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When did plants become important to leaf-nosed bats? Diversification of feeding habits in the family Phyllostomidae

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Abstract

A great proportion of bats of the New World family Phyllostomidae feed on fruit, nectar and pollen, and many of them present adaptations to feed also on insects and small vertebrates. So far, attempts to examine the diversification of feeding specialization in this group, and particularly the evolution of nectarivory and frugivory, have provided contradictory results. Here we propose a molecular phylogenetic hypothesis for phyllostomids. On the basis of a matrix of feeding habits that takes into account geographical and seasonal variation, we tested different hypotheses of the evolution of feeding specializations in the group. We find strong support for the evolutionary model of a direct dietary diversification from insectivory. The estimates of divergence times of phyllostomid bats and the reconstruction of ancestral states with a Bayesian approach support the parallel evolution of frugivory in five lineages and of nectarivory in three lineages during the Miocene. On the basis of these findings, and recent dietary studies, we propose that during the evolution of phyllostomids switches to new feeding mechanisms to access to abundant and/or underexploited resources provided selective advantages that favoured the appearance of ecological innovations independently in different lineages of the family. We did not find evidences to support or reject the hypothesis that the insectivorous most recent common ancestor of all phyllostomids was also phytophagous.

Keywords: BayesTraits, frugivory, nectarivory, phyllostomids

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Introduction

Leaf-nosed bats (Chiroptera: Phyllostomidae) have undergone a remarkable evolutionary diversification among mammals. This radiation has been linked to four evolutionary shifts in diet from a presumably insectivorous ancestor (Freeman 2000). In the last two decades the dietary diversification of this family has been the subject of a few albeit seminal investigations (Ferrarezi & Gimenez 1996; Wetterer *et al.* 2000; Cruz-Neto *et al.* 2001; Datzmann *et al.* 2010). Phyllostomid species that feed on plant material, either as main or complementary resource, represent over 75% of the family (calculated from Muscarella & Fleming 2007; Fleming *et al.* 2009; see also Supporting Information). Moreover, it has been proposed that a shift to frugivory probably triggered the diversification of the family (Freeman 2000). It is important, then, to elucidate the evolutionary patterns of dietary diversification of phyllostomids for a better understanding of the process of adaptive radiation that took place in this group.

The interest on this topic is reflected in a group of studies that have tried to reconstruct the phylogenetic relationships of the family (e.g. Carstens *et al.* 2002; Jones *et al.* 2002, 2005) in order to make inferences about the evolutionary steps that have conducted the process of feeding specialization. However, studies so far have shown conflicting results. The evolution of feeding specializations in Phyllostomidae was first

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reconstructed in a phylogenetic framework by Ferrarezi & Gimenez (1996). According to these authors frugivory and nectarivory evolved only once in the family; frugivory evolved from insectivory and in turn gave rise to nectarivory. In the reconstruction of Wetterer *et al.* (2000) frugivory evolved as main feeding habit from a complementary insectivorous, nectarivorous and frugivorous ancestor, while nectarivory appeared four times within a monophyletic clade. Recently, Datzmann *et al.* (2010) conducted a phylogenetic reconstruction of feeding strategies on a well resolved and strongly supported molecular phylogeny of the family. The authors found that frugivory evolved in a clade comprising the subfamilies Rhinophyllinae, Carolliinae, Stenodermatinae and Glyphonycterinae, and that nectarivory evolved in two independent lineages (Glossophaginae and Lonchophyllinae). However, the feeding habit of the most recent common ancestor of omnivorous and phytophagous phyllostomid bats remained equivocal.

Rex *et al.* (2010) have provided evidences for insectivory in a group of phyllostomid species considered as specialized nectarivorous or frugivorous. The authors have suggested that the radiation in the family has been possible because the capability of phyllostomids to exploit a variety of food types, i.e. to behave as opportunistic omnivorous. Baker *et al.* (in press) have hypothesized that a mixed feeding habit (primarily insectivorous and complementary phytophagous) in the ancestor of all phyllostomids could explain the evolution to all the specialized feeding strategies. These authors point out to a prevailing trend from insectivory to phytophagy in feeding diversification of phyllostomids. The processes by which this diversification occurred need to be clarified.

