Molecular systematics of the genus Artibeus (Chiroptera: Phyllostomidae)

Rodrigo A.F. Redondo, Letícia P.S. Brina, Ricardo F. Silva, Albert D. Ditchfield, Fabrício R. Santos

Abstract

A molecular phylogeny of the genus Artibeus using 19 of the 20 recognized species, many with samples from a broad geographic range, is presented. The analysis shows a clear distinction between the two subgenera (or genera), the ‘large’ Artibeus and the ‘small’ Dermanura, in both mitochondrial and nuclear genes. The placement and status of A. concolor remains inconclusive and is presented as the third subgenus Koopmania. The phylogenies and divergence time estimates show a marked influence of the Andes in the formation of the subgenera and the main lineages inside each subgenus. Nuclear genes showed a highly incomplete lineage sorting among species inside subgenera Artibeus and Dermanura. Indeed, shared alleles were also found between Artibeus and Koopmania, which are presumed to have split apart during the Miocene, showing that great care should be taken in using these markers. Cytochrome-b gene divergences and monophyly analyses suggest that A. lituratus and A. intermedius are indeed conspecifics. These analyses also suggested the existence of at least four ‘new’ species revealing a significant cryptic diversity inside the genus.

1. Introduction


Based on morphological evidence, Owen (1987, 1991) suggested that the genus should be divided into three taxa: Artibeus, including the large species, Dermanura, for the small species, and a new monospecific genus, Koopmania, for the Artibeus (Dermanura) concolor. Former molecular evidence from the mitochondrial Cytochrome-b gene and nuclear EcoR1 defined satellite DNA (Van den Bussche et al., 1998) showed an inconsistent support for the Koopmania genus, which they included in Artibeus, and also suggested Dermanura and Artibeus as sister groups. The differences between the Artibeus and the Dermanura, come also from morphology (Smith, 1976), karyology (Baker, 1973) and restriction site data (Van den Bussche et al., 1993). However the recognition of these two genera is not widely accepted (Simmons, 2005), and in most publications Dermanura is considered as a subgenus of Artibeus.

Although Artibeus in the broad sense (including the Dermanura and Koopmania subgenera) is widely accepted as monophyletic (Simmons, 2005), the relationships among the species inside both subgenera (or genera) is a matter of intense debate (Van den Bussche et al., 1998; Lim et al., 2004; Guerrero et al., 2004; Guerrero et al., 2003; Marques-Aguia, 1994; Owen, 1987, 1991; Handley, 1987).

Most of the works on phylogenetic relationships of Artibeus have centered on the ‘large’ species (Patterson et al., 1992; Marques-Aguia, 1994; Guerrero et al., 2003; Guerrero et al., 2004; Lim et al., 2004). So far, the largest taxon sampling including most of the species recognized in the genus (using few individuals from each species) was presented by Van den Bussche et al. (1998), but it missed one of the large species (A. amplus) and apparently misidentified A. jamaicensis using two individuals of A. planirostris in their analysis as pointed out by some authors (Guerrero et al., 2004; Lim et al., 2004).

The subsequent work of Lim et al. (2004) concentrated on elucidating the relationships of the ‘large’ species, challenging a close relationship (and the synonymization) of A. planirostris and A. jamaicensis as proposed by other authors (Handley, 1987; Marques-Aguia, 1994; Simmons, 2005) and firmly recognizing A. amplus (Handley, 1987; Lim and Wilson, 1993) as a separate species. However they used few individuals of each species and the relationships among A. obscurus, A. planirostris, A. amplus and A. lituratus presented low support values in their analysis, so a more consistent phylogenetic approach is still needed.

The complex taxonomy and the use of different species’ names have made it difficult to establish precise species’ distributions and
to analyze the full variation and phylogenetic relationships within the genus *Artibeus*.

The use of molecular data to help defining and discovering species have been widely discussed in the taxonomic community (Tautz et al., 2002, 2003; Lipscomb et al., 2003; Seberg et al., 2003; Dunn 2003), especially the DNA-barcoding initiative (Hebert et al., 2003, 2004; DeSalle et al., 2005). Although DNA sequence-based identification may have many problems, an integrated approach of phylogenetic methods with DNA sequences, morphological analysis and other evidence (e.g. geographic ranges, behavior, etc.,) can be extremely powerful (DeSalle et al., 2005). Molecular data could complement traditional taxonomy and, in some cases, point out outliers as possible incipient species deserving further investigation (Bradley and Baker, 2001).

Using a combination of molecular analyses following the phylogenetic and genetic species concept (Mishler and Theriot, 2000; Bradley and Baker, 2001), this manuscript intends to investigate in detail the phylogeny and the systematics of the species in the genus *Artibeus*. Furthermore, we use molecular taxonomy to point out potential new species, for some of which additional evidence is also presented.

2. Materials and methods

2.1. Samples

Specimens examined are listed in Appendix, with sampling localities and museum/field numbers of the vouchers. All DNA samples collected in this study were deposited in the DNA bank of the Laboratório de Biodiversidade e Evolução Molecular, Universidade Federal de Minas Gerais, Brazil (Santos et al., 2002).

We sequenced the entire segment (1140 base pairs, bp) of the mitochondrial Cytochrome-b (*Cyt-b*) gene of 429 bats of the genus *Artibeus* and retrieved another 111 sequences from GenBank. Altogether, the 540 sequences cover 19 of the 20 recognized *Artibeus* species, spanning 15 countries of South, Central and North America, plus the Lesser Antilles, covering almost all of their distribution (Fig. 1). In addition we sequenced 501 bp of the mitochondrial Cytochrome Oxidase I gene (*COI*) in a subset of 168 bats (12 species sampled and one additional species retrieved from GenBank), and two fragments of the nuclear genes *RAG2* (650 bp) in 42 individuals (plus 4 GenBank retrieved sequences, 13 species sampled in total) and *BrCA1* (700 bp) in 37 specimens (12 species sampled plus one species with a deposited sequence in GenBank).

All *Cyt-b* and *COI* sequences produced in this study were deposited on GenBank under Accession Nos: EU160667–EU161064.

2.2. Molecular methods

DNA extractions were performed with a standard phenol–chloroform–isoamyl alcohol protocol (Sambrook et al., 2001) or with DNeasy® extraction kits (Qiagen®).

