisy et al. 2014), also in the Central Amazon both species correspond to two entirely distinct phonic types, with non-overlapping FME, which enables their reliable bioacoustic separation. As Thoisy et al. (2014) demonstrated, echolocation calls from hand-recorded *Pteronotus* cf. *rubiginosus* do not differ from those of free-flying bats, which suggests that echolocation differences can be used to reliably differentiate between these species in the field. Acoustic divergence allows bats to target different prey and hence promotes resource partitioning (Russo et al. 2011). In general, bats calling at higher frequencies tend to target smaller prey, leading to the emergence of disruptive selection (Houston et al. 2004). However, due to the relatively small difference in FME between the sympatric populations of these two lineages of *Pteronotus* it seems unlikely that these differences are related to prey size. Jiang et al. (2013) and Lin et al. (2014) suggested that variations of 5-7 kHz in FME do not impact prey detection ability and thus, might not directly affect resource use.

According to Clare et al. (2013) interspecific divergence in echolocation found between *Pteronotus* sp. 3 and *P. rubiginosus* is more likely to be a consequence of primarily drift in allopatric populations (Puechmaille et al. 2012, Puechmaille et al. 2011) or selection for non-interference in sympatric groups due to local adaptation and restrictive social interactions (Kingston et al. 2001). These social interactions occur when, for instance, some populations specialize in using lesser-used harmonics of their fundamental calls (a process known as "harmonic hopping"), creating an almost instantaneous method of reproductive isolation among conspecifics (Kingston and Rossiter 2004).

Our results support the findings of Thoisy et al. (2014) and Clare et al. (2013) from French Guiana that, despite *Pteronotus* sp. 3 being slightly smaller than *Pteronotus rubiginosus*, external morphological measurements due to great overlap are not useful for the separation of the two sympatric species in the field. Although the skull measurements seem to overlap less than external

morphological variables, the differences are small and, as shown by the low accuracy of the craniodental DFA, they might not be sufficient for reliable species identification.

*Intraspecific divergence (geographical variation)*

*Pteronotus* cf. *rubiginosus* includes some of the few Neotropical bat species with high-duty cycle echolocation with very constant frequency calls. Due to the physical nature of this type of pulses, they tend to be less affected in structure and shape by environmental variables (e.g., weather condition or clutter) than other types of pulses, thus being more suitable for assessing intraspecific geographical variation. We provide the first insights into geographical variation in echolocation call characteristics for the genus *Pteronotus* from the Amazon. Intraspecific acoustic differences found in *Pteronotus* sp. 3 and *P. rubiginosus* between French Guiana and the Central Amazon are very distinctive with very little overlap in some acoustic measurements, especially FME. However, this variation present within both species does not compromise our ability to differentiate between them acoustically, independently of the location where they have been recorded. Nevertheless, this intraspecific geographical variation should be taken into consideration to improve the performance of automatic classification algorithms and should be the subject of further study, incorporating a wider range of localities in the analysis. We provide evidence that intraspecific variation has a unique acoustic signature for each locality, forming two clearly separated clusters, as shown in the DFA, with 96 and 86% of classification accuracy and with most of the variables showing significant differences between locations.

Describing intraspecific variation in echolocation across localities and environments is crucial for bat research and conservation. In fact, unravelling geographic variation in bat calls fundamentally aligns with the concerns recently raised by many acoustic experts about the deficiencies of many bat call libraries, and highlights some important requirements to be considered when designing new reference libraries. Compiling a wide range of recordings from the same species, covering as many localities and environmental conditions as possible, is essential to better train and increase

343 the accuracy of automatic classifiers, which are becoming more and more widely used (Russo and
344 Voigt 2016) and are currently being developed by several companies worldwide, including for
345 Neotropical species.

346 Acoustic signals are known to vary geographically due to the combined effects of genotype and
347 environmental characteristics (Wilczynski et al. 1999). The similar environments in which both
348 *Pteronotus* sp. 3 and *P. rubiginosus* occur and the consistency in the variation of their
349 echolocation calls in French Guiana and Brazil suggest the potential existence of a certain degree
350 of isolation between these species in both regions.