In the light of such results and statements the following questions have not been yet satisfactorily addressed: (i) Did nectarivory and frugivory evolve independently from insectivory or one of these phytophagous habits gave rise to the other? (ii) Did the shifts to nectarivory and frugivory occur only once or in different moments during the evolutionary history of the family? To this end, we assess different models for the evolution of feeding habits and for the evolution of frugivory and nectarivory. Then, we reconstruct ancestral states with a Bayesian approach on the basis of a mitochondrial and nuclear phylogenetic hypothesis of the family and a matrix of feeding habits that incorporates geographical and seasonal variation of diet. Besides, the divergence times of the phyllostomid genera were estimated to assess whether shifts to frugivory and nectarivory were related to changes in diversification rates of the family and/or to other biological and climatic events that took place during the evolution of phyllostomids.

Methods

Taxa, sequences and alignments

We followed the classification of the family Phyllostomidae proposed by Baker *et al.* (2003) for suprageneric taxa (see Supporting Information), and from Simmons (2005) for generic and infrageneric taxa. DNA sequence data from *Pteronotus* (Mormoopidae) and 56 phyllostomid taxa were downloaded from GenBank (see Supporting Information) using MEGA4 (Tamura *et al.* 2007). All genera of the family but *Lichonycteris*, *Neonycteris*, *Platalina*, *Scleronycteris* and *Xeronycteris* were sampled. The sampled genes were the nuclear recombination activating gene 2 (rag2), and four mitochondrial genes: cytochrome *b* (cytb), and the adjacent genes 12S rRNA, tRNA-Val and 16S rRNA (treated hereafter as mtrDNA).

MUSCLE 3.6 (Edgar 2004) was used for complete individual alignments of the loci. The alignment of mtrDNA was improved in GBLOCKS 0.91b (Castresana 2000), allowing smaller final blocks, gap positions within the final blocks, and less strict flanking positions. The minimum number of sequences for a conserved position and for a flanking position was 28 in both cases. The maximum number of contiguous non-conserved positions was eight while the minimum length of a block was five. Gap positions were allowed with half.

Phylogenetic analyses

To determine the best fit model of sequence evolution for each gene, three substitution schemes were selected in JMODELTEST 0.1.1 (Posada 2008), including invariable sites and rate variation among sites, for a total of 24 models. According to the Akaike Information Criterion, the general time reversible model of substitution with allowance for gamma distribution (Γ) of rate variation and for proportion of invariant sites (I) best fits the data of each locus.

We generated four data partitions for the concatenated sequences of mtrDNA, cytb and rag2: (i) no partitioning, (ii) mitochondrial and nuclear loci separately, (iii) each locus separately, and (iv) each locus separately with partitioning into codon positions for rag2. We selected the fourth partitioning strategy to conduct the maximum-likelihood and Bayesian analyses (see Brown & Lemmon 2007). The file with the final alignment is available in NEXUS format from the authors upon request.

Maximum-likelihood analyses were conducted in RAXML 7.0.3 with the rapid hill-climbing algorithm

(Stamatakis 2006). We used the general time reversible + Γ model with four rate categories. Support values were obtained through a rapid bootstrap (BS) algorithm (Stamatakis *et al.* 2008) with 2500 iterations. We also performed two independent Markov chain Monte Carlo analyses in MrBayes 3.1 (Huelsenbeck & Ronquist 2001). Each analysis was allowed to run for 10 million generations sampling from the chain every 1000 generations. Convergence was assessed on examination of the standard deviation of the split frequency among parallel chains, and with Tracer 1.5 (Rambaut & Drummond 2007) after a burn-in value of 10% of the samples. Results of the Bayesian analyses were combined and summarized with a 50% majority-rule consensus tree with posterior probability values for each node. We excluded from the analyses all 3rd codon positions in cytb because they showed a high degree of homoplasy (homoplasy index = 0.83 according to a parsimony analysis of the 3rd codon position of cytb in paup 4.0 beta – Swofford 2002).

