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Integrating multiple evidences in taxonomy: species diversity and phylogeny of mustached bats (Mormoopidae: *Pteronotus*)

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ABSTRACT

A phylogenetic systematic perspective is instrumental in recovering new species and their evolutionary relationships. The advent of new technologies for molecular and morphological data acquisition and analysis, allied to the integration of knowledge from different areas, such as ecology and population genetics, allows for the emergence of more rigorous, accurate and complete scientific hypothesis on species diversity. Mustached bats (genus *Pteronotus*) are a good model for the application of this integrative approach. They are a widely distributed and a morphologically homogeneous group, but comprising species with remarkable differences in their echolocation strategy and feeding behavior. The latest systematic review suggested six species with 17 subspecies in *Pteronotus*. Subsequent studies using discrete morphological characters supported the same arrangement. However, recent papers reported high levels of genetic divergence among conspecific taxa followed by bioacoustic and geographic agreement, suggesting an underestimated diversity in the genus. To date, no study merging genetic evidences and morphometric variation along the entire geographic range of this group has been attempted. Based on a comprehensive sampling including representatives of all current taxonomic units, we attempt to delimit species in *Pteronotus* through the application of multiple methodologies and hierarchically distinct datasets. The molecular approach includes six molecular markers from three genetic transmission systems; morphological investigations used 41 euclidean distances estimated through three-dimensional landmarks collected from 1628 skulls. The phylogenetic analysis reveals a greater diversity than previously reported, with a high correspondence among the genetic lineages and the currently recognized subspecies in the genus. Discriminant analysis of variables describing size and shape of cranial bones support the rising of the genetic groups to the specific status. Based on multiples evidences, we present an updated taxonomic arrangement composed by 16 extant species and a new and more robust phylogenetic hypothesis for the species included in the genus *Pteronotus*. Studies developed under such integrative taxonomic approach are timely for a deeper and wider comprehension of Neotropical diversity, representing the first step for answering broader questions on evolutionary and ecological aspects of Neotropical life history.

1. Introduction

The field of systematics has experiencing a major shift regarding how to infer evolutionary patterns as well as recognize and delimit species. The introduction of molecular data in phylogenetic studies provided many conceptual challenges in the area, affecting not only methods for reconstructing phylogenies, but also the relationship of systematics with other disciplines, such as phylogeography (Avise et al., 1987) and coalescent theory (Kingman, 1982). The integration of concepts from adjacent areas to elucidate taxonomic issues in systematic studies (Dayrat, 2005; Will et al., 2005; Schlick-Steiner et al., 2010) has also proven fruitful, allowing the emergence of more rigorous, accurate and complete scientific hypothesis. This integrative approach is particularly important for biological groups whose species boundaries are not obviously correlated with morphological changes (Bickford et al., 2007; Adams et al., 2009), which are traditionally used in taxonomy as main evidences for delimiting species (Wiens, 2007).

Bat diversity in the Neotropical region is steadily increasing due to more frequent and powerful systematic investigations that have been developed recently. According to Simmons (2005) the order Chiroptera harbors 18 families, 186 genera and around 1100 species, while this estimate was 10% smaller five years before (~1001 species *sensu* Hutson et al., 2001). Phylogenetic studies

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with bats have been published in large numbers, with well-supported phylogenies being currently available for many groups. This increasing dataset relies mostly on molecular information, which suggests that several bat taxa actually include more species than previously believed or sometimes do not even represent monophyletic groups (Clare, 2011; Pavan et al., 2013; Velazco and Patterson, 2008, 2013; Parlos et al., 2014). These more refined evolutionary hypotheses are offering important opportunities for reconstructing historical patterns of change in several bat lineages. Likewise, they are central to investigations in many related areas, such as biogeography, ecology and evolution.

The studies investigating the phylogenetic relationships within Mormoopidae reflect such pursuit. The interest on this small neotropical family of insectivorous bats increased rapidly in the last years, including studies with morphological (Simmons and Conway, 2001), molecular (Lewis-Oritt et al., 2001; Van Den Bussche and Weyandt, 2003) and combined analyses (Van Den Bussche et al., 2002; Dávalos, 2006). The taxonomy of Mormoopidae is controversial and through the 20th century it was even classified as one subfamily of Phyllostomidae (Chilonycterinae). Smith (1972) raised it to family rank, recognizing within the group two genera – *Mormoops* and *Pteronotus*, three subgenera – *Pteronotus*, *Chilonycteris* and *Phyllodia* (all in the genus *Pteronotus*), and eight species (two in *Mormoops*, six in *Pteronotus*), most of them with associated geographical variation described as subspecies (Table 1). Two extinct species are also described in the family, *Mormoops magna* and *Pteronotus pristinus* (Silva-Taboada, 1974), and a new genus and species is being described (Morgan and Czaplewski, 2012). A brief synopsis on the natural history of mormoopid species is provided by Simmons and Conway (2001) and Patton and Gardner (2007).

The subsequent studies published for Mormoopidae confirmed independently the monophyly of the family and genera, although did not provide an unambiguous and well supported phylogeny for this group. The initial investigations on the genetic diversity of Mormoopidae reported divergent lineages within many *Pteronotus* species (Lewis-Oritt et al., 2001; Dávalos, 2006), suggesting an underestimated diversity in this genus. Recently, more comprehensive studies were performed, and results suggest a much more complex evolutionary history for the genus *Pteronotus* than previously described (Clare et al., 2011, 2013; Thoisy et al., 2014). Thus, additional species have been recognized in the subgenus *Phyllodia*, elevated from the subspecies rank by such recent studies (*P. paraguanensis*, Gutiérrez and Molinari, 2008; *P. mesoamericanus*, Clare et al., 2013; *P. pusillus* and *P. portoricensis*, Thoisy et al., 2014).

A deeper knowledge on the diversification pattern of this bat group has also being sought because of their echolocation system. While the majority of mormoopids as well as all the remaining Neotropical bat species are low duty cycle (LDC) echolocators, the extant representatives of subgenus *Phyllodia* (henceforth mentioned as *P. parnellii* complex) have evolved the high-duty cycle (HDC) echolocation, which is also present in the Old World families Rhinolophidae and Hipposideridae. HDC bats use long, narrowband echolocation calls that improve their ability to detect, lock onto and track flying prey, probably in areas of high clutter (for a review about this topic see Fenton et al., 2012). The evolution of this

Table 1
Current taxonomic diversity in the family Mormoopidae according to the last systematic review (Smith, 1972) with updates provided by Simmons (2005).

Species	Subspecies	Type locality	Geographic range
Pteronotus parnellii	*P. p. parnellii*	Jamaica (unspecified locality)	Cuba and Jamaica
	P. p. pusillus	Arroyo Salado, Dominican Republic	Dominican Rep. and Haiti
	P. p. gonavensis[b]	En Café, La Gonave Island, Haiti	Gonave Island, Haiti
	P. p. portoricensis	Cueva di Fari, Pueblo Viejo, Puerto Rico	Puerto Rico
	P. p. mexicanus	San Blas, Nayarit, Mexico	Sonora and Tamaulipas to Oaxaca and Veracruz, Mexico
	P. p. mesoamericanus	Yepocapa, Chimaltenango, Guatemala	Southern México and Pacific coast of Central America to Panama
	P. p. fuscus	Las Quiguas, Puerto Cabello, Venezuela	Caribbean coast of Colombia and Venezuela, north of Orinoco river
	P. p. rubiginosus	Caiçara, Mato Grosso, Brazil	From Honduras southward along the Caribbean coast of Central America; Trinidad; Amazonian lowlands and Guiana Shield, south of Venezuela, Peru and Central Brazil
Pteronotus paraguanensis[a]	–	Pueblo Nuevo, Falcón, Venezuela	Paraguana peninsula, Venezuela
Pteronotus personatus	*P. p. psilotis*	Tehuantepec, Oaxaca, Mexico	Caribbean Coast of Honduras and El Salvador to Sonora and Tamaulipas, Mexico
	P. p. personatus	São Vicente, Mato Grosso, Brazil	Central Brazil to pacific coast of Costa Rica
Pteronotus davyi	*P. d. fulvus*	Las Peñas, Jalisco, Mexico	Caribbean Coast of Honduras and El Salvador to Sonora and Tamaulipas, Mexico
	P. d. davyi	Island of Trinidad	Caribbean Coast of South America and Lesser Antilles to south of Nicaragua
	P. d. incae	Suyo, Piura, Peru	Peru
Pteronotus gymnonotus	–	Cuiabá, Mato Grosso, Brazil	Central Brazil to Veracruz, Mexico
Pteronotus macleayi	*P. m. macleayi*	Guanabacoa, Habana, Cuba	Cuba
	P. m. griseus	Phoenix Park, Westmoreland Parish, Jamaica	Jamaica
Pteronotus quadridens	*P. q. quadridens*	Baracoa, Oriente Province, Cuba	Cuba
	P. q. fuliginosus	Port au Prince, Haiti	Jamaica, Haiti, Dominican Rep., Puerto Rico
Mormoops megalophylla	*M. m. megalophylla*	Parrás, Coahuila, Mexico	USA to Honduras and El Salvador
	M. m. tumidiceps	Point Gourde Caves, Trinidad	Colombia, Venezuela and Trinidad
	M. m. intermedia	Hatto, Curaçao, West Indies	Dutch Islands
	M. m. carteri	La Paz, Carchi Province, Ecuador	Ecuador
Mormoops blainvillei	–	Jamaica	Greater Antilles

[a] Subspecies described by Linares and Ojasti (1974) and recently ranked to the species level (Gutiérrez and Molinari, 2008).
[b] Extinct.

system allowed a distinct foraging strategy by *P. parnellii* complex regarding its congeners, and involved many morphological and physiological adaptations (Fenton et al., 2012; Mancina et al., 2012). Several studies point to a dual function of calls in resource acquisition and communication in HDC bats, suggesting ecological selection on frequency might lead to assortative mating and ultimately reproductive isolation and speciation (Kingston et al., 2001; Kingston and Rossiter, 2004). This hypothesis led Clare et al. (2013) to propose a similar process shaping diversity in mainland populations of *P. parnellii* complex and assume the existence of four different species in this taxon, based on molecular, morphometric and bioacoustic evidences.

Despite the number of studies on mormoopids, previous works did not aim to produce an updated phylogenetic hypothesis for the genus *Pteronotus* as a whole, whose taxonomic diversity is doubtful since its last systematic review (Smith, 1972). To date, no work merging multilocus genetic data and morphometric variation along the entire geographic range of this group, sampling representatives of all taxonomic units, was realized. The present study attempted to delimit species in *Pteronotus* through the application of multiple methodologies and included a wide exploration of data at different hierarchical levels. The use of this integrative approach allowed us to place the evidences presented by last studies into a broad and detailed investigation, with direct taxonomic implications. For this, we started from the taxonomic arrangement proposed by Smith (1972) as our null hypothesis, since it represents the most complete (geographically and taxonomically) study on this genus. In addition, the proposal of a robust phylogenetic hypothesis for the genus *Pteronotus* is timely since it represents a very interesting model for addressing broader evolutionary and ecological questions in Neotropical Region.