351 In contrast to what has been found in birds, where genetic discontinuities are due to dialect
352 boundaries (Slabbekoorn and Smith 2002), acoustic divergence within bat species is unlikely to be
353 a barrier to gene flow between geographically dispersed bat populations or a limitation for their
354 ecological adaptations. Indirect ecological selection and cultural drift has been previously
355 suggested to explain geographic variation in *Hipposideros armiger* in south China (Lin et al.
356 2014). The same pattern was observed by Puechmaille et al. (2011) in bats from Thailand, that
357 showed acoustic divergence due to local adaptations. Because of the physical nature of acoustics,
358 small morphological differences in the vocal apparatus, or even in body size, could also be
359 responsible for producing different frequencies (Lin et al. 2014). For instance, it has been shown
360 that a larger larynx produces lower frequencies than a smaller one (Jones 1999). Despite the fact
361 that these differences have been detected at the family level (Jones 1999) they are rarely found
362 intraspecifically (Lin et al. 2014).

363 In bats, intraspecific acoustic communication is essential for most social interactions, individual
364 recognition and sexual selection (Wilczynski et al. 1999), which would suggest that geographical
365 variation might potentially represent a starting stage of divergence (Clare et al. 2013). Analysis of
366 social calls could unravel some intraspecific behavioural isolation barriers as has already been
367 found in other bats with similar echolocation such as *Rhinolophus ferrumequinum* (Sun et al.

368 2013) and *Rhinolophus monoceros* (Chen et al. 2009). Unfortunately, social calls have not been
369 well documented yet for the target species. Further, *CytB* and COI haplotypes from the Central
370 Amazon are very similar or identical to those from the Brazilian state of Amapá and French
371 Guiana suggesting that, at least for these mitochondrial markers, there is no evidence of genetic
372 isolation between these populations. Given the relative lack of barriers preventing contact between
373 bat populations from the Central Amazon and French Guiana, the documented acoustic variation
374 needs to be further studied in geographically intermediate locations to understand the potential
375 isolation processes that could be causing the described divergence in echolocation and to
376 determine whether this variation is either discrete or continuous.

377

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## Tables

Table 1. Echolocation, external morphology and craniodental data for *Pteronotus rubiginosus* and *Pteronotus* sp. 3 from French Guiana (Barataud et al. 2013, Thoisy et al. 2014), Guyana (Clare et al. 2013) and the Central Amazon (current study). Values are shown as mean ± std (min-max). Abbreviations are specified in the methods. *First values correspond to Thoisy et al. 2014 and second values to Barataud et al. 2013.