Divergence date estimation was performed in beast 1.6.1 (Drummond & Rambaut 2007). The uncorrelated log-normal relaxed clock was selected to account for lineage-specific rate heterogeneity (Drummond *et al.* 2006). Substitution models were unlinked for the three loci. We applied the general time reversible + Γ model with four rate categories to the mitochondrial loci. For rag2 we used the Hasegawa-Kishino-Yano + Γ model with four rate categories, with 1st and 2nd codon positions linked and 3rd positions allowed to have a different relative rate of substitution, transition-transversion ratio and gamma distributed rate heterogeneity. The Yule process was used as the tree prior. Two calibration points were incorporated: the lower limit of the Whitneyan stage, from which the oldest known fossil of Mormoopidae has been recovered (30.8 Ma; Morgan & Czaplewski 2002), and the lower limit of the Laventan stage, where the oldest fossils of the tribe Phyllostomini were found (11.8 Ma; Czaplewski *et al.* 2003). These values were taken as minimum age constraints of log-normal distributions. In the first case, maximum age constraint was set to the upper limit of divergence between Mormoopidae and Phyllostomidae (40.99 Ma), as estimated by Teeling *et al.* (2005). In the second case, the estimated upper limit of divergence between *Lophostoma* and *Mimon* (17.1 Ma; Hoffmann *et al.* 2008) was set as maximum age constraint.

We conducted four independent analyses in beast 1.6.1 with 40 000 000 steps sampled at 4000 steps. In each analysis, convergence of the chain to the stationary distribution was confirmed by inspection of the Markov chain Monte Carlo samples with the program Tracer 1.5. We combined the last 2500 trees from each independent analysis. From the final sample of 10 000 trees we built a maximum clade credibility tree with the program TreeAnnotator 1.6.1.

The resulting divergence times estimated for the phylogeny were used to construct a lineages-through-time plot (LTT). Numbers of lineages were tallied at sequential time points (0.5 Ma intervals). The relationship between mean divergence dates and 95% highest posterior density intervals (HPD) was assessed with a Spearman rank correlation test (significance level = 0.05).

Reconstruction of ancestral states

We built a data matrix of feeding habits of phyllostomid bats. Literature was carefully surveyed in order to identify the relative importance of items (e.g. insects, fruits) in the diet of bats. We used four states for each feeding habit (see Ferrarezi & Gimenez 1996): absent (0), complementary (1: the food source is a secondary component of the diet, i.e. no more than 40%), predominant (2: the food source is the most important component of the diet, i.e. no less than 60%) and strict (3: only the food source is consumed). When quantitative data were not available we relied on qualitative reports. We used the categories predominant and strict to finally code each taxon as insectivorous, sanguivorous, carnivorous, nectarivorous or frugivorous. Those taxa that exploit different items as main dietary resources (e.g. insects and nectar or insects, nectar and fruits) were classified with such mixed habits (e.g. insectivorous–nectarivorous, insectivorous–nectarivorous–frugivorous, etc.) incorporating the variation of feeding specializations known for each taxon. The data matrix and a more detailed explanation of coding the dietary data are provided in Supporting Information.

We used the program BayesTraits 1.0 (Pagel *et al.* 2004; Pagel & Meade 2006), which combines Bayesian and maximum likelihood based approaches, to reconstruct ancestral states of feeding habits. Previous analyses were limited to parsimony (Wetterer *et al.* 2000) or maximum-likelihood (Datzmann *et al.* 2010) approaches. The module MultiState of the program fits continuous-time Markov model to discrete character data. The model allows changes from one state to another at any given time over very small intervals (Pagel 1994). Different evolutionary models can be tested and also can be applied to samples of trees such that the model parameters can be estimated and the evolutionary hypotheses can be tested taking phylogenetic uncertainty into account. Besides, the program allows including in the analyses more than one state of a trait for each taxon, i.e. it is assumed that the taxon can take with equal probability any of the assigned states.

The inference of ancestral character states depends on the phylogenetic relationships among the taxa of interest, the satisfactory knowledge of the states of the characters among the extant taxa, and the model of character evolution that includes the direction, order and reversibility of state changes (e.g. Nosil & Mooers 2005). The simplest model is the unconstrained parsimony or Brownian motion model that assumes equally likely changes in character states in any direction. A variant of this model assumes a single rate parameter of change between any two states (Lewis 2001). This model was assumed in the most recent ancestral reconstruction of feeding habits of phyllostomids (Datzmann *et al.* 2010). As different models lead to different state reconstructions it is important to assess alternative models and identify the most probable (e.g. Keever & Hart 2008).

For the five-state character 'main feeding habit' we compared four evolutionary models. The first was a null model (H0) where transitions between any pair of states have its own probability, estimated as the most probably Bayesian fit to the tree topology, branch length and tip distribution of character states. This model was tested against three alternative hypotheses. The first alternative (F) resembled the hypothesis of Ferrarezi & Gimenez (1996): sanguivory, carnivory and frugivory would have evolved from insectivory, and nectarivory evolved from frugivory. The second alternative (N) assumed that sanguivory, carnivory and nectarivory evolved from insectivory, and then frugivory would have evolved from nectarivory. The third alternative (I) assumed that all feeding habits evolved directly from insectivory.