2. Materials and methods

2.1. Molecular sampling

Tissue samples of 411 individuals of *Pteronotus* were obtained by loans from Brazilian and foreign research collections (Table S1). The molecular sampling represented 16 out of 18 current nominal taxa within the genus (Smith, 1972) and a wide geographic range (Fig. 1). Total genomic DNA was extracted from muscle or liver tissues by a NaCl/SDS/Proteinase K protocol (Bruford et al., 1992). Alternatively, for those samples provided in low quantities, DNA was extracted using a Qiagen DNeasy Blood & Tissue Kit (Qiagen, Inc.) following the manufacturer's protocol. Six molecular markers were selected for this study, representing three different genetic transmission systems: the entire *Cyt b* gene (CYTB) and a fragment of the COI gene (COI) in the mitochondrial DNA; a non-coding fragment of the *Dby* gene in Y chromosome (DBY); fragments of the autosomal genes STAT5A (non-coding), PRKC1 (non-coding) and RAG2 (coding). The amplification of selected markers was performed via Polimerase Chain Reaction (PCR) with primers and protocols described previously (Baker et al., 2000; Eick et al., 2005; Martins et al., 2007; Borisenko et al., 2008; Lim et al., 2008; Pavan et al., 2013).

Sequences were assembled and checked for quality using the programs Phred, Phrap and Consed (Ewing and Green, 1998; Ewing et al., 1998; Gordon et al., 1998) or, alternatively, using Geneious v.7.1 (Biomatters). Coding regions (CYTB, COI and RAG2) were aligned by eye, whereas the alignment of intron sequences (STAT5A, PRKC1, DBY) was carried out using the ClustalW tool available in MEGA 6 (Tamura et al., 2013). The mitochondrial dataset was also analyzed for saturation with DAMBE5 (Xia, 2013). For

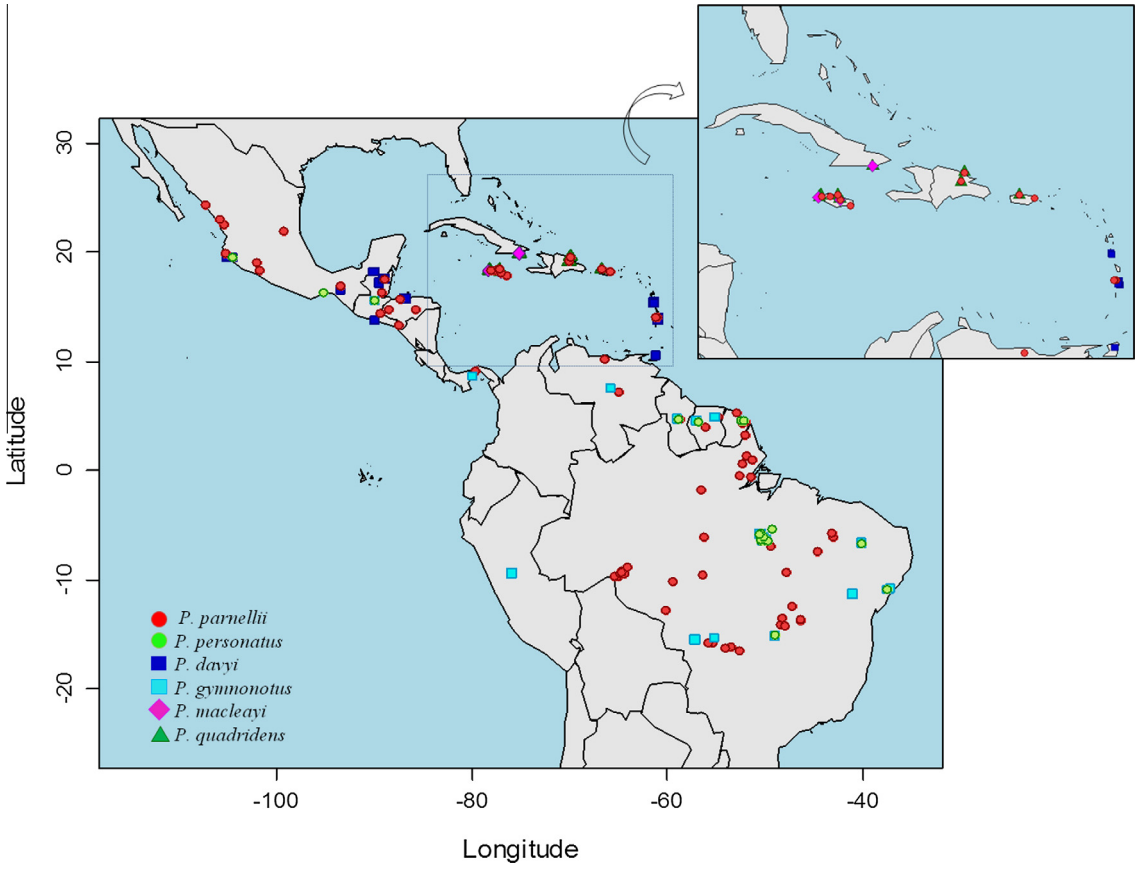
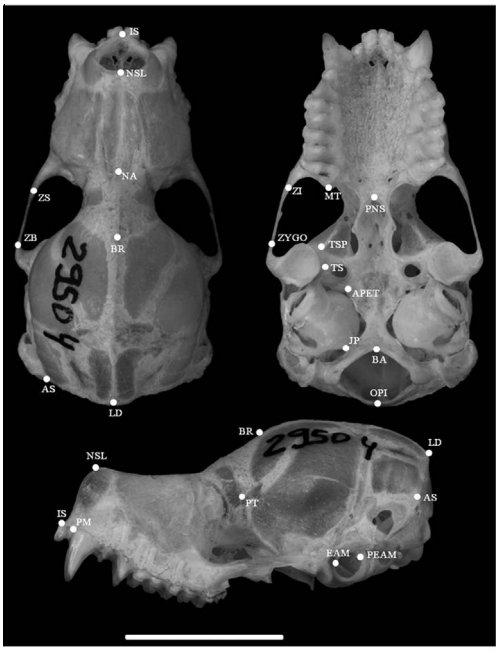


Fig. 1. Geographic distribution of molecular samples used in the present study. Taxonomic names displayed in the legend follow traditional classification (Smith, 1972; Simmons, 2005).

autosomal genes analysis, the presence of heterozygous nucleotide positions was represented in the dataset as ambiguous IUPAC codes. For the introns PRKC1 and STAT5A, heterozygous individuals for the insertion/deletions (INDELS) of bases were also found, and these samples were represented in the phylogenetic dataset by the longer sequences of each individual.

2.2. Morphological sampling and cranial landmarks

The morphometric investigation was performed with 1628 adult specimens from 13 museum collections in Brazil and USA: IEPA (Macapá-Brazil), MZUSP (São Paulo-Brazil), UFMG (Belo Horizonte-Brazil), INPA (Manaus-Brazil), UFPE (Recife-Brazil), UFPB (João Pessoa-Brazil), AMNH (New York-USA), TCWC (College Station-USA), KUM (Lawrence-USA), NMNH (Washington-USA), FMNH (Chicago-USA), LACM (Los Angeles-USA) and TTU (Lubbock-USA). Sampling included localities along the geographic range of all current species (Table S2). We used a Microscribe MX digitizer (Microscribe, IL) to collect three-dimensional (3D) coordinates from anatomical points (bones sutures or other discrete cranial features; named henceforth landmarks) in *Pteronotus* skulls. Twenty-two landmarks were established in the skull of *Pteronotus* species (Fig. 2) and used to estimate 41 Euclidean distances (Table S4) describing size and shape within the genus. This methodology for collecting data provides a more complete description on the skull variability than caliper measurements (Reig, 1996). The adoption of 3D coordinates instead of fixed measurements in the skull may provide a better performance in morphometric analysis since it yields a larger number of distances to the morphometric dataset with similar time effort and associated error. In addition, the use of 3D data allows the storage of skull coordinates for each specimen, which can be further used to create new distances that prove to be useful for morphometric analyses. Furthermore, the distances used here are meant to capture local developmental/functional processes while at the same time represent the whole skull (Cheverud, 1982, 1996). Thus, traits are not redundant and do not capture general growth process as usual in the case of caliper measures since individual bones are represented (Cheverud, 1996). All landmarks were collected twice per specimen. Because asymmetry between the right and left sides of the skull is not of interest in this study, measurements present in both sides were treated as their average. Whenever one side of the skull was damaged, the other was considered as the mean. Normality and outlier analyses were also performed to check the quality of the collected data. All subsequent analyses were carried out using the average of repeated measurements.



Landmarks description

1) Intradentale superior (IS)
2) Premaxillary suture at the alveolus, right and left (PM)
3) Nasale (NSL)
4) Nasion (NA)
5) Bregma (BR)
6) Pterion, right and left (PT)
7) Zygomaxilare superior, right and left (ZS)
8) Zygomaxilare inferior, right and left (ZI)
9) Maxillary tuberosity, right and left (MT)
10) Posterior nasal spine (PNS)
11) Anterior petrous temporal, right and left (APET)
12) Básion (BA)
13) Opistion (OPI)
14) Anterior external auditory meatus, right and left (EAM)
15) Posterior external auditory meatus, right and left (PEAM)
16) Inferior zygo-temporal suture, right and left (ZYGO)
17) Temporo-spheno-parietal junction, right and left (TSP)
18) Temporo-sphenoidal junction at the petrous, right and left (TS)
19) Jugular process, right and left (JP)
20) Lambda (LD)
21) Asterion, right and left (AS)
22) Zigomatic breadth, right and left (ZB)

Fig. 2. Anatomical position (dorsal, ventral and lateral views) and description of the 22 landmarks collected in the skulls of *Pteronotus*.

2.3. Phylogenetic analyses

The phylogenetic analyses were initially performed for each marker separately in order to observe congruence among topologies. Four different datasets were then constructed for evaluating the importance of different factors such as the genetic transmission systems and the number of base pairs in the performance of analysis: (1) mitochondrial dataset (mtDNA: CYTB and COI); (2) fast-evolving nuclear dataset (nDNA: introns PRKC1 and STAT5A); (3) five-gene dataset (CYTB, COI, PRKC1, STAT5A and DBY); (4) complete dataset (CYTB, COI, PRKC1, STAT5A, DBY and RAG2).