PCR amplifications were carried out in 15 µL reactions containing 40–80 ng of DNA, Buffer 1B (Phoneutria®—1.5 mM MgCl₂, 10 mM Tris–HCl (pH 8.4), 50 mM KCl, 0.1% Triton X-100), 200 µM dNTPs set (Amersham-Biosciences®), 0.5 µM of primers and 1.25 U of *Taq* DNA polymerase (Phoneutria®). The primers used for *Cyt-b* amplifications were L14121 (GACTAATGACATGAAAAATCA) and H15318 (TATTCCCTTTGCCGGTTTACAAGACC) both developed in this study. *COI* amplifications used primers LCO1490 and HCO2198 (Folmer et al., 1994), *RAG2* used primers RAG2F220, RAG2R995, and *BrCA* used BRCA1f126, BRCA1r3012, both described by Teeling et al. (2000).

Most PCRs used the following cycling scheme: 5’ at 94°C followed by 35 cycles of 30’ at 94°C, 30’ at 50°C (*Cyt-b*) or 55°C (*BrCA1* and *RAG2*) for primer annealing and 1’ 20’ at 72°C for extension, and a final 9’ extension at 72°C after the last cycle. *COI* amplifications used the cycling conditions described by Folmer et al. (1994).

PCR products were purified using a solution of 20% Polyethylene-glycol 8000 in 2.5 M of NaCl using a protocol described by Sambrook et al. (2001).

Sequencing of both strands were carried out in a MegaBACE 1000® (GE Healthcare) automated sequencer using DYEnamic ET® Terminator kits (GE Healthcare®) and the same primers used in PCR amplifications, except for *Cyt-b* where two additional internal primers were also used: MVZ4 (Kocher et al., 1989) and L14881 (GACATAATTCCATTCCACCCCTAC) developed in this study.
Sequences were assembled and checked for quality using a combination of the programs Phred v.0.20425 (Ewing et al., 1998; Ewing and Green, 1998) and Phrap v.0.990319 (Green, 1994), and the assembled chromatograms were verified and edited (when it was necessary) using Consed 12.0 (Gordon et al., 1998).

In the analysis of nuclear genes, the presence of heterozygous nucleotide positions was identified in the Consed program and the haplotypes (also called here alleles) were inferred using the software Phase 2.1 (Stephens et al., 2001).

2.3. Phylogenetic analysis

Out of the 540 Cyt-b sequences we used 413 unique haplotypes (complete Cyt-b data set) in the analyses plus three Enchisthenes hartii sequences as outgroups. We also used a reduced (103 haplotypes) data set in some computationally intensive analyses. The sequences in this reduced set were chosen arbitrarily, using individuals representing the internal clades of the species studied.

These two data sets were the most explored because they contain samples of all species and a broad geographic range. The COI sequences revealed 93 unique haplotypes that were used in a complementary phylogenetic analysis or combined with Cyt-b to investigate complicated groups, using 12 species (114 individuals). We also used all nuclear gene sequences generated, although they presented some complex results (see Section 3).

Sequences were aligned using the Clustal-W algorithm implemented in MEGA 3.1 (Kumar et al., 2004). MEGA 3.1 was also used to calculate intraspecific and interspecific sequence divergence with a Kimura 2-parameter (K2P) model to compare with published data. Saturation of the sequences in the datasets was tested using DAMBE 4.5.35 (Xia and Xie, 2001).

Modeltest 3.7 (Posada and Crandall, 1998) was used to select the best fit model of sequence evolution using the Bayesian Information Criterion (Posada and Buckley, 2004). The selected model for Cyt-b data set was the Hasegawa–Kishino–Yano model (HKY) with a transition/transversion ratio $R = 6.397$ and gamma distributed ($I^2$) rates variation across sites ($\alpha = 0.895$) and a proportion of invariant sites ($I = 0.436$). For the COI data set the same model (HKY) was selected with transition/transversion ratio $R = 7.438$ and gamma distributed rates ($\alpha = 1.559$) and invariant sites ($I = 0.593$).

We used four methods of phylogenetic inference: Neighbor Joining (NJ), Maximum Parsimony (MP), Maximum Likelihood (ML) and MCMC Bayesian inference (BI).

Neighbor joining analyses were performed in Paup*4.0b10 (Swofford, 1998) using maximum likelihood distances with the model selected by Modeltest 3.7 with associated parameters. Confidence in the clades was assessed by 1000 bootstrap replications (Felsenstein, 1985).

We used the PhyML (Guindon and Gascuel, 2003) algorithm for maximum likelihood inference of large phylogenies with the model selected above. Two methods for estimating the confidence in the clades were used: the approximate likelihood ratio test (aLRT) method (Anisimova and Gascuel, 2006) with SH-like and $\chi^2$ probability tests and traditional bootstrap analysis with 1000 replicates (only used with the reduced Cyt-b data set and the combined Cyt-b and COI analysis). The aLRT method assesses the gain in the likelihood of a clade against a null hypothesis of the collapse of that clade, and tests its significance. This method seems to be more conservative than bootstrap (Anisimova and Gascuel, 2006).

Maximum parsimony trees were searched with the Parsimony Ratchet method (Nixon, 1999) performed on Paup*-4.0b10 using a batch file produced with the PaupRat software (Sikes and Lewis, 2001) with 18 runs of 200 iterations and two runs of 1000 iterations; half of the runs with 10% of re-weighted characters and half with 20% re-weighted characters. We also calculated the Bremer support values (decay index—DI) for the clades in strict consensus trees using the ratchet method (Müller, 2004).

MrBayes 3.1.2 (Huelsenbeck and Ronquist, 2001) was used for the Bayesian inference using two independent runs of four Markov Chains (1 cold and 3 heated) with $2 \times 10^8$ generations and sampling every 100 generations. The first 25% of the sampling trees and estimated parameters were burned off to allow the chains to reach stationarity for the reduced Cyt-b data set, and two runs of four Markov Chains (1 cold and 3 heated) with $10^7$ generations and sampling every 100 generations, and again burning the first 25% for the complete data set.

Testing of topologies, parsimony and likelihood scores were also performed in Paup*4.0b10.