To obtain a more detailed picture of the evolution of nectarivory and frugivory in phyllostomid bats we built two data matrix incorporating the relative importance of nectar, pollen and fruits in the diet. A four-state matrix was generated for frugivory and a three-state matrix was generated for nectarivory (we did not find any report of strict nectarivory for the taxa included in the phylogeny). For each of the two characters we tested a null model (H0) against three alternative models. All transition probabilities of the null model (6 for nectarivory, 12 for frugivory) were estimated, and the model was tested against three alternative models. The first alternative hypothesis (OI) was an ordered and irreversible model of evolution: the habit evolved from absent to predominant (nectarivory) or to strict (frugivory). The second alternative (PR) was an ordered and partially reversal model. The reversal was from predominant to complementary in nectarivory and from strict to predominant in frugivory. The third alternative (OR) was an ordered and fully reversible model.

We tested the evolutionary models in a sample of 1000 phylograms that were randomly chosen from the final sample of 10 000 trees of the four independent analyses conducted in BEAST 1.6.1. Posterior probabilities for transitions probabilities were obtained from the reversible-jump Markov chain Monte Carlo approach (Pagel & Meade 2006). We used a gamma prior for the three characters (main feeding habit, nectarivory and frugivory). Priors were seeded from a uniform hyperprior, which allows values of the prior to be estimated from the data (Pagel & Meade 2006). The range for the hyperprior was informed by maximum likelihood parameter estimates previously obtained with the Bayes-Multistate method. The amount of change in rate coefficients among generations in the Markov chain Monte Carlo (i.e. the ratedev parameter) was set to achieve acceptance rates in the range 20–40%, in order to ensure adequate mixing among chains. Markov chains were run four times for 10 000 000 sampling every 1000 steps and a burn-in of 20% to ensure convergence of likelihood and probability values.

We calculated the Bayes factor (BF) as $2 \times$ [ln margL(alternative) – ln margL(null)] to test among evolutionary models for each character. The term margL refers to the marginal likelihood of the null and alternative models. These values are well approximated by the harmonic mean likelihood values from the posterior distribution in the MultiStates analysis. According to Pagel & Meade (2006) a BF value > 2 is a positive evidence of support of the alternative model, > 5 is strong evidence and > 10 is very strong evidence. Under the selected model, characters were mapped over the 1000 phylograms with a gamma prior seeded on a uniform hyperprior, with the same number of generations, sample frequency and burn-in values that were used for model selection.

Results

Phylogeny of the family Phyllostomidae

The maximum-likelihood and Bayesian inferences of the concatenated mitochondrial and nuclear DNA data set show congruent results. The branching pattern and the relatively high BS and Bayesian (BPP) support of the main lineages of the family were consistently recovered with both reconstructions (Fig. 1). The genus *Macrotus* is the basal ingroup lineage of phyllostomid bats. The subfamilies Micronycterinae and Desmodontinae are basal to its clades (Karyovarians and Victivarians, respectively). The clade Phyllovarians (BS 97, BPP 1.00) split up in the subfamily Phyllostominae (BS 89, BPP 1.00) and the clade *Lonchorhina* + Hirsutaglossa (BS 89, BPP 0.97). Glossophaginae is the sister taxa of the clade