Additional sequences of *Pteronotus* species from GenBank were included in the datasets when available (Table S1). GenBank sequences from *Peropteryx kappleri* (Emballonuridae), *Noctilio albiventris* (Noctilionidae), *Mormoops megalophylla* (Mormoopidae), *Glossophaga soricina* and *Trachops cirrhosus* (Phyllostomidae) were also used as outgroups in phylogenetic analysis. Since there are no available sequences of DBY gene for *M. megalophylla* and *N. albiventris* at GenBank, these species were represented as missing data for this locus in the five-gene and complete datasets.

Nucleotide substitution models that best explained the variation observed in each dataset were estimated by PartitionFinder v.1.1.0 (Lanfear et al., 2012). These models of molecular evolution allowed the setting of partitioned schemes in the datasets for subsequent multilocus analysis via Bayesian and Maximum Likelihood methods. Bayesian Inference (BI) was implemented in MrBayes 3.2.2 (Ronquist and Huelsenbeck, 2003) using four Markov chain Monte Carlo (MCMC) in two independent runs at 10 million generations each for the *mtDNA dataset*, and 5 million generations for the others. Sampling of chains occurred every 1000 generations and the first 25% of the sampled trees and estimated parameters were discarded as burn-in. Stationarity of runs were checked in Tracer v.1.6 (Rambaut et al., 2013) by examining the average standard deviation of split frequencies (Ronquist et al., 2010). For Maximum Likelihood (ML) analyses, five to eight independent searches with 5 million generations were performed and compared for each dataset in Garli 2.0 (Zwickl, 2006). The tree with the smaller likelihood (Ln) value was kept as the best topology and used to plot the result

of 100 bootstrap replicates in each dataset. BI and ML analyses were run on the CIPRES Science Gateway. Maximum parsimony (MP) analyses were performed in TNT v.1.1 (Goloboff et al., 2003) by 1000 replicates of heuristic searches using a combination of tree-searching algorithms (*sectorial searches, drift, ratchet* and *tree-fusing mixed trees*). Node supports in MP analysis were accessed by resampling via bootstrap and the values were plotted in the majority consensus tree obtained by MP searches.

2.4. Species tree

In addition to phylogenetic analyses, a Bayesian framework for species tree estimation from multilocus data was adopted. This approach considers the incomplete lineage sorting as one of the major causes of gene tree heterogeneity and gene tree/species tree conflicts (Heled and Drummond, 2010). The complete dataset was used for the species tree estimate, adopting independent nucleotide substitution models for each dataset partition according to the result obtained by Partition Finder. The analysis was performed in *BEAST (StarBEAST), an extension within the software package BEAST v.1.8 (Drummond and Rambaut, 2007). The settings included a run for 50 million generations, with MCMC sampling every 5000 generations. A Yule speciation model was used as tree prior, with an exponential mean of 0.8 (yule.birthRate). Substitution rate prior for each partition (ucld.mean) followed a lognormal distribution model with mean 0.05 substitutions/site/million years (subs/site/my) for mtDNA and mean 0.02 for introns, with a standard deviation of 2. A total of 10,000 trees were generated and summarized to produce the topology with most credibility based on data, which was visualized in the software FigTree v.1.4 (Rambaut, 2012).

2.5. Morphometric analyses

Exploratory analyses were done in the complete dataset in order to observe the distribution of values for each variable and deviations from normality. Principal Component Analysis (PCA) was subsequently performed to investigate the distribution of each current taxon (*sensu* Smith, 1972) within the total cranial space variation of the genus. Then, Multivariate Analysis of Variance (MANOVA) and Discriminant Function Analysis (DFA) were applied in *a priori* defined categories. These categories were established according to results obtained by molecular data analyses. Therefore, the grouping hypothesis for morphometric analyses was based on the phylogeny found, which allowed the identification of operational taxonomic units (OTUs) congruent with the evolutionary history of the genus (evolutionary lineages, *sensu* de Queiroz, 1998). MANOVA was performed in defined categories to identify the existence of sexual variation. In case of statistical significance ($p < 0.05$), variation between genders was controlled before dataset's using in subsequent analysis. DFA was applied in each *Pteronotus* species group in order to test if cranial morphometrics could discriminate specimens according to the grouping hypothesis based on molecular data. MANOVA analyses were performed in R software environment (R Development Core Team, 2013) while SYSTAT 11 (SYSTAT Software, Inc. 2004) was used for PCA and DFA analyses.

3. Results

3.1. Molecular variation and evolutionary models

From the total available tissue samples, 384 were sequenced for, at least, one fragment of mtDNA (Table S1). This allowed the assignment of these samples in the evolutionary lineages identified in the genus *Pteronotus*, even if they were not used in final data analysis. Complete sequences of CYTB (1140 bp) and COI (651 bp) were obtained for 328 and 350 specimens, respectively, while *mtDNA dataset* was represented by 314 individuals. Both mitochondrial markers presented similar levels of genetic variation and non-significant results in the test of substitution saturation. Due to the high number of available tissue samples, we obtained a genetic variation overview by preliminary analysis with mtDNA only. The remaining molecular markers were then sequenced for a subset of the available samples, which was representative of the genus phylogenetic diversity. Introns of PRKC1 (462 bp) and STAT5A (602 bp) were sequenced in 128 specimens and presented a similar variation to that observed for the 64 males sequenced for DBY (510 bp). The nuclear exon RAG2 (804 bp) was sequenced in 27 specimens and, as expected, showed the smaller variation within data. The complete information about molecular variation and evolutionary models of each analyzed dataset is available in Table S3.

3.2. Phylogenetic inferences

Phylogenetic analysis of each marker separately showed highly congruent, recovering similar patterns of relationship among specimens. As a general result, molecular data points to the existence of four major clades (subsequently referred to as Clades 1–4) within the genus, regardless which dataset and searching method was adopted: Clade 1 comprises *P. davyi* and *P. gymnonotus*; Clade 2 includes *P. macleayi* and *P. quadridens*; Clade 3 corresponds to *P. personatus*; Clade 4 encompasses all samples of *P. parnellii*. All phylogenies confirmed the genus monophyly and presented the same arrangement of individuals within these clades. Analysis of mtDNA and nDNA datasets alone did not generate topologies with highly supported basal nodes in *Pteronotus*, with independent searching methods exhibiting alternative relationships of clades. More robust datasets (five-gene and complete), on the other hand, yielded a highly concordant result, depicting the same phylogenetic relation for the major clades with high support values in all tree nodes (Fig. 3).

3.2.1. mtDNA dataset

A total of 17 mitochondrial independent lineages were identified within *Pteronotus* (Fig. S1). More than one lineage was found in three *Pteronotus* taxa recognized as species by current taxonomy: *P. parnellii* (eight), *P. personatus* (four) and *P. davyi* (two). The geographic range of these lineages generally corresponds to those of subspecies within each species (Smith, 1972). The observed genetic divergence among lineages, however, overcomes values currently described as intraspecific variation in Neotropical bat species (Bradley and Baker, 2001; Baker and Bradley, 2006). The estimate of nucleotide distance among *Pteronotus* lineages from the same mtDNA major clade varies from 3% up to 15%. In some cases comparison between two genetic lineages of the same current species (e.g. *P. parnellii*) presents higher levels of divergence than estimates between two recognized species in the genus (e.g. *P. davyi* × *P. gymnonotus*).

In Clade 1 (*P. davyi* and *P. gymnonotus*), mtDNA points to the existence of two paraphyletic and allopatric lineages within *P. davyi* (Figs. S1a and S2a): one of them (N = 18) is distributed in the Lesser Antilles (Santa Lucia, Dominica and Trinidad) and coincides with the range of the South American subspecies *P. d. davyi*; the second (N = 10) occurs from Mexico to Honduras and agrees with the distribution of the Central American subspecies *P. d. fulvus*. Each genetic lineage includes one sample from the type locality of the respective subspecies (Table 1). The mtDNA between these two lineages of *P. davyi* is 7% divergent, the

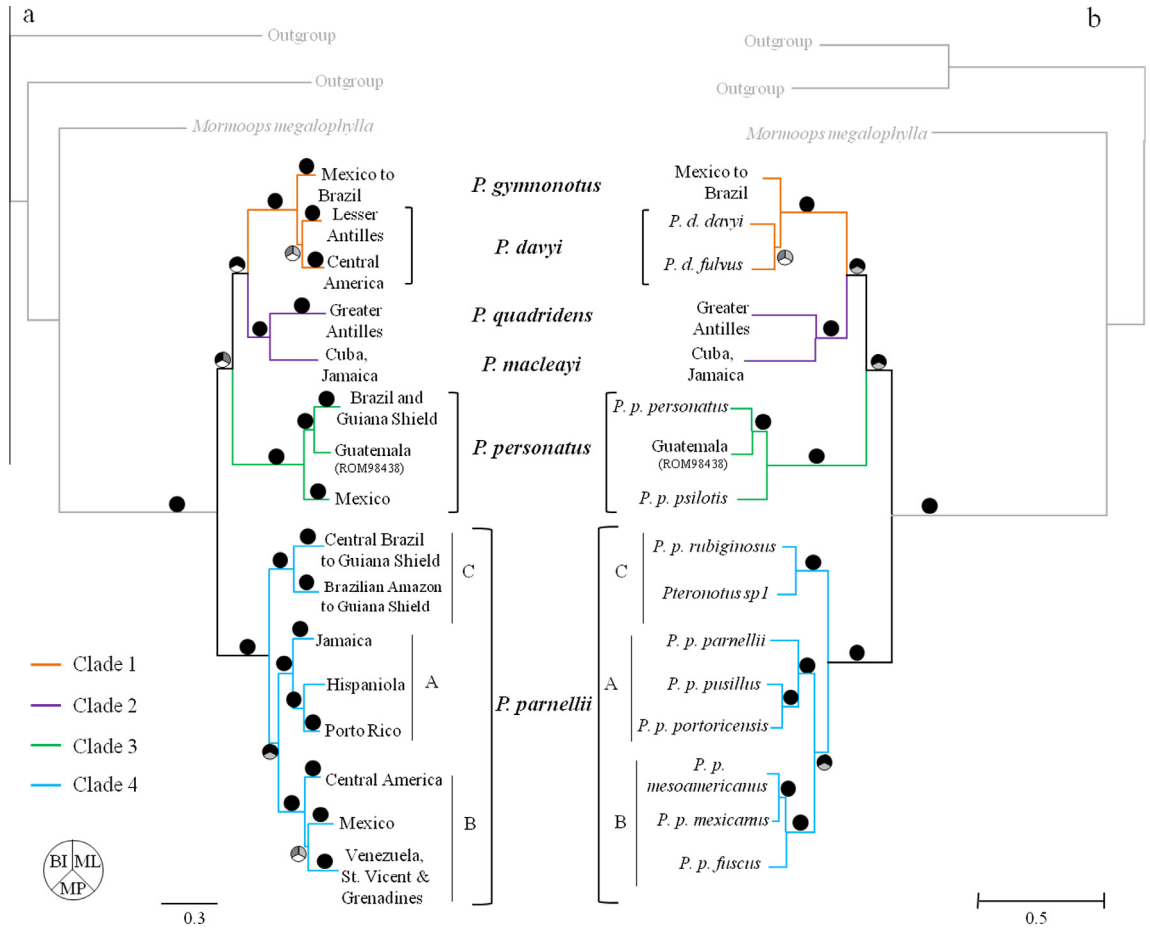


Fig. 3. Phylogenetic relationships in *Pteronotus* inferred from (a) five-genes and (b) complete datasets. Topologies of BI approach are presented in both cases and reflect exactly the same relationships among terminals. Symbols on the nodes represent values of bayesian posterior probabilities (bpp), ML and MP bootstraps according to the legend. For ML and MP, white = bootstrap frequencies < 50% or a relation not recovered by such method; gray = 50% < bootstrap frequencies < 70%; dark gray = 70% < bootstrap frequencies < 90%; black = bootstrap frequencies ⩾ 90%. For BI, dark gray = 0.8 < bpp < 0.95; black = bpp ⩾ 0.95. Species names in black displayed in the center of the figure correspond to those adopted by traditional taxonomy (Smith, 1972; Simmons, 2005). Terminals at the right tree were labeled with the correspondent subspecies names of the geographic ranges presented in the left tree.

same distance observed among them and their sister-species *P. gymnonotus* (N = 44).