Divergence time estimates were performed using two methods: (i) using linearized trees by re-estimating branch lengths under the assumption of constant rate of evolution (Takezaki et al., 1995) implemented in MEGA 3.1; and (ii) using a Bayesian method described by Thorne et al. (1998) and modified by Kishino et al. (2001) implemented in the program MultiDivTime that does not assume a constant rate of evolution and incorporates variation of rates across lineages. In both methods, outgroups from other sub-families of Phyllostomidae bats (Desmodus, Tonatia and Glossophaga) were included in the analyses to permit constraints in the time of splits based in fossil calibrations and previous divergence time estimates (Teeling et al., 2005).

3. Results

3.1. Mitochondrial DNA analyses

We found 413 unique Cyt-b haplotypes for Artibeus and three for Enchisthenes hartii (Table 1). The complete Cyt-b data set (1140 bp alignment) shows 595 constant and 545 variable positions, from which 135 present singletons (autapomorphic) and 410 present parsimony informative characters. The COI data set (93 haplotypes) had 501 bp, 333 constant sites, 168 variable sites, 136 of which were parsimony informative sites and 32 singletons.

Fig. 2 shows the maximum likelihood tree using the complete data set. Haplotypes that grouped separately from their respective ‘expected’ clades were named as ‘sp2’ (see Section 3.3 and Section 4).

Maximum Likelihood (ML) and Bayesian (BI) trees presented identical interspecific topologies, while Maximum Parsimony (MP) trees differed from them only in the placement of A. cinereus sp2 and A. concolor (Figs. 3 and 4).

The ML tree using the complete data set presented a likelihood score of ln L = 17,569.301 and the topology showed in Figs. 2 and 3. In this analysis, A. concolor clusters with the ‘small’ Artibeus (Dermanura) with low (aLRT $\chi^2 = 52$) to very low values (aLRT SH-like = 18), and A. cinereus sp2 is basal to a clade formed by A. cine- reus, A. glaucus, A. gnomus and A. anderseni.

In the MP trees, A. cinereus sp2 appears as a basal group to the ‘small’ Artibeus (Dermanura), and A. concolor is an external group sister to all Artibeus.

The BI analysis showed the same topology of the ML tree, grouping A. concolor with the ‘small’ Artibeus displaying a Bayesian posterior probability of 0.57.

The parsimony ratchets found 2536 MP trees of 2940 steps, with ensemble consistency index (CI) of 0.238 and ensemble retention index (RI) of 0.853. All rearrangements in MP trees with the complete Cyt-b dataset were intraspecific swaps as it was shown by the absolute frequency of bipartitions between the species on the majority rule consensus tree (Fig. 4).

The NJ tree presented a different topology from all other methods, but almost all clades showed very low support values (not
shown). In this analysis A. concolor clusters with the ‘large’ Artibeus group with a low bootstrap value of 47%, and A. cinereus sp2 is a sister group to a cluster of A. cinereus and A. anderseni, with a very low bootstrap support (34%). Many other species’ relationships are also different in this tree but always with low support values. However, all entities called as ‘sp2’ remain as separate monophyletic groups, independent of their ‘expected’ clades, no matter which method or mitochondrial gene was used for phylogenetic reconstruction.

The combined COI-Cyt-b dataset clustered A. concolor with the ‘large’ Artibeus group in all methods (data not shown) with support values ranging in ML trees from low (SH-like = 43 and bootstrap-ML = 55) to high ($\chi^2 = 80$), and the remainder part of the trees shows a topology compatible with the trees of their respective methods (Fig. 3). When only the COI gene is used, A. concolor also clusters with the ‘large’ Artibeus in all methods used (SH-like = 63 $\chi^2 = 60$ in ML trees and bootstrap-NJ = 38). The COI phylogenies could also discriminate all species studied, but the support values for interspecies relationships were very low in most cases (data not shown).

We tested the alternate topologies concerning the placement of A. concolor and A. cinereus sp2 (the only discrepancies among optimization methods used) and also the probability of existence of the groups labeled as ‘sp2’ against the null hypothesis of they belonging to their respective ‘expected’ species. For this, we constrained the concurrent topologies and then searched the 95% credible set, the first one in the posterior distribution of the trees, the clade found on MP trees with 99% credible trees. The alternative topologies can be found in the 95% credible set, the first one in 6.51% of the trees and the second in 17.97%.

For A. cinereus, the first alternative hypothesis (MP), with A. cinereus sp2 being a sister clade to all other ‘small’ Artibeus, could not be rejected and was also quite frequent (17.94%). However, the second one (NJ), with A. cinereus sp2 as a sister group to only A. anderseni and A. cinereus, was rejected even in the interval of 99% credible trees.

As a complement to the topology tests concerning the placement of A. concolor and A. cinereus sp2, we took one of the most parsimonious trees of the Cyt-b dataset and constrained the topologies with the tree around different placements of Fig. 4, retaining the rest of the tree, and recalculated the number of steps of these trees.

Only one more step (2941 steps total) is required to let A. concolor become part of the ‘large’ Artibeus group, and two steps (2942 steps) to place it together with the ‘small’ group showing that very few steps separates the topologies in a parsimony-based decision. The same procedure was repeated with the two different placements of A. cinereus sp2, we constrained the topology discrepant with the MP tree. The alternative topology (the same of the ML tree for this group) is two steps longer than the MP tree, while the NJ tree had so many different groupings that is difficult to constrain the topology around A. cinereus sp2 and involved taxa without changing the rest of the tree, however it is at least 15 steps longer than the MP tree.

We also tested the monophyly of A. cinereus, A. obscurus and A. jamaicensis, when including their ‘sp2’ counterparts and also the odd case of the two A. phaeotis (see below). Both A. phaeotis and A. obscurus monophilies were rejected, while A. jamaicensis and A. cinereus were not, but the frequencies of occurrence were very low, 0.18% and 1.32%, respectively.

### 3.2. Nuclear DNA analyses

The BrCa1 exon 11 fragment (700 bp) presented only four parsimony-informative sites. Eight alleles were found and shown to
cluster in two distinct groups (plus the outgroup *E. hartii*), one with the 'small' *Artibeus* alleles and one with the 'large' *Artibeus*. Alleles were also shared among species inside the groups. The most impressive example was the allele BrCA1-1 found in *A. lituratus*, *A. planirostris*, *A. obscurus*, *A. fimbriatus*, *A. concolor* and the GenBank sequence of *A. jamaicensis* (AY834647).