| | ***Pteronotus* sp. 3** (55kHz) | | | ***Pteronotus rubiginosus*** (60kHz) | | |
|---|---|---|---|---|---|---|
| | **Central Amazon (N=45)** | **French Guiana (N=83, 19) *** | | **Central Amazon (N=20)** | **French Guiana (N=91, 22) *** | |
| FME | 55.12 ± 0.63 (53 - 56.6) | 53.1 ± 0.6 & 52.6 ± 0.5 | | 60.08 ± 0.5 (58.3 - 61.5) | 59.2 ± 0.7 & 58.4 ± 0.7 | |
| Minfreq | 45.49 ± 2.69 (39.2 - 54.4) | | | 48.33 ± 2.14 (41.5 - 53.4) | | |
| Maxfreq | 56.64 ± 1.08 (52.5 - 62.1) | | | 61.54 ± 1.07 (53.8 - 64.7) | | |
| Startfreq | 52 ± 1.67 (42.1 - 55.3) | 49.4 ± 3.8 | | 56.77 ± 1.38 (53.3 - 59.4) | 55.5 ± 1.1 | |
| Endfreq | 45.52 ± 2.7 (39.2 - 54.3) | 45.1 ± 1.6 | | 48.33 ± 2.13 (41.4 - 53.5) | 48.4 ± 1.8 | |
| Duration | 18.51 ± 6.01 (3 - 48) | 25.2 ± 3.8 | | 18.58 ± 5.09 (7 - 33) | 23.2 ± 4.2 | |
| | **Central Amazon (N=42)** | **French Guiana (N=43)** | | **Central Amazon (N=26)** | **French Guiana (N=65)** | |
| FA | 64.91 ± 0.97 (63.3 - 66.6) | 64.2 ± 0.13 | | 61.92 ± 1.24 (60.4 - 64.5) | 61.8 ± 0.12 | |
| Weight | 26.45 ± 2.67 (23.25 - 35) | 23.9 ± 0.19 | | 22.58 ± 2.37 (20 - 26.5) | 21.7 ± 0.16 | |
| Tibia | 26.07 ± 0.6 (24.7 - 27) | 25.6 ± 0.18 | | 24.58 ± 0.82 (23.7 - 26.4) | 24.0 ± 0.11 | |
| | **Central Amazon (N=7)** | **French Guiana (N=8)** | **Guyana (N=60)** | **Central Amazon (N=8)** | **French Guiana (N=14)** | **Guyana (N=74)** |
| ONLI | 23.18 ± 0.27 (22.8 - 23.4) | 23.54 (22.94 - 24.30) | | 21.95 ± 0.33 (21.6 - 22.4) | 22.38 (21.74 - 22.83) | |
| ONL | 22.34 ± 0.23 (22 - 22.5) | 22.85 (22.25 - 23.30) | | 20.89 ± 0.43 (20.2 - 21.3) | 21.80 (21.46 - 22.20) | |
| CBLI | 22.78 ± 0.28 (22.5 - 23.2) | 22.55 (22.22 - 22.90) | 21.38 (20.61 - 22.65) | 21.54 ± 0.28 (21.1 - 21.9) | 21.48 (21.03 - 22.11) | 22.45 (20.84 - 23.23) |
| CBL | 22.22 ± 0.23 (21.9 - 22.5) | 21.48 (20.96 - 21.90) | | 20.96 ± 0.3 (20.5 - 21.3) | 20.27 (19.90 - 20.7) | |
| MTL | 9.62 ± 0.26 (9.2 - 9.9) | 10.21 (10 - 10.44) | 9.44 (9.11 - 10) | 8.8 ± 0.23 (8.4 - 9.1) | 9.54 (9.22 - 9.83) | 9.96 (9.22 - 10.32) |
| ZW | 13.22 ± 0.24 (12.9 - 13.5) | 13.56 (13.30 - 13.85) | 12.81 (12.28 - 13.80) | 12.71 ± 0.42 (12.2 - 13.4) | 12.98 (12.50 - 13.4) | 13.39 (12.52 - 14.01) |
| MDL | 10.72 ± 0.13 (10.6 - 10.9) | 11.56 (11.35 - 11.77) | 10.03 (9.68 - 10.59) | 10.08 ± 0.39 (9.7 - 10.9) | 10.87 (10.57 - 11.11) | 10.58 (9.86 - 10.99) |
| PL | 11.04 ± 0.32 (10.5 - 11.3) | 11.47 (11.15 - 11.72) | | 10.46 ± 0.27 (10 - 10.8) | 10.82 (10.58 - 11.08) | |
| MMW | 8.24 ± 0.17 (8.1 - 8.5) | 8.76 (8.55 - 8.94) | | 7.64 ± 0.17 (7.3 - 7.8) | 8.35 (8.14 - 8.8) | |
| MCCL | 16 ± 0.21 (15.7 - 16.2) | 16.71 (16.45 - 17.07) | | 15.01 ± 0.2 (14.7 - 15.3) | 15.59 (15.20 - 15.91) | |
| ZRL | 16.5 ± 0.27 (16.2 - 16.9) | | 16.16 (15.48 - 17.36) | 15.7 ± 0.29 (15.3 - 16.2) | | 17.16 (15.79 - 17.7) |
| BD | 10.2 ± 0.47 (9.4 - 10.6) | | 9.13 (8.59 - 9.64) | 10.08 ± 0.21 (9.8 - 10.5) | | 9.33 (8.78 - 9.69) |
| BW | 10.62 ± 0.11 (10.5 - 10.8) | | 10.77 (10.27 - 11.33) | 10 ± 0.33 (9.5 - 10.4) | | 11.07 (10.61 - 11.42) |
| RW | 8.38 ± 0.13 (8.2 - 8.5) | | 8.56 (8.16 - 8.94) | 7.99 ± 0.27 (7.7 - 8.4) | | 8.92 (8.09 - 9.35) |
| IOW | 4.12 ± 0.04 (4.1 - 4.2) | | 4.61 (4.25 - 5) | 4.06 ± 0.2 (3.8 - 4.3) | | 4.54 (4.17 - 4.92) |