In Clade 2, mitochondrial haplotypes were grouped into two highly divergent (~13%) clades, which correspond to *P. quadridens* (N = 25) and *P. macleayi* (N = 7) samples. A discrete structuring in the mitochondrial haplotypes was observed within each of these clades: in *P. macleayi* we can find Cuban and Jamaican samples arranged separately for each other; in *P. quadridens*, specimens from Dominican Republic and Puerto Rico are grouped together, with Cuban and Jamaican haplotypes basal to them (data not shown). Even so, the overall nucleotide divergence within these Antillean species is smaller than 1%, suggesting they are genetically cohesive groups.

In Clade 3 (*P. personatus*) four divergent lineages (4–9%) were identified through mitochondrial data (Fig. S1). The first lineage (N = 52) is widely distributed in Brazil and Guiana Shield (Fig. S2b), including specimens from Brazilian localities close to the type locality of *P. p. personatus* in Mato Grosso state (Smith, 1972), probably corresponding to this subspecies. The second lineage (N = 8) encompasses specimens from Mexico (sequenced at the present study) and Guatemala (available only as mtDNA GenBank sequences), which agrees with the geographic range of the subspecies *P. p. psilotis* (Fig. S2b). As further evidence, this lineage includes haplotypes from Oaxaca (Mexico), the type locality of *P. p. psilotis* (Smith, 1972). The third lineage of *P. personatus* comprises a few specimens from Guiana Shield (N = 4; obtained through COI or CYTB GenBank sequences only) and the fourth lineage is represented by a unique sample from Guatemala (ROM 98438). These two last lineages have no available name in the current taxonomy of *P. personatus* (Fig. S1b). Given their high mtDNA divergence from the two named lineages of *P. personatus*, they may represent new species, but the incomplete sampling prevented us from making a deeper investigation.

Clade 4 (*P. parnellii*) is composed by eight different mitochondrial lineages structured in three main clades (Figs. S1 and 3). These clades are present in all performed analysis, but relationships among them are different in MP, BI and ML topologies. The overall K2P nucleotide distance between genetic lineages from different clades ranges from 11.5 to 14.6% while K2P divergences from 3 to 8.9% are observed among lineages within the same clade. Mitochondrial lineages found in *P. parnellii* also seem to present geographic ranges (Fig. S2) overlapping with subspecies currently recognized within the taxon (*sensu* Smith, 1972), although some disparities will be further discussed.

The first clade (4-A) includes three allopatric lineages distributed in the islands of Jamaica (N = 6), Dominican Republic (N = 6) and Puerto Rico (N = 7), which correspond to the distribution of the Antillean subspecies *P. p. parnellii*, *P. p. pusillus* and *P. p. portoricensis*, respectively (Smith, 1972). The second clade (4-B) is composed by three parapatric lineages spread in Western

Mexico (N = 17), Southern Mexico and Central America (N = 38), and Northern South America including Lesser Antilles (N = 26), showing very similar geographic ranges, in this order, to the subspecies *P. p. mexicanus*, *P. p. mesoamericanus* and *P. p. fuscus*. In addition, each of the lineages in Clade 4-B includes one or more samples close to the type locality of the correspondent subspecies (Table 1). The third clade (4-C) is represented by two South American sympatric lineages, occurring in Brazil and Guiana Shield. Our dataset contains several localities where these lineages were sampled together, i.e., in syntopy (Fig. S2c). One of them (N = 106) extends further southward in Brazilian Cerrado, including all samples from Mato Grosso state, the type locality of the subspecies *P. p. rubiginosus* (Smith, 1972), while the other (N = 27) encompasses only Amazonian localities and represent an unnamed taxon, hereafter mentioned *Pteronotus* sp1 (corresponding to *P.* sp3 *sensu* Clare et al., 2013; Thoisy et al., 2014).

Some differences between subspecies and genetic lineages ranges deserve consideration. Smith (1972) originally assigned *P. parnellii* specimens from Trinidad to the South American subspecies *P. p. rubiginosus.* However, the molecular results undoubtedly place Trinidad and Saint Vicent and Grenadines samples in the same clade of Venezuelan samples (Figs. 3 and S1), which corresponds to the subspecies *P. p. fuscus*. Another difference is the contact zone postulated by Smith (1972) between *P. p. mesoamericanus* and *P. p. rubiginosus* along Central America. According to the author, a zone of intergradation would exist in eastern Honduras, southward more or less along the continental divide. Therefore, populations of *P. parnellii* in Central America southward Honduras should be assigned to *P. p. mesoamericanus* when located in the Pacific versant or to *P. p. rubiginosus* if occurring in the Atlantic (Caribbean) versant (see Table 1). According to our results, however, all specimens from Central America (including a large sampling across Honduras) belong to the same genetic lineage (Fig. S2), which was identified under the name *P. p. mesoamericanus*, similarly to the findings of Clare et al. (2013). These evidences led us to limit the north distribution of *P. p. rubiginosus* group to Guiana Shield and Southern Venezuela.

3.2.2. nDNA dataset

Introns presented a very congruent result with mtDNA dataset, although a smaller phylogenetic structure within each of the *Pteronotus* major clades is observed (Fig. S3). In Clade 1, both *P. davyi* and *P. gymnonotus* are recovered as monophyletic groups. Within *P. davyi*, one of the mitochondrial lineages (*P. d. davyi*) is placed as a nested clade within the other lineage (*P. d. fulvus*) for all phylogenetic analyses (ML, BI and MP trees). This same nested pattern is observed in ML and BI phylogenies for *P. personatus* (Clade 3): Mexican samples (*P. p. psilotis*) are grouped as a highly supported clade inside the major clade containing remaining samples of *P. personatus* (Brazil, Guiana Shield, Guatemala - Fig. S3a). MP tree, however, displays the two mitochondrial lineages equivalent to *P. p. psilotis* and *P. p. personatus* as monophyletic groups (Fig. S3b). Regarding the two unnamed mitochondrial lineages within Clade 3, the specimen ROM98938 from Guatemala was placed together with *P. p. personatus* samples for nuclear sequences while the Guiana Shield specimens could not be included in nDNA analyses since only mtDNA sequences were available for them at the GenBank. The Antillean species composing Clade 2 (*P. quadridens* and *P. macleayi*) were recovered as reciprocal monophyletic groups, reinforcing the pattern observed for mtDNA dataset. For *P. parnellii* (Clade 4) the three main mtDNA clades above described (A, B and C) were recovered by the nDNA dataset in all analyses, but monophyly of mitochondrial lineages was not always observed (Fig. S3b).

In general, the overall nucleotide divergence among *Pteronotus* nDNA lineages is one order of magnitude smaller than in mtDNA, suggesting lower evolutionary rates for the introns when compared to the mitochondrial markers. Lacking of reciprocal monophyly among *Pteronotus* lineages diagnosed by mtDNA is therefore expected in nuclear data for recent diversification events (Maddison, 1997), such as those within Clades 4-B and 4-C. Also, nDNA dataset contains only 1053 bp, which are less informative than the 1791 bp of the mtDNA dataset. Evidences for the separation noticed in mtDNA are present as lineage-specific SNP's and INDEL's in nuclear data for these lineages (data not shown).

3.2.3. Five-gene and complete datasets

Two distinct multilocus analysis, using five markers (62 specimens, 3368 base pairs) and the complete dataset (32 specimens, 4172 base pairs), were performed and compared as regards their robustness in solving the basal relationships within the genus (Fig. 3). Both datasets resulted in the same topology through ML and BI methods, with some nodes displaying better support values with the inclusion of the sixth maker (RAG2) in the analysis (Fig. 3b). The basal position of Clade 4 (*P. parnellii* samples) in the genus and a closer relationship between Clades 1 and 2 were strongly supported. The MP search also found a similar relationship among lineages, though the support values for basal nodes were still below 70%.

Regarding intraclade diversification, all lineages previously identified through mtDNA were strongly supported in the five-gene dataset analysis, which sampled two or more specimens for most groups (Fig. 3a). The more robust analyses suggest the taxon *P. davyi* (henceforth referred as *P. davyi* complex) as a monophyletic group comprising two divergent lineages in Clade 1 (Fig. 3). However, support values for the sister relationship between these two lineages (*P. d. davyi* and *P. d. fulvus*) were not conclusive. The close relationship among Antillean species *P. quadridens* and *P. macleayi* in Clade 2 remained stable along all phylogenetic inferences. In Clade 3 (*P. personatus* complex), the Mexican lineage (*P. p. psilotis*) exhibited a basal position, with the South American (*P. p. personatus*) and the Guatemalan specimen being sister groups in all resulted topologies. Within Clade 4 (*P. parnellii* complex), ML and BI results agreed with a sister relationship between Antillean (Clade 4-A) and Central American (Clade 4-B) clades, leaving South American lineages (Clade 4-C) in a basal position within the *P. parnellii* complex. Relationships among lineages of Clade 4-B (*P. p. mesoamericanus*, *P. p. mexicanus* and *P. p. fuscus*) were resolved and well supported only when the larger dataset was analyzed, which may be consequence of the recent splitting within this group.