Fig. 2. Maximum Likelihood tree using all 413 *Artibeus* Cyt-b haplotypes of *Artibeus* plus 3 outgroup haplotypes. Haplotype sequences of the same species were 'condensed' in triangles with the base size proportional to sample size and height proportional to the depth of species divergence. Numbers near the branches represent SH-like test support values.
The RAG2 sequences (650 bp) were more informative than BRCA1, presenting 15 parsimony informative sites and again showing two distinct clusters of *Artibeus*. Once more, shared alleles were observed, but only among species of the “large” *Artibeus*. One of the *A. concolor* specimens shares alleles with *A. lituratus*, *A. obscurus*, *A. fimbriatus* and the GenBank sequence AF316433 for *A. hirsutus*.

Because of the shared alleles among species for the two analyzed autosomal genes, the topology of the tree inside the two groups cited previously is unreliable, and a more extensive analysis could not be performed with these datasets.

### 3.3. Molecular taxonomy

Using the complete Cyt-b dataset, *A. fraterculus*, *A. phaeotis*, *A. jamaicensis*, *A. obscurus* and *A. cinereus* showed up as monophyletic (non-monophyletic) taxa and *A. intermedius* haplotypes were mixed among the high number of *A. lituratus* haplotypes (Fig. 5). All other species showed monophyletic status.

All *A. intermedius* haplotypes downloaded from GenBank (see Appendix for accession numbers) fall inside a large clade of *Artibeus lituratus* and do not form a monophyletic clade themselves (Fig. 5). Some of the haplotypes clustered with individuals of *A. lituratus* collected as far as southern Brazilian Atlantic Forest, Brazilian and Peruvian Amazon and Central America.

Both Van Den Bussche et al. (1998) and Lim et al. (2004) found low values for Cyt-b sequence divergence between *A. intermedius* and *A. lituratus* (2.5% and 2.8%, respectively; we found a smaller value of 1.8%) but they only have one sequence of each species in their work, so they appeared to be sister clades in these papers. Because of the polyphyletic status, small sequence divergence and the lack of supporting morphological evidence, we do not
recognize A. intermedius as a proper species and the A. intermedius haplotypes are considered as part of the A. lituratus species diversity.

The A. fraterculus haplotype Afra_6 (GenBank Accession No. AY684772) grouped inside the clade of A. fimbriatus. Since it was collected in Peru and is endemic to the Western slope of the Andes (voucher FMNH 129105, see Appendix) while A. fimbriatus is endemic of the Atlantic Forest in coastal Brazil and south of Paraguay (Simmons, 2005), we believe it was likely a mistake made when uploading (or handling) data to GenBank, since, this specimen (FMNH 129105), displays an A. fraterculus haplotype (and differed from A. fimbriatus) in Patterson et al. (1992) study using mitochondrial ATPase 6 and 8.

Similar cases include AY684768 (voucher MVZ185677, also named AD519), which is an A. fimbriatus in the Museum of Vertebrate Zoology (MVZ) database (also in the field notes of A.D. Ditchfield), but the sequence in GenBank clusters with A. obscurus (and it is identical to AY684767, an A. obscurus in the MVZ database). Moreover, we also sequenced this specimen (AD519) and it shows a common A. fimbriatus haplotype. A. obscurus AY684765 and A. hirsutus AY684764 also display identical sequences.

This illustrates that great care must be taken in both uploading sequences in a public database and in using them in analyses and publications. None of these sequences were used in our analysis.

Thus, if we exclude the haplotype Afra_6, A. fraterculus becomes a monophyletic species.

A. phaeotis represents another issue with haplotypes Apha_1 (APU66514, Van Den Bussche et al., 1998) and Apha_2 (AY157584, Porter and Baker, 2004) that are clearly different (11% sequence divergence) and cluster with distinct species (Figs. 2–4): Apha_1 with A. toltecus (5.4% divergence) and Apha_2 with a clade formed by A. watsoni and A. aztecus (8.8% divergence).
In *A. jamaicensis*, two haplotypes, Ajam_28 (AY572353) and Ajam_30 (AY572355) (Carstens et al., 2004), are also divergent from all other *A. jamaicensis* haplotypes (5.4%). Besides, they form a monophyletic clade basal to *A. amplus*, *A. obscurus*, *A. planirostris* and *A. lituratus* with short branch length but with moderate to high support values in all tests (Fig. 2). A similar situation happens with *A. obscurus* sp2, where two individuals collected in the Acre state of the Brazilian Amazon presented the same haplotype (Aobs_38). It displays 7.4% sequence divergence from all other *A. obscurus* haplotypes and appears to be basal to *A. jamaicensis* sp2 (Figs. 2–4), also with short branch length but with relatively good support values.

*Artibeus cinereus* sp2 haplotypes Acin_20, Acin_21 and Acin_22 also form a monophyletic group that is highly divergent from *A. cinereus* (8.2%). These haplotypes were found in two localities in the Serra do Mar, a mountain chain in the Brazilian Atlantic Forest. They were called *Artibeus sp1* in a phylogeography paper by Ditchfield (2000) due to morphological and Cyt-b differences (see Section 4).

The graph on Fig. 6 shows two plots of the maximum intraspecific divergence against the minimum interspecific divergence for all species studied. In the first graph (Fig. 6A) the divergences were calculated using the current *Artibeus* taxonomy and the identity of the specimens assigned to the species by traditional methods (species keys) and/or identifications in databases (GenBank or museums), excluding obvious mistakes of public databases (as some described above). The Fig. 6B shows the graph recalculated taking the species called as ‘sp2’ in the trees as true species. Note that these species and *A. gnomus* (marked as triangles in the graph) are more dispersed in Fig. 6A, ranging over 6.5% of intraspecific sequence divergence, than in Fig. 6B where all species are more aggregated with a maximum of 5.4% divergence.