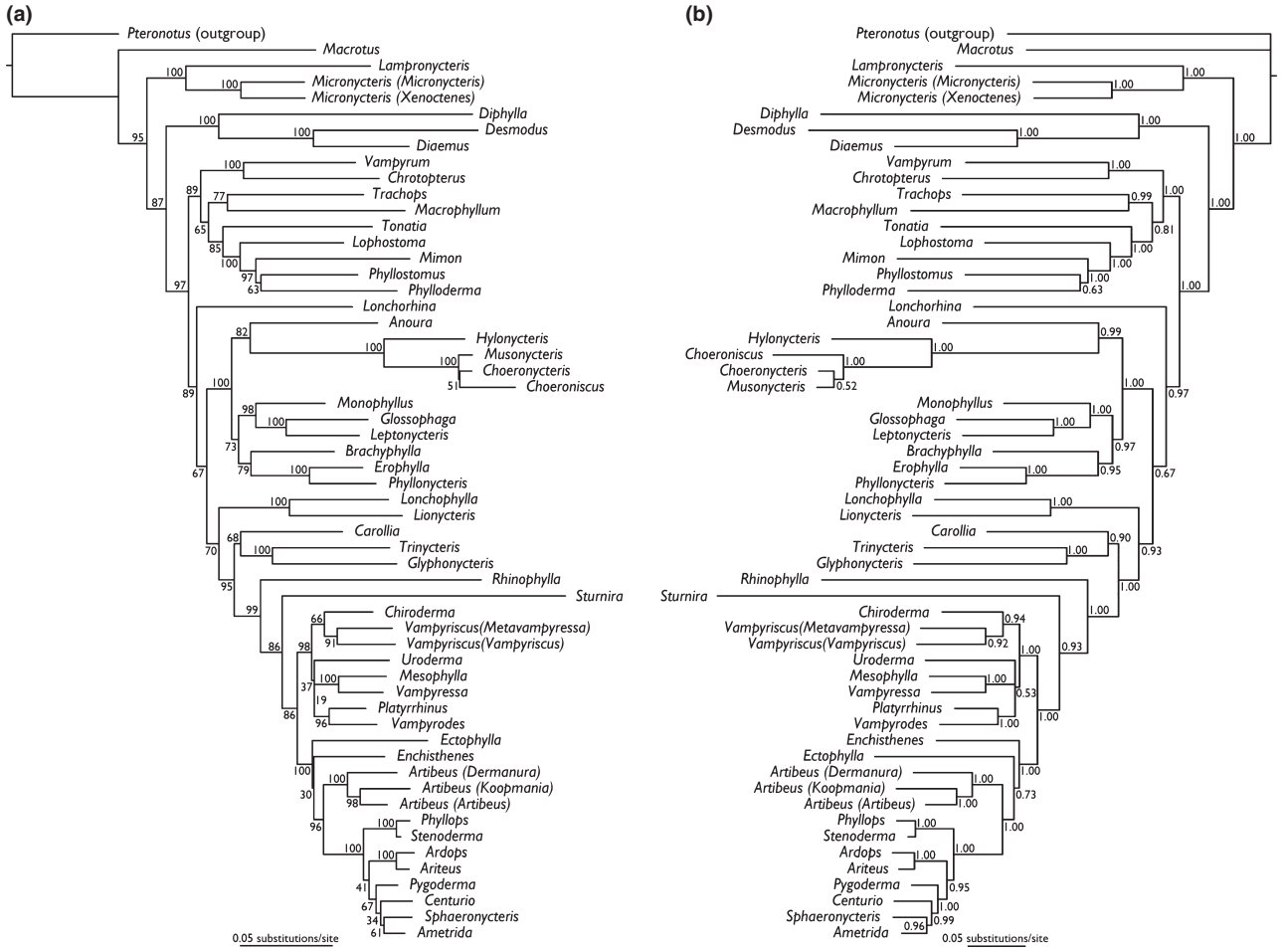

Fig. 1 Maximum-likelihood and Bayesian reconstructions of phyllostomid phylogeny. (a) Best maximum-likelihood tree with BS support values. (b) Bayesian majority-rule consensus tree with posterior probability values.

Dulcivarians (BS 70, BPP 0.93), which includes the subfamilies Lonchophyllinae, Carolliinae + Glyphonycterinae (BS 68, BPP 0.90) and Rhinophyllinae + Stenodermatinae (BS 99, BPP 1.00), in this order of divergence. Differences between the maximum-likelihood and Bayesian reconstructions occur in the clades *Choeroniscus–Choeronycteris–Musonycteris* and *Uroderma–Mesophylla–Vampyressa–Platyrrhinus–Vampyrodes* (the latter not fully resolved in the Bayesian inference), and in the relative position of *Enchisthenes* and *Ectophylla* within Stenodermatinae.

Divergence times

BEAST parameters had very large effective sample sizes, between 711.21 and 6978.61. The coefficient of variation of the branch rates was 0.3167 ± 0.0007. Therefore the strict molecular clock was rejected. The covariance between parent and child branches spanned zero (0.0761 ± 0.0014) which indicates that fast rates and slow rates were next to each other, and that there was no strong evidence of autocorrelation of rates in the phylogeny. Approximately 41% of the 95% HPD intervals comprise 5 Ma or less. There was a significant although weak increasing relationship between the estimated divergence times and the 95% HPD intervals (Spearman $r = 0.69$, $P < 0.0001$).