| | | | | |
|---|---|---|---|---|
| MW | 12.08 ± 0.22 (11.9 - 12.4) | 12.06 (11.59 - 13.03) | 11.7 ± 0.32 (11.3 - 12.1) | 12.36 (11.54 - 12.91) |

**Figure captions**

Figure 1. Kernel density plot of FME values recorded for 87 individuals from the Central Amazon and 257 from French Guiana. Yellow and green: *Pteronotus rubiginosus*; Red and orange: *Pteronotus* sp. 3

Figure 2. Principal Component Analysis (PCA) based on the echolocation data.

Figure 3. Comparison between standard echolocation call parameters for the two cryptic species (*Pteronotus* sp. 1 and *Pteronotus rubiginosus*) recorded in French Guiana and the Central Amazon. The median is represented by a thicker horizontal line, the box limits denote the lower (Q1) and upper (Q3) quartiles, and the vertical extending lines are standard deviations. Outliers are plotted as individual dots. Significant intraspecific differences are indicated by an asterisk. Variable abbreviations as specified in the methods.

Figure 4A. Phylogenetic tree using both COI and *CytB* genes for both *Pteronotus rubiginosus* and *Pteronotus* sp. 3 (Pavan & Marroig, 2016).

Figure 4B. Enlargement of the mitochondrial lineage A) *Pteronotus rubiginosus* and B) *Pteronotus* sp. 3. Above right: Map displaying the geographic ranges of both species in South America.