Although the haplotypes of *A. gnomus* form a monophyletic group in the trees of all methods, they can be resolved as two sister clusters (Fig. 7): one with haplotypes from the Amazon (Agno_1, Agno_2, Agno_3, Agno_4, Agno_5 and Agno_10) and another with Brazilian Cerrado haplotypes (Agno_7, Agno_8, Agno_9) plus one Amazonian haplotype (Agno_6). The divergence between the two clusters is high, about 7.1%, and there are some morphological differences between the individuals of different clusters (see Section 4).
Fig. 6. Scatter plot of the maximum intraspecific divergence against the minimum interspecific divergence found in Artibeus species. (A) Divergences calculated using the ‘traditional’ species scheme. (B) Divergences calculated taking the taxa called here as ‘sp2’ as ‘true species’. A triangle represents species that have ‘sp2’ counterparts.

Fig. 7. A detail of the ML tree showing the divergence between the two clades of Artibeus gnomus that could represent different species. Numbers below the branches represent SH-like test support values, numbers above the branches of each clade of A. gnomus are the Bremer Decay Index support values found on the maximum parsimony strict consensus tree.

3.4. Divergence times

Times of splits were calibrated with both fossil and previous genetic estimates (Teeling et al., 2005) for the Phyllostomidae phylogeny. Both methods presented similar results.

The split between Phyllostominae (here represented by the genus Tonatia) and the group formed by Glossophaginae (represented by Glossophaga) plus Stenodermatinae (represented by Artibeus and Enchisthenes) was about 22.4 million years ago (m.y.a., max = 26.1, min = 21), in the Oligocene–Miocene transition. Stenodermatinae diverged from Glossophaginae around 20.4 (24.4–17) m.y.a. in the Miocene.

The Artibeus diversification started around 13.2 m.y.a. (17.9–8.8) in the Miocene, splitting into the large (Artibeus) and small (Dermanura) clades. The two sub-genera started diversifying soon afterward at 11.7 (16.5–7.5) m.y.a. and 12.2 (16.9–7.9) m.y.a., respectively, also in the Miocene epoch.

4. Discussion

4.1. Artibeus (Koopmania) concolor

The taxonomic status of A. (K.) concolor was always a matter of hot debate in bats’ systematics. When first described by Peters in 1865 (voucher from Paramaribo, Suriname), it was described as an Artibeus (Owen, 1891). In 1908, Andersen suggested that it could be related to the ‘large’ species of the genus, based largely on the presence of 3/3 molars (third molar present in both jaws) (Owen, 1991). Despite its ‘intermediate’ size, A. concolor presented a shortened rostrum and other morphological characteristics, differentiating it from small and large species of Artibeus (Owen, 1991). In the following years, many authors aligned A. concolor with the small species instead of the large ones based on other morphological evidences (Owen, 1987; Handley, 1987). Owen (1991) concluded that A. concolor (Dermanura concolor in that paper) should be included in its own genus, Koopmania, with K. concolor as the only species.

In the first molecular analysis of the Artibeus genus, Van den Bussche et al. (1998), using the complete sequences of the Cyt-b gene and variation in the EcoRI defined satellite DNA, concluded that there is no support for the Koopmania genus and A. concolor should be recognized as a member of the large Artibeus group. Later, Lim et al. (2004) also analyzed Cyt-b variation, but using more individuals and species of the large group, and reached the same conclusion that A. concolor is more related to the ‘large’ group.

We found three different placements for A. concolor in our trees, varying with the phylogenetic method, gene or number of samples used. However none of the methods presented high support values for the association of A. concolor with any other groups (Figs. 2–4). There is a relatively large number of homoplasious (and autopomorphic) characters in the trees (CI = 0.238), but also a very large amount of synapomorphies (RI = 0.853) and no saturation was ver-
ified for both COI and Cyt-b (data not shown). Beyond the existence of several synapomorphic sites that clearly define A. concolor, there are no sites that unambiguously connect it to any of the large or small Artibeus species.

The most compelling evidence of a closer relationship of A. concolor with the large Artibeus group comes from the nuclear genes studied. In both genes we found alleles shared between A. concolor and other species of the large species group, but never with the small one. However this evidence is relatively weak, since A. concolor appears sometimes as a basal group to all other Artibeus among the trees obtained, raising the possibility of these alleles being plesiomorphic.

Because of the ambiguous results and low support values in the phylogenetic methods used, and the lack of concrete morphological evidence favoring Artibeus or Dermamura groups, we recommend a provisional allocation of A. concolor to the subgroup or genus Koopmania, as proposed by Owen (1991).

4.2. The large Artibeus group

The resulting trees presented usually the same topology for the large species group in all methods, with the exception of small incongruences (with very low support) in the NJ-tree.

Apart the possibility of A. concolor belonging to the large group, this cluster branches into two clades, one formed by A. inopinatus, A. hirsutus and A. fraterculus and another with all other large species.

The first clade is highly supported (ML SH-like = 95, \(\chi^2 = 99\), boot-ML = 96; DI = 6) with A. inopinatus as sister taxon to a group formed by A. hirsutus and A. fraterculus with moderate to low support values (Figs. 2–4). Lim et al. (2004) found this same grouping but the entire group branched after A. fimbriatus (the first of the 'large' Artibeus to diverge just after A. concolor in their analysis). In the work of Van den Bussche et al. (1998), the group also branches after A. fimbriatus but the clade is formed by A. fraterculus as sister taxon to A. hirsutus plus A. inopinatus. A. hirsutus and A. inopinatus are considered closely related (Webster and Jones, 1983) and, in fact, Marques-Aguiar (1994) scored both identical in her morphological phylogenetic analysis, despite recognizing them as separate species. They have very furry uropatagium (Webster and Jones, 1983) and were among the smallest of the large Artibeus group; indeed A. inopinatus overlaps with A. aztecus (the largest Dermamura) in size (Lim et al., 2004). Our data indicates that A. fraterculus is more closely related to A. hirsutus, but with low support values in most of the analyses. These three species share distributions that lie mainly west of the Andes (Koopman, 1982; Patterson et al., 1992) and little is known about the biology of this group. We estimate the divergence time between this clade and other large Artibeus in 11.7 (16–7.5) m.y.a., a time compatible with the elevation of the Andes during the middle Miocene.