Phyllostomid bats arose between the end of the Lutelian (Eocene) and the beginning of the Rupelian (Oligocene) stages (33.4–43.1 Ma), most probably at the end of the Bartonian (37.8 Ma), in the Late Eocene (Fig. 2a; see also Supporting Information). The appearance of new lineages through time increased just after the Late Oligocene Warming (23.5–26.7 Ma), as it can be seen in the change of slope of the lineages-through-time chart (Fig. 2c). The clades Micronycterinae, Desmodontinae, Phyllostominae and Hirsutaglossa began its diversification just 1 Ma after this event. At the end of the Mid-Miocene Climatic Optimum (15–17 Ma) 24 of 52 genera had appeared. Diversification of Stenodermatinae

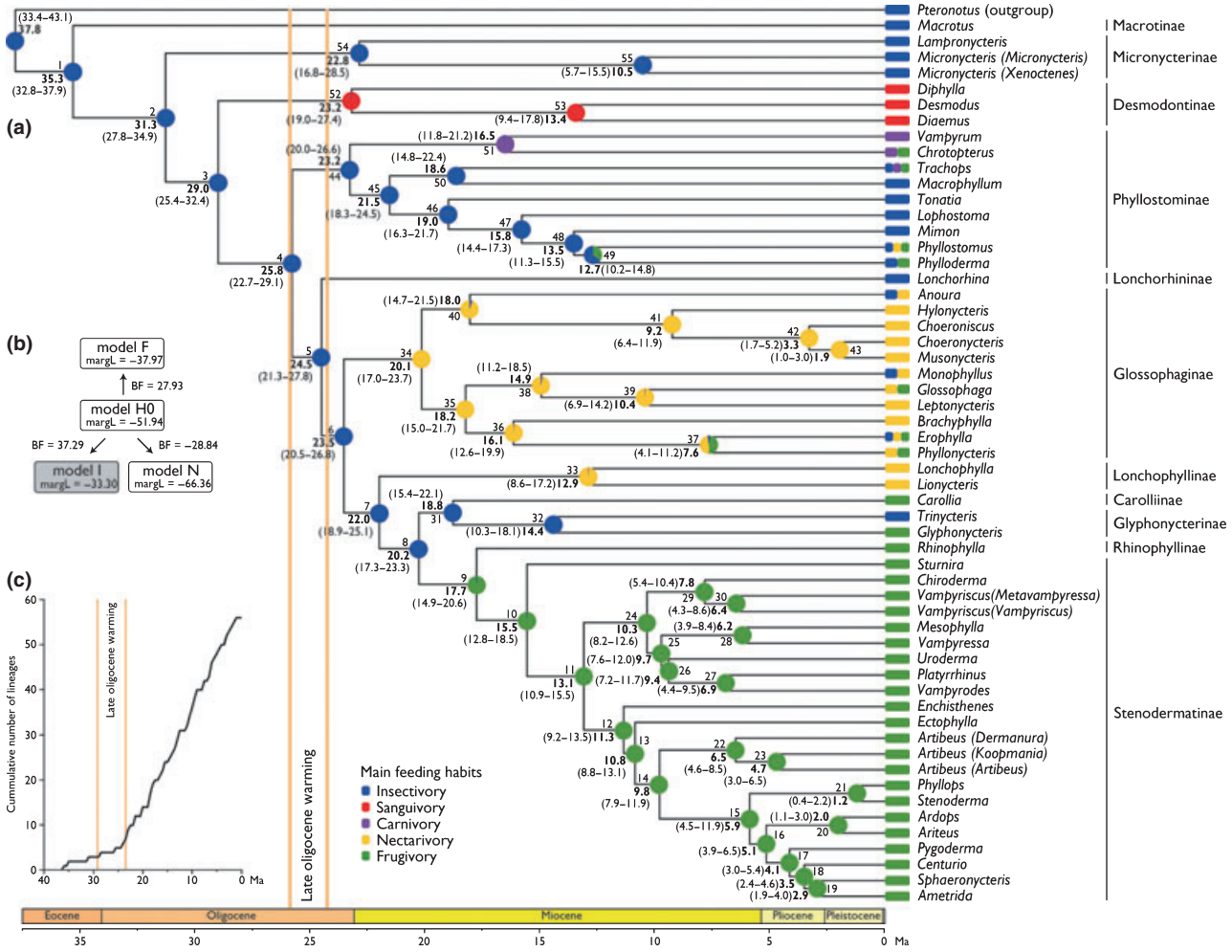

Fig. 2 Evolution of feeding habits in phyllostomid bats. (a) Bayesian phylogeny of the family and Bayesian ancestral character reconstruction under the evolutionary model I (see Methods). Main unranked taxa as follows: Karyovarians (node 2), Victivarians (node 3), Phyllovarians (node 4), Hirsutaglossa (node 6), Dulcivarians (node 7), Nullicauda (node 8), Carpovarians (node 9). (b) Support of the competing evolutionary models. margL = marginal likelihood, BF = Bayes factor. (c) Lineages-through-time plot of the family Phyllostomidae.

occurred after this event until the Calabrian stage (Pliocene).

Three important splits occurred in the evolution of the family: (i) during the Chattian stage (25.8 Ma) resulting in the common ancestors of Phyllostominae and Lonchorhininae + Hirsutaglossa; (ii) around 23.5 Ma resulting in the common ancestors of Glossophaginae and Dulcivarians; and (iii) within Stenodermatinae, at the beginning of the Serravallian stage (13.1 Ma), deriving in the common ancestors of Vampyressina and the clade composed by Enchisthenina, Ectophyllina, Artibeina and Stenodermatina. In two clades (tribe Glossophagini and subtribe Stenodermatina) the taxa restricted to the Caribbean islands (*Monophyllus*, *Stenoderma*, *Phyllops*, *Ardops* and *Ariteus*) arose before the sister taxa distributed in mainland (Fig. 2a).

Diversification of feeding specializations in phyllostomid bats