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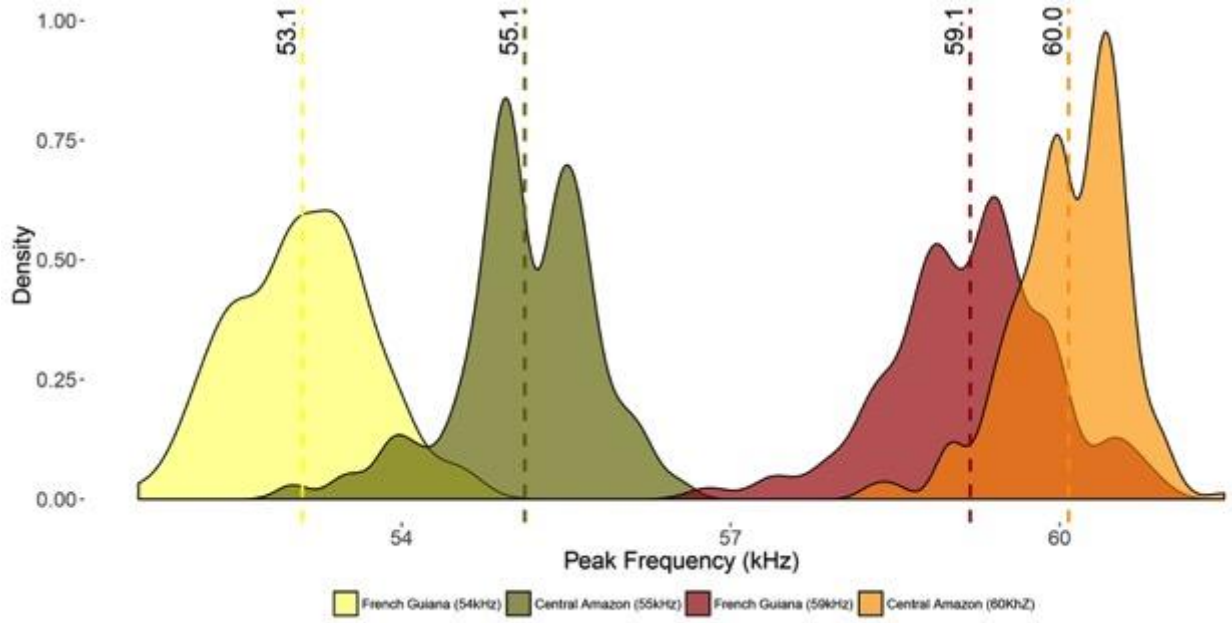
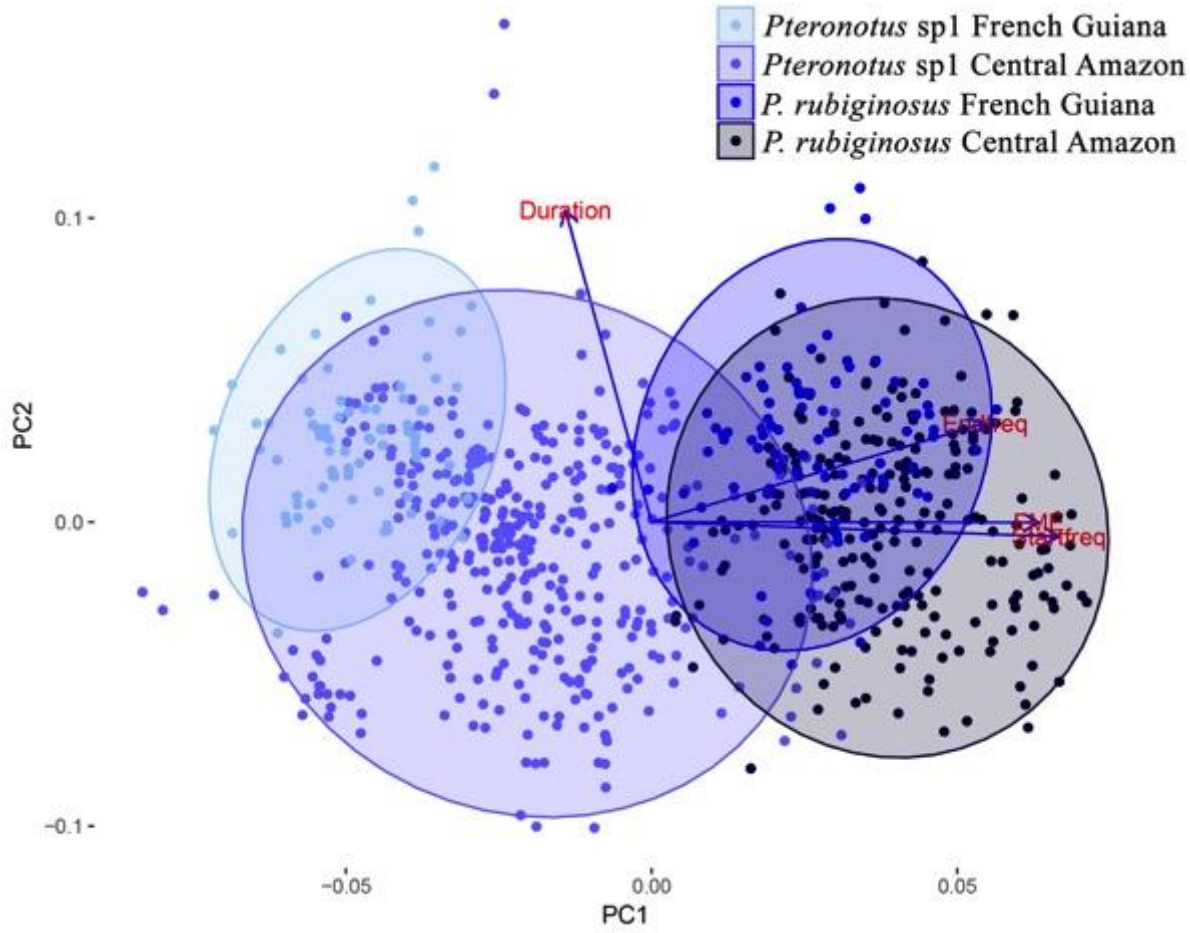

53.1
55.1
59.1
60.0
1.00
0.75
0.50
0.25
0.00
Density
54
57
60
Peak Frequency (kHz)
French Guiana (54kHz)
Central Amazon (55kHz)
French Guiana (59kHz)
Central Amazon (60KhZ)