3.3. Species tree

For performing this analysis, each divergent lineage identified in the complete dataset topology (Fig. 3) was considered a different species under the multispecies coalescent model, designating a group of individuals that have no history of breeding with individuals outside that group (Heled and Drummond, 2010). Three of these lineages are equivalent to current *Pteronotus* species according to Fig. 3: *P. gymnonotus*, *P. quadridens* and *P. macleayi*. Eleven lineages received names from correspondent subspecies in the traditional taxonomy of *P. parnellii*, *P. davyi* and *P. personatus* complexes (Fig. 3b) but were ranked to the specific status given their higher distinctiveness in phylogenetic analysis. One last lineage has no available name in the current taxonomy and was referred as *Pteronotus* sp1 (one South American lineage in *P. parnellii* complex). The lineage identified in Clade 3 (*P. personatus* complex) by a unique specimen from Guatemala was not included in the species tree approach since we do not have satisfactory evidence it represents a distinct species. The topology resulted from this analysis

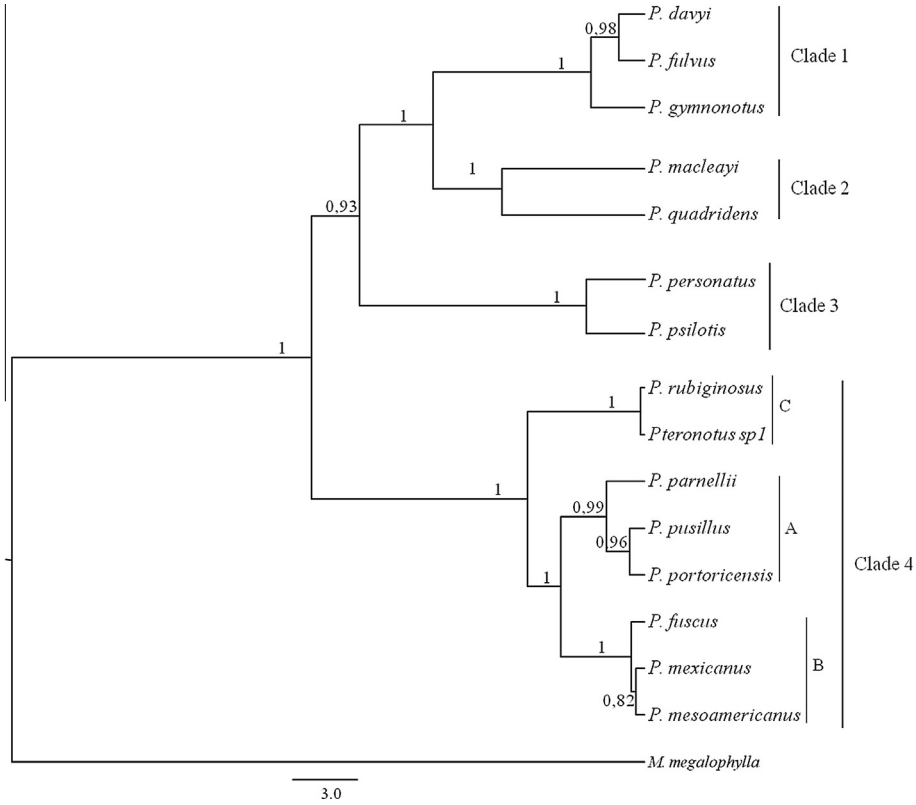


Fig. 4. Species tree for the genus *Pteronotus* estimated from the complete dataset, depicting the relationships among major clades according to the multispecies coalescent approach. Names of terminals incorporate results of Fig. 3 and correspond to our new proposed hypothesis of species diversity in the genus *Pteronotus* (for more details, see text).

(Fig. 4) presented the same relationship among lineages of genus *Pteronotus* than phylogenetic analysis above described.

3.4. Morphometrics

The morphometric dataset was divided in 17 categories (or grouping hypothesis) to be tested, corresponding to the currently described subspecies within the genus *Pteronotus* (Smith, 1972). Variables means and standard deviations in each category are presented in Table S4. Although the same number of categories in morphometric analyses was tested in relation to the genetic lineages found, they are not strictly equivalent. Some of the genetic lineages identified (e.g. the unnamed mtDNA lineages of *P. personatus* and *P. parnellii* complexes) could not be established as morphological categories because they overlapped geographically the lineages assigned to current subspecies. Since we lacked information on skull diagnostic characters for each of these lineages (this was not a goal of the present study), the assortment among them was not possible. Therefore, specimens belonging to such lineages, if sampled for morphometric data, were classified in the subspecies recognized for that given locality according to the species taxonomy. On the other hand, some taxonomic units not identified as independent lineages (subspecies of *P. quadridens* and *P. macleayi*) or not included in the molecular component (*P. d. incae*) were tested in the morphometric dataset as distinct groups.

3.4.1. Principal component analysis

The PCA included all *Pteronotus* specimens sampled and allowed a general outline of the skull morphological variation within the genus (Fig. S4). The first and second principal components (respectively, PC1 and PC2) explained 95% of the total variation of this group. PC1 represents a size vector while PC2 describes different ratios of rostrum and braincase along the skulls morphotypes. The contrast between these two PCs established two main groups in *Pteronotus*: the first group is composed by *P. parnellii* complex specimens, which presents the higher scores in PC1; the second group includes all the remaining species, with smaller PC1 scores than *P. parnellii*. The smaller *Pteronotus* species are differentiated along the PC2, exhibiting a continuum of variation which departs from a small braincase e elongated rostrum in *P. macleayi* to a robust braincase and shorter and wider rostrum in *P. gymnonotus*. It is noticeable that the total skull variation occupied by *P. parnellii* complex in the principal component space is equivalent to several species in the second group of *Pteronotus*.

3.4.2. Sexual variation

Since we missed information on the geographic distribution/contact zones of the genetic lineages within species complexes, we probably incurred in some errors during morphological groups delimitation, which primarily followed the available information on subspecies geographic range (Smith, 1972). In order to avoid false positive tests of sexual dimorphisms due to the existence of interspecific variation within some categories, the MANOVA tests were performed twice. The first test evaluated groups covering the complete geographic range of the correspondent subspecies. Once such groups returned significant differences ($p < 0.05$) between genders, a second test with geographically restricted groups, defined from one or some adjacent localities undoubtedly assigned to the same genetic lineage, was performed to confirm result. The second MANOVA test resulted in non-significant differences for some groups initially considered dimorphic (Table S5), which allowed the inclusion of both male's and female's original data for DFA. For the categories with significant results, we adjusted the female's values adding the observed main difference

for each variable between males and females (Marroig and Cheverud, 2004) and this corrected dataset was henceforth used in DFA.

3.4.3. Discriminant analysis

DFA was performed in each of the major clades found in the molecular results. This multivariate analysis provides a description of differences among *a priori* groups through the extraction of classification (discriminant) functions from the raw morphometric data. It generates tables showing the success classification rates of specimens in each of the defined groups, i.e., the power of all discriminant functions in assigning specimens into discrete morphological categories. Small differences in the original and jackknifed classification matrices values are expected when discriminant functions (DFs) allow robust sorting of specimens in the *a priori* groups (Klecka, 1980). Together with molecular data, results described by such analyses were our guidance for species delimitation in the genus *Pteronotus*. In general, the extracted functions showed a satisfactory to high discrimination among the majority of the morphological groups established.

The DFA performed among the four categories established in Clade 1 (*davyi*, *fulvus*, *incae* and *gymnonotus*) was highly significant (Wilk's lambda = 0.016, df = 123, 1208, $p < 0.001$). The three individual discriminant functions (DF1 to 3) extracted from the data represented 92%, 6% and 2% of the total variation, respectively. The correlations between DF scores and skull measurements indicate the DF1 as a size factor because all significant values are positive, whereas DF2 represents the ratio of the zygomatic components in the total cranial length (data not shown). Post hoc classification of cases in the defined categories presented accuracy in the classification rate superior to 85% both in original and jackknifed matrices (Fig. 5; Table S6). The morphometric differentiation among categories defined within Clade 1 is also represented in Fig. 5 by the first two DF space plot. We observe a clear separation of categories *fulvus* and *gymnonotus*, while *davyi* and *incae* show a slight overlap in their DF scores, suggesting a larger similarity between them. It is important to highlight, however, that interpretation of the groups' morphological distinctiveness based solely on their position in the plot may be inaccurate, since there is a third DF that also contributes to classification rates in the DFA (4 groups tested = 3 DFs). If we repeat the DFA without the species *P. gymnonotus*, for example, position of the three groups from the *P. davyi* complex in the DF1 × DF2 plot changes due to the decreasing in DF1 variation axis caused by removal of the *gymnonotus* group, which has a larger size.

Within Clade 2 (*P. macleayi* and *P. quadridens*), we considered the two subspecies currently recognized within each species (Table 1) as independent categories in the DFA in order to investigate their morphological differentiation (Smith, 1972). In both cases the extracted discriminant function (DF1) was significant (*P. macleayi*: Wilk's lambda = 0.074, df = 33, 10, $p = 0.015$; *P. quadridens*: Wilk's lambda = 0.134, df = 41, 35, $p < 0.001$) and represents size variation between the subspecies. Fig. 6 shows the success classification rates and the differences in DF1 values exhibited by the two morphological categories within each species. Post hoc classification of cases in the defined categories (subspecies) was higher in *P. quadridens* than in *P. macleayi* (Fig. 6; Table S7). In both cases, however, the values reported by jackknifed classification matrices were much smaller than the original matrices, suggesting an unsatisfactory assignment of data to the categories, likely due to the low sampling values.

The DFA within Clade 3 (*P. personatus* complex) was performed between two categories: *personatus* and *psilotis*. As said previously, we had no information regarding geographic range and cranial diagnostic characters for testing the unnamed genetic mtDNA lineages from Guiana Shield and Guatemala as independent

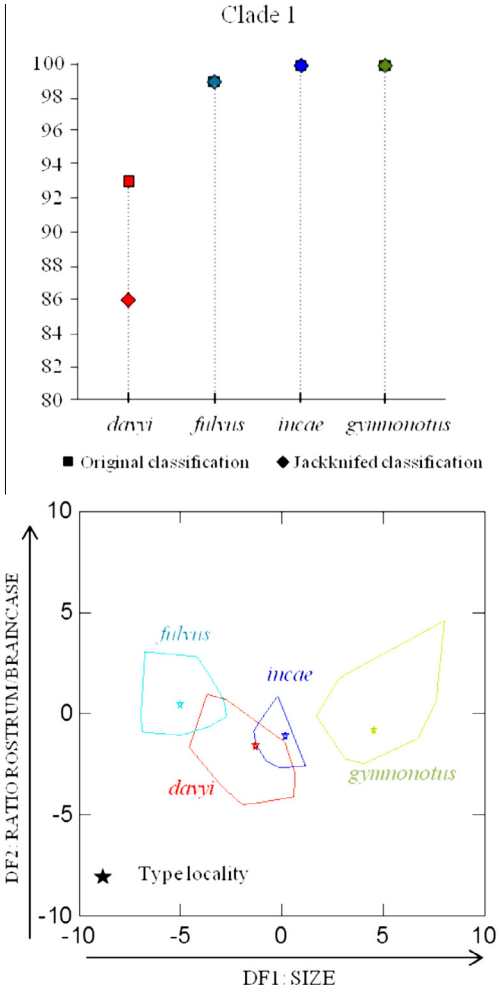


Fig. 5. [above] Rates of correct classification of specimens in the morphological categories defined for the DFA within Clade 1; [below] Plot of Clade 1 morphological groups scores against first two discriminant functions (DF1 and DF2).

morphological groups for DFA analyses. Therefore, all specimens measured from Brazil to Costa Rica were included in the category *personatus* and the same was done for *psilotis* regarding specimens from Honduras to Mexico (Smith, 1972). The DFA resulted in one highly significant discriminant function (Wilk's lambda = 0.103, df = 41, 228, $p < 0.001$), which describes the size difference between categories. Both original and jackknifed classification matrices show correct assignments of specimens to the categories close to 100% (Fig. 6; Table S8), suggesting a robust separation of *P. personatus* complex in two morphologically distinct groups.