The second clade is also highly supported (ML SH-like = 77, \(\chi^2 = 86\), boot-ML = 85; DI = 4), and A. fimbriatus is the first species to branch out. A. fimbriatus is also considered a basal species to all large Artibeus (excepting A. concolor) in other morphological (Marques-Aguiar, 1994) and molecular analyses (Lim et al., 2004; Van den Bussche et al., 1998), with low to moderate support values (MP-bootstraps <50 and 76 and decay index of 2 and 22, respectively, in the molecular studies). This species seems to be endemic to the Atlantic Forest and ranges from southern Brazil and Paraguay to coastal northeast Brazil in Bahia state (Handley, 1989; Simmons, 2005).

The next species to branch out is A. jamaicensis, with very high support values (ML SH-like = 97, \(\chi^2 = 99\), boot-ML = 94; DI = 4). A. jamaicensis was once considered a very variable and widely distributed species with many subspecies, including A. planirostris (Handley, 1987; Marques-Aguiar, 1994). Van den Bussche et al. (1998) found little divergence between A. jamaicensis and A. planirostris (3.9%), which were sister groups in their analysis. However other molecular works (Lim et al., 2004; Guerrero et al., 2004) found that A. planirostris is more closely related to A. obscurus, and based on geographic distributions concluded that Van den Bussche et al. used only A. planirostris sequences in their study. Lim et al. (2004) suggested that A. jamaicensis is only distributed north of Orinoco River into Central America, although the limit of the species in the Llanos region of Venezuela could not be traced for sure. Our results agree partially with Lim et al. (2004), but a detailed analysis suggests that A. jamaicensis does not exist in South America even north of Orinoco River in the Llanos region in Venezuela. We retrieved from GenBank one sequence supposedly of A. jamaicensis collected in Calabozo, north of the Orinoco River in Venezuela (AY684477) and many of the A. planirostris sequences used by Lim et al. (2004). The A. jamaicensis sequence also showed an A. planirostris haplotype suggesting that either the species is sympatric in this region or A. jamaicensis does not exist in South America. Therefore, it is crucial to analyze individuals of both species from Venezuela, Colombia, Panama and Ecuador West of the Andes to determine precisely their distribution limits. Despite the molecular (Lim et al., 2004; Guerrero et al., 2004) and morphometric (Guerrero et al., 2003) evidences, some authors still think of A. planirostris as conspecific with A. jamaicensis (Handley, 1987; Simmons, 2005). Moreover, the mixed use of the names A. jamaicensis planirostris and A. planirostris in the literature or GenBank entries causes many problems, since “A. jamaicensis” may be used to refer to any of the two species. As discussed in Lim et al. (2004) and also supported by our results, this practice should be discontinued because inclusion of A. planirostris in A. jamaicensis generates a paraphyletic taxon.

A. obscurus sp2 is the next to branch out with low support values (ML SH-like = 55, \(\chi^2 = 71\), boot-ML = 43; DI = 1) but it is recognizable as a distinct entity of true A. obscurus in all phylogenetic trees and Bayesian monophyly tests (100% rejection), they also presented 7.34% divergence with Cyt-b. We examined two specimens identified as A. obscurus sp2 (see Appendix) from the Acre state in the Brazilian Amazon, very near the border with Peru. Both specimens presented the same haplotype, distinct from other A. obscurus as described above. Morphologically, they are a little larger than the common A. obscurus (forearm with 64 mm against ~60 mm), have a larger and broader rostrum and mandibular arc (data from Redondo and Aires, in preparation). Ditchfield (1996) found a ‘strange’ haplotype in three individuals identified as A. obscurus from Venezuela. They did not form a group with the other A. obscurus and were external groups to both A. lituratus and A. planirostris in his analysis. We reanalyzed his data (402 bp of Cyt-b) including all our haplotypes, and found that his ‘strange’ haplotypes cluster with our A. obscurus sp2 with strong support (ML-SHlike = 90) and little divergence (2.8%). These results suggest that they are also A. obscurus sp2.

In 1989, Handley reanalyzed A. fuliginosus Gray and A. obscurus Schinz and concluded that A. fuliginosus was one of the many variant forms of what he considered to be A. jamaicensis (here A. planirostris because of the locality in Peru), and A. obscurus was the valid name for the small blackish Artibeus. The A. obscurus sp2 individuals that we analyzed are similarly sized and have five warts on either side of the chin, as in Gray's A. fuliginosus (A. obscurus have 3–4 warts) and lack the soft long fur, typical of A. obscurus (Handley, 1987). Although there are morphological (and molecular) differences between A. obscurus and A. obscurus sp2, their morphological characteristics were not exclusive, being found also in A. jamaicensis and A. planirostris. A detailed morphological and molecular analysis of individuals from the area between Venezuela and Peru is needed to likely resurrect the name Artibeus fuliginosus (or to name it as something else) for what we name here as A. obscurus sp2.
The next branch is A. jamaicensis sp2 with high and some low support values (ML SH-like = 83, $\chi^2 = 98$, boot-ML = 68; DI = 2). These two haplotypes were first described by Carstens et al. (2004), who found a very distinct group of three haplotypes (we removed haplotype W of Carstens et al. because it was an incomplete sequence) diverging 7.2% from other A. jamaicensis from the Lesser Antilles (only 5.4% divergence is found in our analysis using many more sequences). Carstens et al. have not included any outgroup species, thus these haplotypes appeared as a sister group to other A. jamaicensis. They also concluded that these haplotypes were more closely related to South American haplotypes (actually A. planirostris) than to other ones found in the Lesser Antilles. These results were similar to ours since the sequence divergence between A. jamaicensis sp2 and A. planirostris (5.48%) is smaller than between A. jamaicensis sp2 and A. jamaicensis (6.86%). However, Bayesian monophyly test analysis could not exclude the possibility of A. jamaicensis sp2 forming a monophyletic group with A. jamaicensis, even if the probability is very low (0.19%) and they did not group together in our trees. More molecular and morphological data are also needed to establish the identity of this taxon.