Fig. 1


*Pteronotus* sp1 French Guiana
*Pteronotus* sp1 Central Amazon
*P. rubiginosus* French Guiana
*P. rubiginosus* Central Amazon
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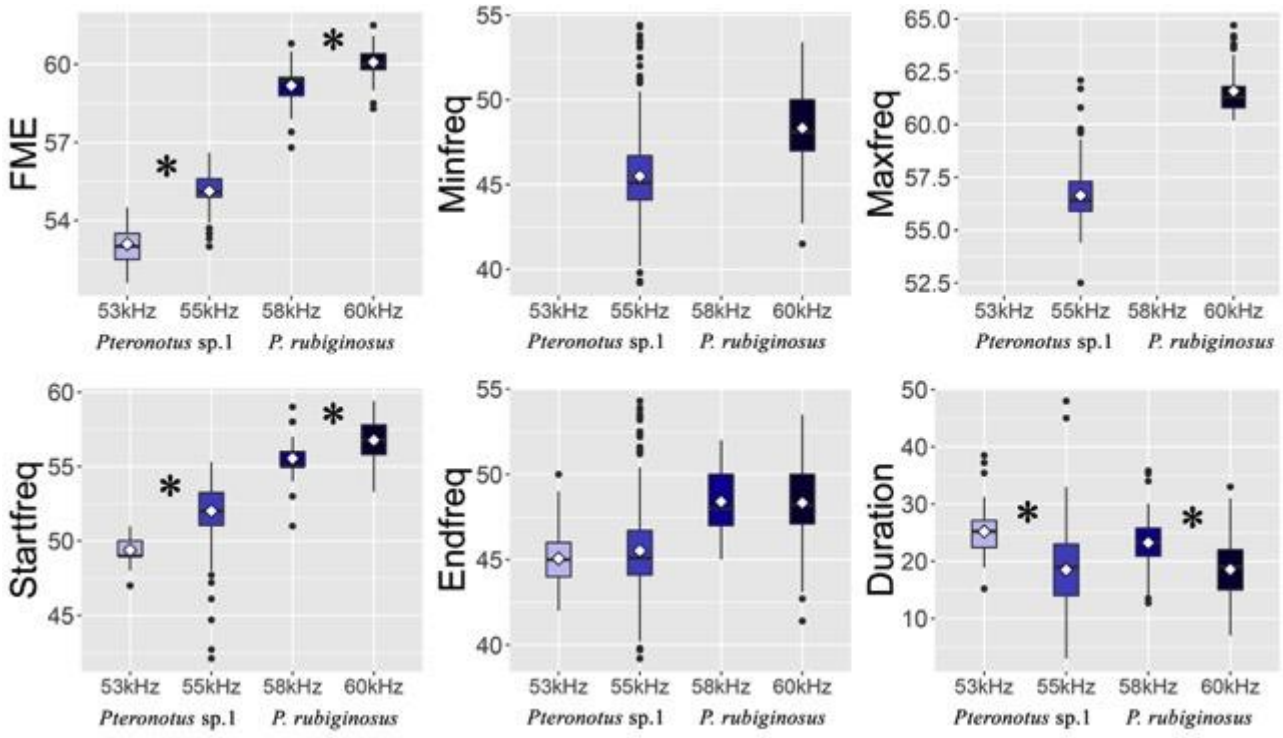
0.0
−0.1
PC2
−0.05
0.00
0.05
PC1