For the morphometric analysis in Clade 4, specimens of *P. parnellii* complex were split into seven categories, three of them in Caribbean Islands - *parnellii*, *portoricensis* and *pusillus* – and four in the continental portion – *mexicanus*, *mesoamericanus*, *fuscus* and *rubiginosus*. Group's delimitation followed primarily the geographic distribution observed for the genetic lineages, with their contacts zones inferred from information provided by the subspecies geographic range (Smith, 1972). As occurred in the *P. personatus* complex, the unnamed South American lineage of *P. parnellii* complex could not be sorted from its sympatric sister-group *rubiginosus* by cranial characters. The unique measured specimens surely classified as *Pteronotus* sp1 were those also sequenced (n = 11). Since we probably have many other specimens belonging to the *Pteronotus* sp1 lineage hidden within the cranial sampling of *P. p rubiginosus*, allocating just the sequenced specimens of *Pteronotus* sp1 in a separate morphological group would

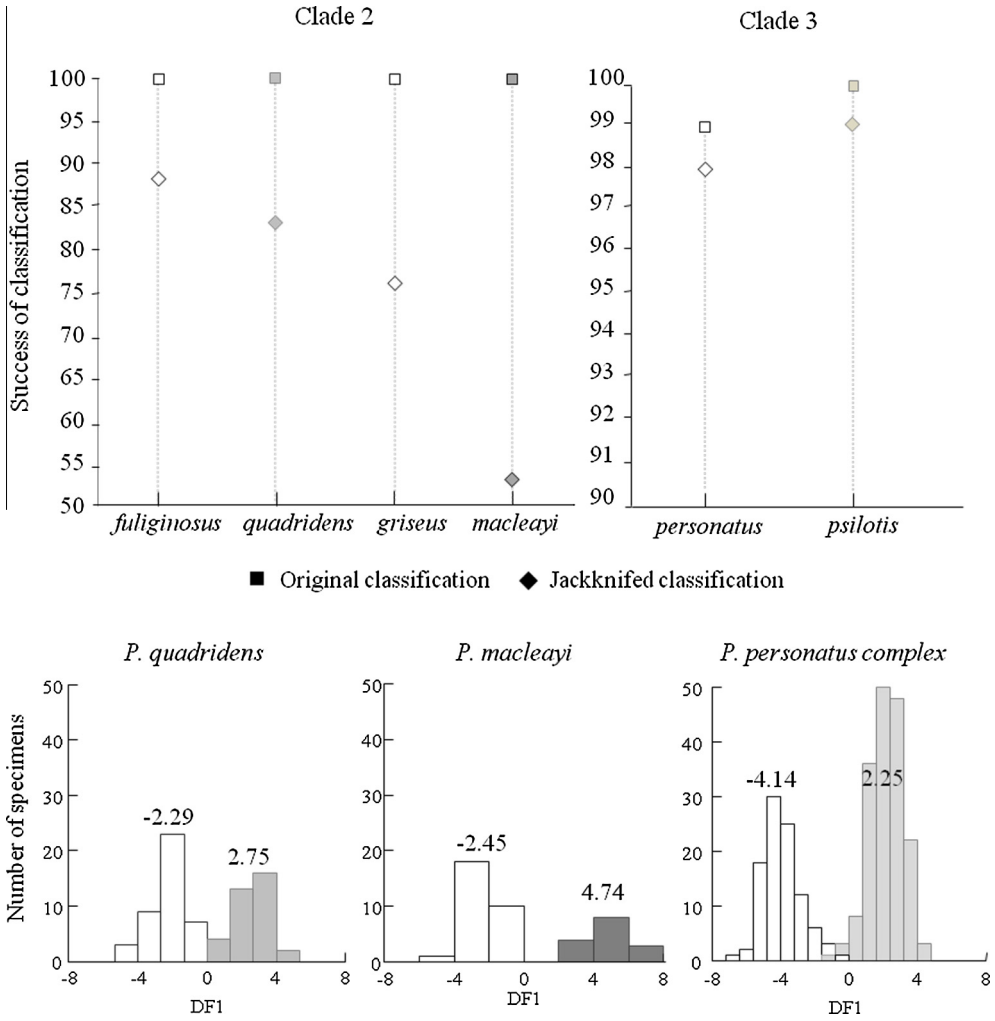


Fig. 6. [above] Rates of correct classification of specimens in the morphological categories defined for the DFA within *P. quadridens* and *P. macleayi* (Clade 2) and *P. personatus* complex (Clade 3); [below] Range of DF1 values exhibited by specimens of each morphological category. Numbers above bars represent canonical scores of group means. Note that, despite their distinctiveness in DF1 axis, jackknifed classification rates of *P. quadridens* and *P. macleayi* groups were low, probably due to closer values in the group's variables means and small sample size.

not make sense. Therefore, all specimens from South America southward Orinoco River were grouped in the category *rubiginosus* and considerations regarding the DFA results will be make further.

The six discriminant functions extracted from DFA were significant (Wilk's lambda = 0.017, df = 287, 4912, $p < 0.001$), with the first three explaining 70%, 17% and 5% of total variation, respectively. The correlations between DF scores and skull variables suggest DF1 as a size vector, which allows a major discrimination between insular and continental lineages (Fig. 7). The DF2 describes the relative proportion of facial components in the total cranial size, representing an important differentiation axis along continental lineages. The post hoc classification scores of specimens in the defined categories were in general smaller than in the other *Pteronotus* clades, although the original and jackknife classification matrices consistently presented close values for most groups (Fig. 7; Table S9). The greater uncertainty observed in the classification results regarded the assignment of specimens to *portoricensis*, which was considered unsatisfactory for our criteria (difference larger than 10% between classification rates of original and jackknifed matrices). This group presented a high overlap of its first two DFs with *parnellii*, suggesting these insular categories are very similar morphological units (Fig. 7). Post hoc classification of cases to *fuscus* by jackknife resampling method was lower than those of remaining continental groups, probably due to the intermediate phenotype between *mesoamericanus* and *rubiginosus* presented by this group, but still satisfactory (Table S9).

Additional exploratory DFAs including just the insular or continental lineages were performed to evaluate the morphological distinctiveness of some specimens (Fig. S5). For both analyses, the specimens investigated were not defined as *a priori* groups in DFA. This enabled us checking their position in the DF1 × DF2 space without assuming the hypothesis they represent independent morphological groups, since this would orientate the analysis in finding discriminant functions maximizing their differences. This is an important detail to be mentioned because the multivariate approach attempts to maximize differences just among the *a priori* defined groups. The DFA comprising only insular lineages included one measured specimen of *P. p. gonavensis* - a subspecies described from fossil specimens found in La Gonave Island, Haiti - to verify its position in the DF space generated by the lineages currently distributed in the Greater Antilles. The analysis included only 37 distances since the specimen of *P. p. gonavensis* is damaged. The result shows *gonavensis* close to specimens of *pusillus*, suggesting a high cranial similarity between these two taxonomic units. The DFA of mainland lineages analyzed specimens of *Pteronotus* sp1 (n = 11) and *P. paraguanensis* (n = 2), plotting their scores within the DF space created by the four mainland categories. The result show *P. paraguanensis* specimens apart from the remaining

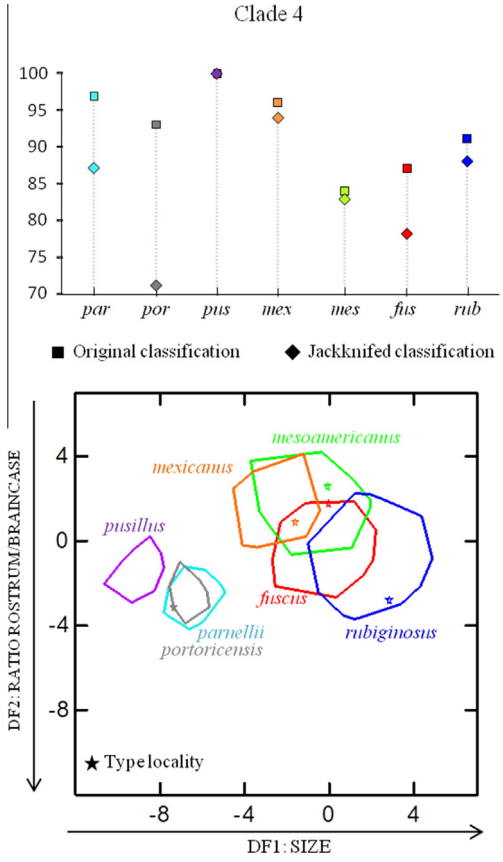


Fig. 7. [above] Rates of correct classification of specimens in the morphological categories defined for the DFA within Clade 4; [below] Plot of Clade 4 morphological groups scores against first two discriminant functions (DF1 and DF2). Additional plots within Clade 4 are presented in Fig. S5.

groups, with their DF scores closer to *mexicanus* and *fuscus*. *Pteronotus* sp1, on the other hand, is completely overlapped with *rubiginosus* in this DF1 × DF2 space.

4. Discussion

4.1. Performance of phylogenetic methods and molecular datasets

The results obtained by probabilistic methods of phylogenetic inference (BI and ML) presented a more similar result than the MP search. This fact may be consequence of the distinct searching strategies adopted by these approaches, where MP search may present a lower performance due to the incomplete lineage sorting in one or more markers or because of the presence of short branches deep in the tree (Kubatko and Degnan, 2007). In general, results obtained by the different datasets were highly congruent in establishing four major clades within *Pteronotus* as well as the genetic lineages they encompass. Although nDNA alone did not strongly support all lineages identified by mtDNA, molecular data investigated through multiloci approaches (with 5 or 6 genes) clearly recover the same evolutionary lineages. Regarding the relationship pattern among these major clades, more robust datasets were necessary to present a confident and accurate hypothesis on the evolutionary history of this group. The topologies found by the five-gene and complete datasets are in accordance with relationships presented by the mtDNA dataset, but higher support values are observed in the deep tree nodes. This result highlights the importance of independent *loci*, with distinct evolutionary rates, for depicting the basal relationships and adequate node supports within a phylogeny (Knowles and Carstens, 2007; Corl and Ellegren, 2013).