The widely distributed A. lituratus branches out next, also with high support values (ML SH-like = 89, $\chi^2 = 99$, boot-ML = 71; DI = 2). A. lituratus and A. intermedius have been usually recognized as distinct species (Davis, 1984; Guerrero et al., 2003) and sometimes as conspecific (Marques-Aguir, 1994). Molecular data have suggested that the divergence between A. lituratus and A. intermedius is very small, ranging from 2.5 to 2.8% (Van den Bussche et al., 1998; Lim et al., 2004), Simmons (2005) argued that there is no morphological character that unambiguously differentiates A. lituratus palmarum (a subspecies from Central America) from A. intermedius (Davis, 1984; Marques-Aguir, 1994) and concluded that A. intermedius represents individuals of A. lituratus palmarum that are in the lower range of normal size variation.

Our results show that A. lituratus and A. intermedius are conspecific according to phylogenetic and generic species concepts (Mishler and Theriot, 2000; Bradley and Baker, 2001). A. intermedius haplotypes display a very small divergence among them and do not form a monophyletic clade, being part of the variation found in A. lituratus (Fig. 5). In addition, no morphological character analyzed so far could be used to differentiate them unambiguously (Marques-Aguir, 1994; Simmons, 2005).

Following A. lituratus, the next species to branch out is A. obscurus (ML SH-like = 77, $\chi^2 = 93$, boot-ML = 54; DI = 2). A. obscurus seems to be associated with tropical moist forests, especially to the Atlantic and Amazon Forests in South America. Although A. obscurus is easily distinguishable from other large Artibeus, the phylogenetic relationship of this species is poorly resolved in morphological analysis (Marques-Aguir, 1994). In the molecular works of Lim et al. (2004), A. obscurus appears to be more related to A. planirostris, although with low support values (MP-bootstrap < 50 and decay index of 2). This result is also shared by Van den Bussche et al. (1998), but they did not include A. amplus in their analysis. Our analysis shows A. obscurus as sister taxon to a clade formed by A. planirostris and A. amplus with large support values (ML SH-like = 80, $\chi^2 = 97$, boot-ML = 92; DI = 2), the last branch of the large Artibeus group.

Morphometric analysis (Guerrero et al., 2003) have grouped A. amplus and A. lituratus as sister taxa, though they resemble more A. planirostris (A. jamaicensis in Handley, 1987) by morphology. The phylogenetic analysis of Marques-Aguir (1994) places A. amplus as an external group to all other large Artibeus, excepting A. fimbriatus and A. concolor. Lim et al. (2004) found A. amplus as a sister taxon to a group formed by A. planirostris and A. obscurus (all with very low support values) and argues that Guerrero et al. (2003) morphometric analysis is biased because of the large sizes of A. amplus and A. lituratus. A. amplus inhabits the Amazon and occurs in Venezuela, Colombia, Guyana and Suriname (Simmons, 2005; Lim et al., 2004). We collected two individuals of A. amplus from Brazil in Barcelos, Amazonas State, near the border with Venezuela somewhat expanding its known distributional range. They were initially identified in the field as A. planirostris fallax, a large subspecies of A. planirostris, but a closer examination showed that it was an A. amplus. Several characters can be used to distinguish both species, including exclusive ectoparasites (Handley, 1987).

### 4.3. The small Artibeus (Dermanura) group

The small Artibeus group showed a more complicated tree structure varying with reconstruction methods (Figs. 2–4), but most of the clades are found in all trees for all genes studied. We will follow the Maximum Likelihood (and Bayesian) branching structure in the following description, pointing out differences among reconstruction methods. Again we are not including A. concolor in this group even though it branches together Dermanura in ML and BI methods, but with low support values (ML SH-like = 23, BP = 67).


Starting with the smaller Mesoamerican clade, A. phaeotis appears as a sister taxon to a clade formed by A. aztecus and A. watsoni with very high support values (ML SH-like = 98, $\chi^2 = 99$, boot-ML = 98; DI = 16; BP = 100). This clade is also observed in the MP trees but as sister group of a clade formed by A. cinereus, A. glaucus, A. gnomus and A. anderseni, with the exclusion of the other species. However, A. phaeotis sequences retrieved from GenBank are clearly not the same species. The monophyly of the two sequences was rejected in Bayesian tests, they do not form clades in the trees of any methods and have a extremely large sequence divergence (~11%). It is more probable that one of the specimens was misidentified. For example, the A. phaeotis sequence AY157584 from Esmeraldas, Ecuador (Porter and Baker, 2004) is identified as Dermanura rosenbergii in Hooper and Baker (2006) (A. glaucus rosenbergii, Handley, 1987).

In any case, which species those sequences belong to? A. phaeotis AY157584 from Ecuador presents 8.8% mean sequence divergence with its closest clade (A. aztecus and A. watsoni) while A. phaeotis APU66514 from Nicaragua presents 5.4% sequence divergence to A. toltecus, its closest relative in all analysis. Both values are in the range expected for typical sister species divergence, but are high for intraspecific divergence (Bradley and Baker, 2001).

We see two possible scenarios to solve this issue. First, Koopman (1982) and Timm (1985) synonymized A. ravus and A. phaeotis (also followed by Handley, 1987). A. ravus was also described as a subspecies of A. toltecus from Ecuador (Webster and Jones, 1982a), showing remarkable external similarities, but was refused by other authors (Handley, 1987; Simmons, 2005). A. ravus’ original description comes also from Esmeraldas, Ecuador and perhaps the sequence AY157584 was indeed an A. ravus sequence, so the synonymy of A. ravus and A. phaeotis must be considered. Second, the sequence APU66514 from Nicaragua probably belongs to A. phaeotis palatinus a clearly demarked subspecies restricted to the western coast of Central America (Timm, 1985); if so, the subspecific status of A. p. palatinus should be reviewed.

A. aztecus is the largest of the small Artibeus and morphologically is closely related to A. toltecus, even with a large size difference (Webster and Jones, 1982b; Handley, 1987). In our analysis,
A. aztecs appears to be more related to A. watsoni with high support values.

The first group to branch out of the second clade of the Dermantura group is formed by A. phaeotis APU66514 and A. toltecs. The situation of A. phaeotis was already discussed; A. toltecs is also distributed in Central and North America (Simmons, 2005).