Fig. 2


FME
Minfreq
Maxfreq
Startfreq
Endfreq
Duration
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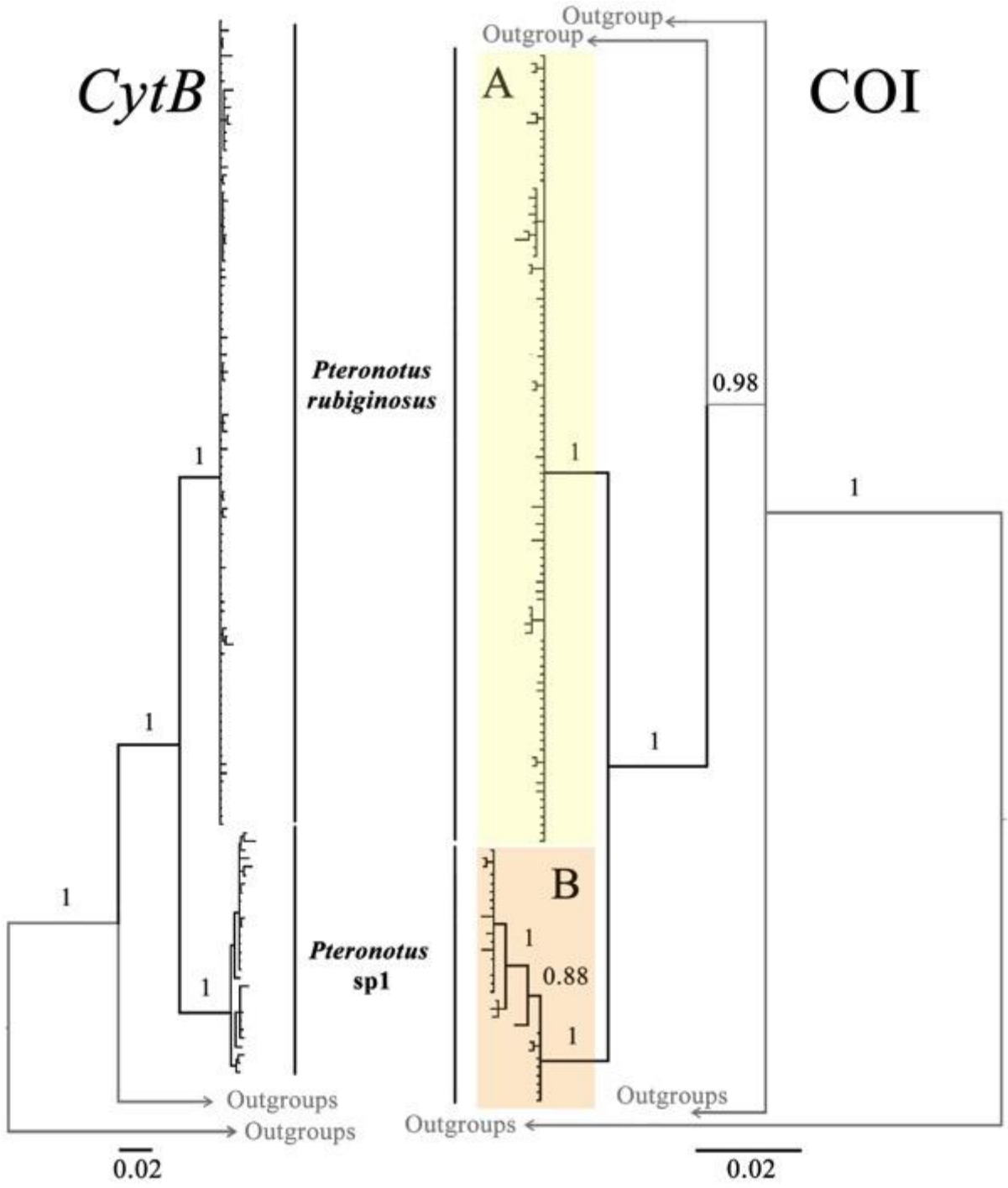
55kHz
58kHz
60kHz
Pteronotus sp.1
P. rubiginosus