4.2. Diversification pattern in the genus Pteronotus

The phylogenetic inference points to a close relationship between the species *P. gymnonotus* and *P. davyi* complex within Clade 1, and *P. macleayi* and *P. quadridens* within Clade 2, which were recovered as sister groups in all performed analysis. Additionally, phylogenetic analyses suggests that Clades 1 and 2 have the most recent common ancestor in the genus, followed by a closer relationship with Clade 3, while Clade 4 is the sister group to all other clades. The multispecies coalescent model also produced the same topology as result, providing independent evidence for such evolutionary pattern within the genus. The basal position of *P. parnellii* complex is already reported by previous molecular investigations (Lewis-Oritt et al., 2001; Van Den Bussche and Weyandt, 2003; Dávalos, 2006) and the relationship among *Pteronotus* species presented here received strong node supports in the final analysis. In accordance with molecular data, the general pattern of cranial variation provided by PCA places all lineages of *P. parnellii* complex in a group detached from the remaining species, mostly due to its larger size (PC1). This dichotomy between *P. parnellii* complex and the other *Pteronotus* species also receives support from other biological aspects, such as morphology (Smith, 1972; Simmons and Conway, 2001) and echolocation calls (Novick, 1963; Fenton et al., 2012; Mancina et al., 2012).

Regarding taxonomy, our phylogeny supports the subgenus *Pteronotus* (Clade 1) and *Phyllodia* (Clade 4) as natural arrangements, whereas points to subgenus *Chilonycteris* (Clade 2 + Clade 3) as an artificial group. This subgenus was described by Smith (1972) including the species *P. macleayi*, *P. quadridens* and *P. personatus*. Simmons and Conway (2001) corroborated the group monophyly based on morphological data, although presented a weak node support for the clade. Molecular evidences, however, does not support this arrangement (Lewis-Oritt et al., 2001; Van Den Bussche et al., 2002; Van Den Bussche and Weyandt, 2003; present work) and we recommend the allocation of *P. personatus* complex (Clade 3) in a new subgenus.

4.3. Integrative taxonomy

Our results also confirm previous reports on the underestimated diversity within the genus (Lewis-Oritt et al., 2001; Dávalos, 2006; Clare et al., 2011, 2013; Thoisy et al., 2014). Three of the major clades herein described include species complexes, entities receiving the same name under current taxonomy but harboring two or more evolutionary lineages: *P. parnellii*, *P. personatus* and *P. davyi*. One impressive result is the equivalence of several current subspecies described in *Pteronotus* with a mitochondrial lineage. These lineages, in turn, could be discriminated by skull morphometrics, suggesting most taxonomic units previously identified as subspecies by Smith (1972) actually deserve the specific status. The existence of discontinuities in morphometric data, combined with other criteria such as geography and biological aspects, were considered solid evidences when integrated to the molecular results for delimiting species in this bat group following the unified proposal of the General Lineage Concept of Species (de Queiroz, 1998). Below we discuss the diversity within each of the clades identified in molecular results based on the available evidences (summarized in Table S10), providing a taxonomic update.

Clade 1 – Corresponds to the subgenus *Pteronotus* described by Smith (1972), being currently composed by *P. davyi* complex and *P. gymnonotus*. The phylogenetic results exhibit all *P. gymnonotus* specimens in a shallow and weakly-structured monophyletic group. The analysis of skull variation also indicates a single

morphological unit within this taxon. The pattern of star-like tree presented by *P. gymnonotus*, with short internal branches and low nucleotide divergences, suggests a rapid population growth or a recent origin for this species (Nordborg, 2001; Wakeley, 2004). For *P. davyi* complex, molecular results show two allopatric, reciprocally monophyletic groups corresponding to the geographic range of current subspecies *P. d. davyi* and *P. d. fulvus*. Cranial morphometrics strengthens this result, pointing to divergent morphogroups for these lineages. The third subspecies, *P. d. incae*, shows morphometric distinctiveness and geographic isolation, suggesting this taxon may represent a third species within this complex, but its absence from the phylogenetic investigation prevents us for making such recognition.

Therefore, we assume the existence of three species within Clade 1: *P. davyi*, *P. fulvus* and *P. gymnonotus* (Tables S10). Morphometric and geographic evidences suggest the isolated population of *P. davyi* complex in Peru (*P. d. incae*) is closer to *P. davyi*, so we consider *incae* as a geographic variation of the specie *P. davyi* until its phylogenetic position and genetic distinctiveness is investigated. The relationship among species within Clade 1 was not well resolved in our phylogenetic analysis: mtDNA suggests a sister relationship between *P. davyi* and *P. gymnonotus*, though the final molecular dataset points to the closer relation between *P. davyi* and *P. fulvus* with low support values. The proposed geographic range for *P. gymnonotus* is being updated by including several Brazilian localities (Fig. S2a, Table S11); ranges of *P. davyi* and *P. fulvus* are provisionally being kept equivalent to the ranges proposed by Smith (1972). While *P. davyi* and *P. fulvus* apparently present allopatric distributions regarding each other, *P. gymnonotus* occurs sympatrically with the other two. Curiously, we have found one specimen of *P. gymnonotus* with introgressed mtDNA from *P. fulvus* (ROM 98442), two clearly established species. More sampling in Central America is therefore necessary to explore the existence of a contact zone between *P. davyi* and *P. fulvus*, as well as to better understand interspecific interactions of *P. gymnonotus* with its sister-groups.

Clade 2 – Comprises the Antillean species *P. quadridens* and *P. macleayi* and should be synonymized to the subgenus *Chilonycteris*, which currently also includes *P. personatus* (Smith, 1972). These two species are displayed as monophyletic groups in all molecular markers investigated. Both *P. macleayi* and *P. quadridens* exhibit a geographical structure in the mitochondrial haplotypes, but the correspondence with subspecies currently described occurs just for *P. macleayi*. Additionally, divergence values observed between such geographic groups are small when compared to the interspecific variation reported for bats (Bradley and Baker, 2001). The morphometric results indicate the existence of size differences between groups within both species, but larger sampling are necessary to better investigate their distinctiveness. Overall, results from molecular and morphometric approaches suggest the variation observed is compatible with population structuring. The diversity in Clade 2 is hence kept to two species, *P. quadridens* and *P. macleayi,* with almost sympatric ranges along the Caribbean islands (Table S11).

The genetic and morphological cohesion observed for the two species of Clade 2 is contrasting given their long branches in the phylogeny, which stand for a relatively old age of these lineages. The similarity among intraspecific mitochondrial haplotypes in *P. macleayi* and *P. quadridens* may reflect a recent history of gene flow among populations occupying different islands within each species range. The morphological similarities, on the other hand, can be consequence of adaptations to the insular habitat or derived from a short period of drift after gene flow was interrupted.

Clade 3 – Mitochondrial data suggests a much more complex pattern in this group than currently recognized by taxonomy, with *P. personatus* complex comprising four distinct lineages. This hypothesis could not be verified in deep due to sample scarcity for two of these lineages, one in the Guiana Shield and other in Guatemala. We were able to identify the existence of two discrete units within this complex through multiloci and morphological data (Table S10). The two well sampled lineages form monophyletic and genetically cohesive groups whose geographic ranges overlap those of the two described subspecies within the taxon traditionally classified as *P. personatus* (Smith, 1972). These groups are corroborated by cranial morphometric analysis, which strongly supports the separation of *P. personatus* complex in two entities (Fig. 6). Based on these results, we recommend the elevation of subspecies *P. p. personatus* and *P. p psilotis* to the species level. Therefore, Clade 3 corresponds to a new subgenus in *Pteronotus* encompassing at least two allopatric species. *P. personatus* comprises populations from Mato Grosso state in Brazil northward to Costa Rica, while *P. psilotis* includes individuals from Honduras to Mexico (Table S11). These geographic ranges essentially follow subspecies distribution proposed by Smith (1972), although we provide new occurrence records of *P. personatus* in Brazil (Fig. S2b). Additional data is nonetheless necessary to better investigate the diversity within this complex as well as to infer species ranges in Central and northern South America.

Clade 4 – Corresponds to the subgenus *Phyllodia* (Smith, 1972) and is composed by distinct populations of *P. parnellii* complex, many of them already recognized as valid species by previous studies (Gutiérrez and Molinari, 2008; Clare et al., 2013; Thoisy et al., 2014). Our phylogenetic results point to three divergent clades within this complex, recovered by all datasets. Molecular data suggests a closer relationship between Clades 4-A (Caribbean Islands) and 4-B (Central and northern South America) while Clade 4-C (exclusively South American samples) diverged first during group diversification. This process apparently occurred in the same time frame, in a rapid radiation event, given the short internal branch splitting Clade 4-C from the other two (see Fig.3).

Mitochondrial data points to eight distinct lineages in *P. parnellii* complex, with nucleotide average distances between 3 and 15%. This result exceeds diversity levels previously reported (Clare et al., 2011, 2013; Thoisy et al., 2014) and shows conspecific lineages of Clade 4 diverging more than species currently described (Bradley and Baker, 2001; Baker and Bradley, 2006). The nDNA reveals a more discrete structure within *P. parnellii* complex, where mtDNA lineages are not always recovered as monophyletic groups. Nevertheless, this result is expected, since nuclear genes have larger effective population sizes (*Ne*) and consequently longer time scales of the coalescent process (Nordborg, 2001; Wakeley, 2004; Degnan and Rosenberg, 2009). Given its reduced *Ne* and relatively rapid rate of evolution, lineage sorting in mtDNA occurs faster than in nDNA. Consequently, diverging patterns in this molecule are anticipated as evidence of speciation (Rosenberg and Nordborg, 2002; Knowles and Carstens, 2007; Weisrock et al., 2010), even enough time has not elapsed to generate the diagnostic phylogenetic pattern of distinct evolutionary paths, i.e. reciprocal monophyly (de Queiroz, 1998), in multiple loci.