The next to branch out is A. cinereus sp2, with moderate to high support values (ML SH-like = 85, $\chi^2 = 99$, boot-ML = 64; BP = 76). A. cinereus sp2 was collected in the Serra do Mar, in the Atlantic Forest, and was recognized as a different species from A. cinereus by Ditchfield (1996, 2000). It is very distinct in the trees made by all methods; in the MP tree it is sister to all small species. Sequence divergence is high, reaching 8.17% when compared to other A. cinereus and 8.11% to A. anderseni, the two closest taxa. Both values are high for intraspecific divergence but commonly found between sister species (Bradley and Baker, 2001). Bayesian monophyly test could not reject the clustering with A. cinereus, but its frequency in the posterior distribution of trees is only 1.32%. Morphometric analysis shows that A. cinereus sp2 is larger than common A. cinereus of both subspecies (A. c. quadrivittatus and A. c. cinereus) and it also presents a darker fur color than typical A. cinereus (data from Ditchfield and Redondo, in preparation). We did not find any possible synonyms for this species and its distribution is perhaps only shared with A. gnomus and A. glaucus (Tavares et al., in press; Simmons, 2005), both easily distinguishable from A. cinereus sp2 by morphology and molecular analysis. We do believe that it is a new species, its formal description will be published elsewhere (Ditchfield and Redondo in preparation).

The last clade is formed (in branching order) by A. cinereus, A. anderseni, A. glaucus and A. gnomus with low (A. cinereus and A. anderseni branching) to high (A. glaucus plus A. gnomus clade) support values. This branching order is the same recovered by Van den Bussche et al. (1998). Prior to the revision by Handley (1987), all of these species (except for A. gnomus described in that work) were considered subspecies of A. cinereus. A. cinereus has a distribution ranging from northern South America in the Amazon to coastal Atlantic Forest in Brazil (Simmons, 2005; Handley, 1987; Ditchfield, 2000), distinct from A. anderseni that ranges in northwest South America (Bolivia, Ecuador, Peru and northwest Brazil). Because of low support values for the A. anderseni branching we searched in the posterior distribution of Bayesian trees for the ones where A. anderseni and A. cinereus form a mixed group. This hypothesis could not be rejected at $P<0.05$ but its probability is only 0.0158. They also form very clearly defined monophyletic groups on the trees and the mean sequence divergence is about 6.96%.

Artibeus glaucus and A. gnomus form a monophyletic clade with very high support values in all analyses (ML SH-like = 97, $\chi^2 = 99$, boot-ML = 98; DI = 10; BP = 100). This also correlates with morphological features shared by the two species (Handley, 1987), even though they are easily distinguishable in external characteristics (Handley, 1987). A. glaucus is thought to have a discontinuous distribution, ranging in northwest South America (not in the Brazilian Amazon), then reappearing in southern Brazil (Simmons, 2005; Tavares et al., in press). Marinho-Filho and Szazima (1998) argued that the species should occur in the Brazilian Amazon, Atlantic Forest and Pantanal. However we found no record for this species in any of these habitats, and it was not collected during the large field survey in Atlantic Forest by Ditchfield (1996) and other expeditions carried out during this work.

Artibeus gnomus has a large, ragged distribution, being found in northern South America (Amazon), as well as central (Cerrado) and southeast (Atlantic Forest) Brazil (Simmons, 2005; Handley, 1987; Tavares et al., in press; Ditchfield, 2000; Gonçalves and Gregorin, 2004). We found two very distinct clades 7.1% sequence divergence, (Fig. 7) with very high support values for each (ML SH-like = 99, $\chi^2 = 99$, boot-ML = 100; DI = 22; BP = 100 and ML SH-like = 99, $\chi^2 = 99$, boot-ML = 100; DI = 18; BP = 100). The first is formed mainly by A. gnomus haplotypes from the Cerrado region plus one from Amazon, and the second is formed only by Amazonian haplotypes. Ditchfield (2000) found 3.3% sequence divergence between one Atlantic Forest haplotype and Amazonian haplotypes, which is an expected value for a species with a marked geographic structure (Bradley and Baker, 2001). However, the large divergence observed between the two A. gnomus clades (7.1%) is high for intraspecific divergence (Bradley and Baker, 2001). There are differences in size and color between individuals of the two clades, the Amazonian individuals have the common size for forearm (mean 37.1 mm) and greatest length of the skull (mean 18.3 mm), while the Cerrado individuals present a mean forearm length of 40.6 mm and skull length of 20.3 mm. When treated as two separate species they fall in the range of between species variation for Cyrt-b (Bradley and Baker, 2001) and congruent with our results (Fig. 7). Although more morphological and molecular analyses are still needed, we are confident that the A. gnomus from Cerrado could be considered a distinct taxon, at least in the subspecies level.

Finally, this study shows a detailed analysis of molecular markers applied to systematics and phylogeny of bat species belonging to a highly diverse genus of the Neotropical region. In addition, it exemplifies the use of molecular data and analysis in conjunction with classical taxonomy studies (morphology and biogeography) to suggest and support the identification of new taxa or to direct new taxonomic studies and revisions.

Acknowledgments

We are grateful to all people who helped in the field works and those who contributed with samples, in special we thank Dr. B. Patterson (FMNH), V.C. Tavares (AMNH/UFGM), F.A. Perini (UFMG), M.H. Marcos, M.O. Garcia-Lopes (UFGM), C. Aires (USP), F. Martins (USP), A.C. Pavan (USP), R.Z. Coutinho (UFES), A.P. de Araújo (UFPE) and K. Santos (UFPE). We also want to thank Dr. B. Patterson again and one anonymous reviewer for their comments and suggestions that greatly improved this manuscript. RAfR, LPSB, RFS and FRS were supported by CNpq (Brazil). This project received grants from FAPEMIG, CNpq, PELD-MCT/CNPq and Fundação o Boticário (FBPN), all from Brazil.

Appendix A

Species, Sample locations (COUNTRY, location), voucher/field numbers or GenBank accession number of samples (between parentheses) used in this study:

A.1. Subgenus Artibeus


A.2. Subgenus Dermanura


A.3. Subgenus Koopmania


A.4. Genus Enshisthenes

Enshisthenes harrii. PERU. Huanuco (AHU66517—). Cuzco: Pau- cartambo (FMNH174703, FMNH174704). GenBank accession numbers.

References