Fig. 3


CytB
COI
Outgroup
Outgroup
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B
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1
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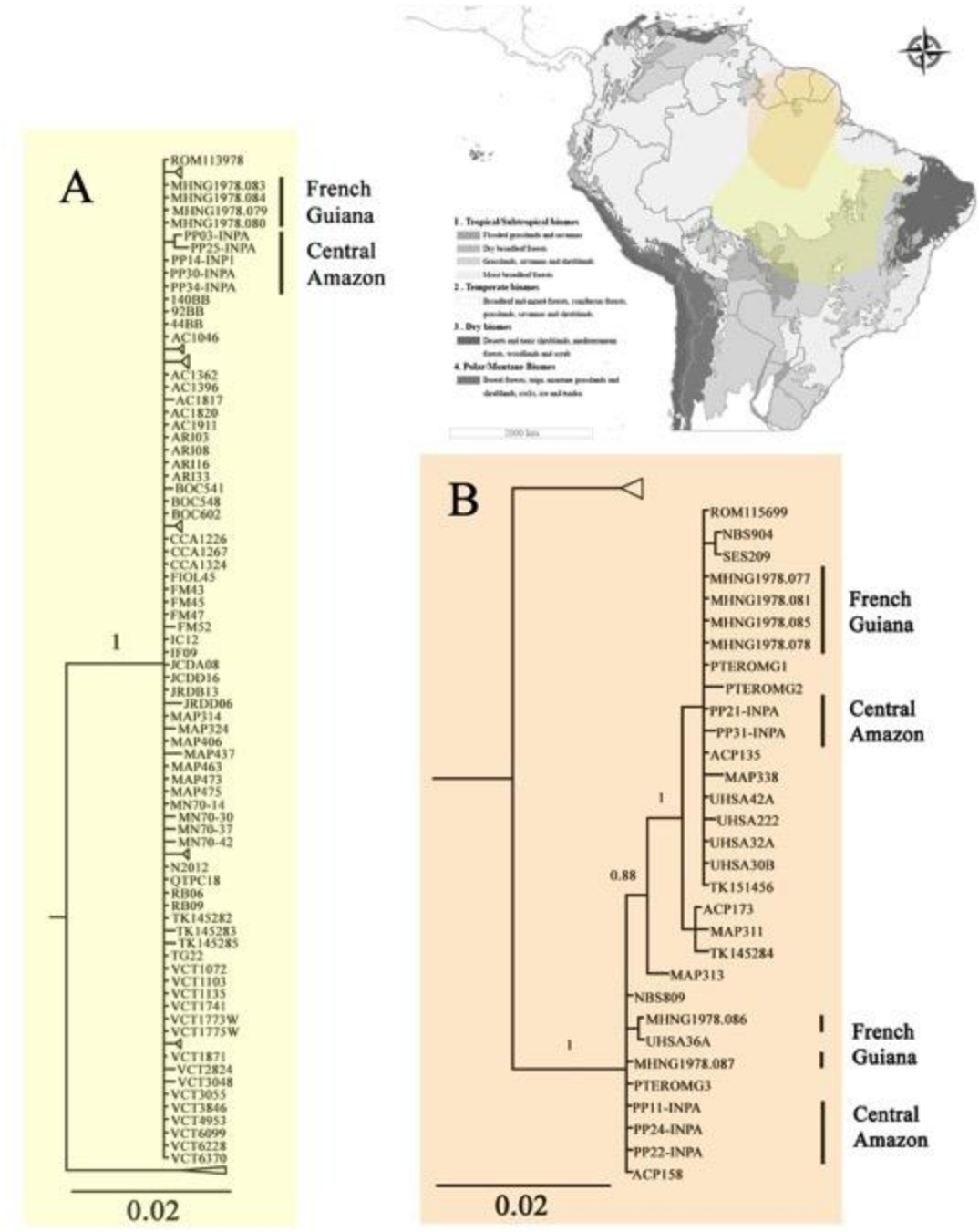
Outgroups
Outgroups
Outgroups
Outgroups
0.02
0.02

Fig. 4A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AC1362
AC1396
AC1817
AC1820
AC1911
ARI03
ARI08
ARI16
ARI33
BOC541
BOC548
BOC602
CCA1226
CCA1267
CCA1324
FIOL45
FM43
FM45
FM47
FM52
IC12
IF09
JCDA08
JCDD16
JRDB13
JRDD06
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MAP406
MAP437
MAP463
MAP473
MAP475
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French Guiana
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1. Tropical/Subtropical biomes
2. Temperate biomes
3. Dry biomes
4. Polar/Montane Biomes
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MHNG1978.081
MHNG1978.085
MHNG1978.078
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MAP313
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0.02
French Guiana
Central Amazon
French Guiana
Central Amazon

Fig. 4B