The skull variation within this complex is characterized by a marked division between Caribbean islands and American continent, as highlighted by PCA (Fig. S4). Insular lineages are smaller, with size increasing gradually in the continent, from *mexicanus* in the north of the geographic range, to *rubiginosus* in the south, where individuals exhibit the largest cranial sizes of *P. parnellii* complex. For the insular groups (Clade 4-A), the single distinct morphological category was *pusillus*. The cranial similarity between *parnellii* and *portoricensis* pointed by our results (Fig. 7) was previously reported by Smith (1972). Notwithstanding, the author justified the recognition of different subspecies for Jamaican (*parnellii*) and Puerto Rican (*portoricensis*) populations due to their geographic isolation by *P. p. pusillus*, a notably smaller

population inhabiting Haiti and Dominican Republic. In addition, molecular evidence also strongly suggests that *parnellii* and *portoricensis* represent independent lineages, not being even sister-groups (Figs. 3 and 4). Thus we recommend the elevation of the taxonomic status of all current insular populations of *P. parnellii* complex to the species level, namely *P. parnellii* (*sensu strictu*), *P. pusillus* and *P. portoricensis*. The taxonomic status of the Cuban population remains uncertain since we did not include samples from this island in our analysis. Following Smith (1972), this population would belong to the species *P. parnellii* (*sensu strictu*) together with Jamaica. However, it may also represent another evolutionary lineage since geographic isolation of the Greater Antilles apparently acted as an effective barrier to gene flow, originating island-specific lineages within this complex. Regarding the subspecies *P. p. gonavensis,* the availability of just one complete skull prevented us from making further interpretations. The DFA suggest this fossil specimen is very similar, but a little smaller, to *P. pusillus* (Fig. S5a). The geographic overlap and morphological resemblance between these two entities might be evidences that they actually belong to the same species, sampled in different moments of its evolutionary history. Based on this, we provisionally consider *P. p. gonavensis* as a temporal variation of *P. pusillus* until a deeper investigation with this taxon is performed.

The DFA comprising just the mainland lineages shows *mexicanus* and *rubiginosus* as discrete, non overlapped morphological units. The specimens from the geographically intermediate groups, *mesoamericanus* and *fuscus*, are more similar between each other and with those in the tip groups, and consequently were misclassified more frequently. Nevertheless, errors in the allocation of specimens to the defined morphological categories might be occurred, due to the lacking of knowledge regarding the contact zones between adjacent lineages. This means that specimens actually belonging to the group *fuscus* may be originally assigned by us to *mesoamericanus* or *rubiginosus* because we do not have full information on the lineage's geographic ranges. These errors may have caused a decreasing of the successful classification rates in DFA. In addition, the two sympatric lineages in South America were included in just one morphological category, *rubiginosus*. Since previous works report a morphometric discrimination between these lineages (Clare et al., 2013; Thoisy et al., 2014), the allocation of all specimens into one single group probably inflated its morphological variation and contributed for a larger overlapping between *rubiginosus* and other groups. Taking these facts into account, the morphometric differentiation among mainland groups of *P. parnellii* complex is probably higher than results in Fig. 7 show.

The available data suggests all mainland lineages of *P. parnellii* complex may represent valid species. The two lineages of Clade 4-C (*rubiginosus* and *Pteronotus* sp1) are represented by clades distributed in South America, with specimens collected syntopically in several localities in the Guiana Shield and Brazilian Amazon (Fig. S2c). Persistency of reciprocal monophyly between sympatric sister-groups constitutes strong evidence that they are in independent evolutionary paths (Coyne and Orr, 2004). Also, previous studies report some morphometric differences and distinct peak frequencies in the echolocation patterns of these two lineages (Clare et al., 2013; Thoisy et al., 2014). The three lineages from Clade 4-C (*mexicanus*, *mesoamericanus* and *fuscus*) occur in a wide geographic range from Mexico to northern South America. They show the smallest between-group mtDNA divergences within the genus (3–5%), suggesting they represent recently diverged lineages. We have observed a direct, strong relation between specimen's geographic origin and mitochondrial clades for the large sampling within this clade (N = 78), demonstrating its higher geographic structuring. We found one single specimen (TK128442) presenting a questionable position in our mitochondrial phylogeny due its divergent CYTB haplotype, though its COI haplotype undoubtedly places it within the *fuscus* clade. The cause of such discrepancy in the two mitochondrial markers regarding this individual was not investigated by us since it does not weaken our interpretations. In general, lineages of Clade 4-B appear to present parapatric distributions regarding each other, although additional data is necessary to explore contact zones among them.

Mormoopids are gregarious and obligatory cave dwellers, which probably restrict their dispersion to new habitats presenting this ecological requirement. Thus, geography in the case of Clade 4-B possibly account as a factor limiting gene flow between distant populations. Besides, distinct echolocation frequencies are reported for many localities within the continental range of Clade 4 (Novick, 1963; Gutiérrez and Molinari, 2008; Clare et al., 2013; Thoisy et al., 2014), which has been shown to have a dual function in the species ecological interactions, ultimately facilitating speciation in other HDC bats (Kingston and Rossiter, 2004). Therefore, adaptation to the echolocation strategy seems to be the key for understanding the diversification process within this complex (Clare et al., 2013). Following this hypothesis, diverging lineages in Clade 4 may have kept very similar cranial features during their evolutionary history given the anatomical and physiological requirements necessary to perform HDC strategy, and this would explain their morphometric similarity.

Based on our results and additional information regarding the group biology (Table S10), we recognize five species in mainland *P. parnellii* complex: *P. mexicanus*, *P. mesoamericanus*, *P. fuscus*, *P. rubiginosus* and *Pteronotus* sp1, which will be further described. Tentative geographic ranges for these species are presented in Table S11, but sampling gaps precluded us from making a complete analysis of their distribution. Our results corroborate the work of Clare et al. (2013) in suggesting the existence of four species of *P. parnellii* in the continent from Southern Mexico to Guiana, and we further include a fifth species in Western Mexico. Another species, *P. paraguanensis*, was not well investigated through our dataset due to the low sample size, but the morphometric distinctiveness of the two measured specimens in our DFA led us to maintain the specific status proposed by Gutiérrez and Molinari (2008). In total, we thereby recommend the recognition of nine species within *P. parnellii* complex, three of them in Caribbean Islands and six in the American continent. However, the inclusion of molecular data is crucial to fully investigate the phylogenetic position and taxonomic status of *P. paraguanensis*. Finally, a deeper knowledge on the acoustic variation along the complex range is needed and will certainly contribute to elucidate the secondary contact zones in this group.

4.4. Species classification and taxonomic arrangement

Most of the *Pteronotus* groups currently recognized at the subspecies rank in taxonomy fulfilled species criteria, possessing a range of diagnostic features including cranial morphometric differences, mtDNA reciprocal monophyly, multiple polymorphisms in nuclear sequences and differences in phonic types (mean constant frequency). Based on an integrative approach, we propose a new taxonomic arrangement for this genus, composed by 16 extant and one fossil species (Table 2). Some of the taxa herein listed were suggested as valid species by previous works (Dávalos, 2006; Clare et al., 2013; Thoisy et al., 2014), which did not depict, however, a full phylogenetic framework of the group.

The present work represents the more complete investigation performed in the genus *Pteronotus* including multiple and independent sources of evidence (maternal, paternal and autosomal genetic systems and morphometrics). The morphological diagnosis of the species herein recognized are however beyond our scope, since it requires the identification of discrete characters representatives of each taxon. The cranial variation we reported was just

Table 2
Changes in taxonomic arrangement proposed for the Family Mormoopidae by the present study when compared to traditional classification (Smith, 1972; Simmons, 2005).

Smith (1972)/Simmons (2005)	Present study
Family Mormoopidae	**Family Mormoopidae**
Genus *Mormoops*	**Genus *Mormoops***
Mormoops blainvillei	*Mormoops blainvillei*
Mormoops megalophylla	*Mormoops megalophylla*
Mormoops m. megalophylla	*Mormoops m. megalophylla*
Mormoops m. intermedia	*Mormoops m. intermedia*
Mormoops m. tumidiceps	*Mormoops m. tumidiceps*
Mormoops m. carteri	*Mormoops m. carteri*
Mormoops magna [b]	*Mormoops magna* [b]
Genus *Pteronotus*	**Genus *Pteronotus***
Subgenus *Pteronotus*	Subgenus *Pteronotus*
Pteronotus davyi	*Pteronotus davyi*
Pteronotus d. davyi	*Pteronotus d. davyi*
Pteronotus d. fulvus	*Pteronotus d. incae*
Pteronotus d. incae	*Pteronotus fulvus*
Pteronotus gymnonotus	*Pteronotus gymnonotus*
Subgenus *Chilonycteris*	Subgenus *Chilonycteris*
Pteronotus quadridens	*Pteronotus quadridens*
Pteronotus q. quadridens	*Pteronotus q. quadridens*
Pteronotus q. fuliginosus	*Pteronotus q. fuliginosus*
Pteronotus macleayi	*Pteronotus macleayi*
Pteronotus m. macleayi	*Pteronotus m. macleayi*
Pteronotus m. griseus	*Pteronotus m. griseus*
Pteronotus personatus	New Subgenus
Pteronotus p. personatus	*Pteronotus personatus*
Pteronotus p. psilotis	*Pteronotus psilotis*
Subgenus *Phyllodia*	Subgenus *Phyllodia*
Pteronotus parnellii	*Pteronotus parnellii*
Pteronotus p. parnellii	*Pteronotus portoricensis*
Pteronotus p. portoricensis	*Pteronotus pusillus*
Pteronotus p. pusillus	*Pteronotus p. gonavensis*[b]
Pteronotus p. gonavensis[b]	*Pteronotus mexicanus*
Pteronotus p. mexicanus	*Pteronotus mesoamericanus*
Pteronotus p. mesoamericanus	*Pteronotus fuscus*
Pteronotus p. fuscus	*Pteronotus paraguanensis*[a]
Pteronotus p. paraguanensis	*Pteronotus rubiginosus*
Pteronotus p. rubiginosus	*Pteronotus sp1*
Pteronotus pristinus [b]	*Pteronotus pristinus* [b]

[a] Taxon not investigated in the present study. Taxonomic status provided by Gutiérrez and Molinari (2008).
[b] Extinct.

quantitative, precluding us from providing such information. While this information is not available, we propose species identification in the genus *Pteronotus* follow provisionally the geographic criteria (Table S11), which delimits satisfactorily most of taxonomic units of the genus but a few contact zones previously discussed.

5. Conclusions

With this study the family Mormoopidae, a small Neotropical group of insectivores bats, has its diversity increased from six species to more than twice this number. Integrated analysis of molecular and morphometric data support the recognition of almost all nominal taxa in the genus *Pteronotus* as valid species. The taxa *P. parnellii*, *P. davyi* and *P. personatus* represent species complexes, as suggested by previous works, encompassing nine, two and two species respectively. We propose a new taxonomic arrangement for the genus *Pteronotus* composed by 16 extant and one fossil species.

Our work comprised a geographically broad sampling and large exploration of data within *Pteronotus*, demonstrating how integrative analysis of multiples sources of evidences contributes to the study of Neotropical bat diversity. Species of *Pteronotus* appear to have kept their cranial and external morphology relatively similar throughout group diversification, despite the deep genetic divergence distinguishing them. A deeper and more detailed study on the taxonomic units proposed is thought necessary, including information of bioacoustics, morphology and geographical variation, to better understand the evolution of this interesting Neotropical bat group. More refined hypotheses of taxa evolutionary history and diversity, like the one above shown, represent the first step for answering broader questions on evolutionary and ecological aspects of Neotropical life history.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2016.07.011.

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