The best model of evolution of feeding specializations was the model I of a direct origin of all feeding habits from insectivory (Fig. 2b). The model had the highest marginal likelihood value (−33.30) and received very strong evidence of support (BF = 37.29). The ancestral state reconstruction under this model suggests that the ancestor of all phyllostomid bats was mainly insectivorous. The reconstruction shows that nectarivory and frugivory as main feeding habits evolved from mainly insectivorous ancestors in independent lineages of the family (Fig. 2a). Furthermore frugivory evolved from a mainly carnivorous ancestor in *Chrotopterus* and from a mainly nectarivorous ancestor in *Glossophaga*. Shifts to

frugivory from equivocal reconstructed ancestors also occurred in *Phyllostomus*, *Phylloderma*, *Erophylla* and *Phyllonycteris*. The rate of evolution to frugivory was the highest (2.53), followed by the rate of evolution to carnivory (2.05).

Nectarivory evolved as main feeding habit in *Phyllostomus*, in the last common ancestor of Glossophaginae (20.1 Ma), and in the last common ancestor of Lonchophyllinae (12.9 Ma). Frugivory evolved in four genera of the subfamily Phyllostominae, in three genera of the subfamily Glossophaginae, in *Carollia*, in *Glyphonycteris* and in the most recent common ancestor of Carpovarians (17.7 Ma).

In the ancestral reconstruction of the character nectarivory the evolutionary model OR presented the highest value of likelihood (−51.92) and a strong support (BF = 7.44) (Fig. 3b). The loss of nectarivory showed the highest rate of evolution (11.27) under this model, while the smallest rate corresponded to the reversion from predominant to complementary (1.90). Predominant nectarivory received a high support in the most recent common ancestor of the glossophagine bats (BPP 1; 20.1 Ma) and in the most recent common ancestor of *Lonchophylla* and *Lionycteris* (BPP 0.98; 12.9 Ma) (Fig. 3a). The reconstruction of the most recent common ancestor of *Phyllostomus* and *Phylloderma* was equivocal (nectarivory as absent: BPP 0.13, complementary nectarivory: BPP 0.63, predominant nectarivory: BPP 0.24). Complementary nectarivory was successfully recovered (BPP > 0.85) in the most recent common ancestor of the following clades: Phyllovarians (BPP 0.88), Phyllostomini (BPP 0.87) and Carpovarians (BSS 0.85). The reconstruction was ambiguous for the most recent common ancestor of all phyllostomids (absent nectarivory: BPP 0.40, complementary nectarivory: BPP 0.59, predominant nectarivory: BPP 0.01).

In the ancestral reconstruction of the character frugivory also the evolutionary model OR obtained the

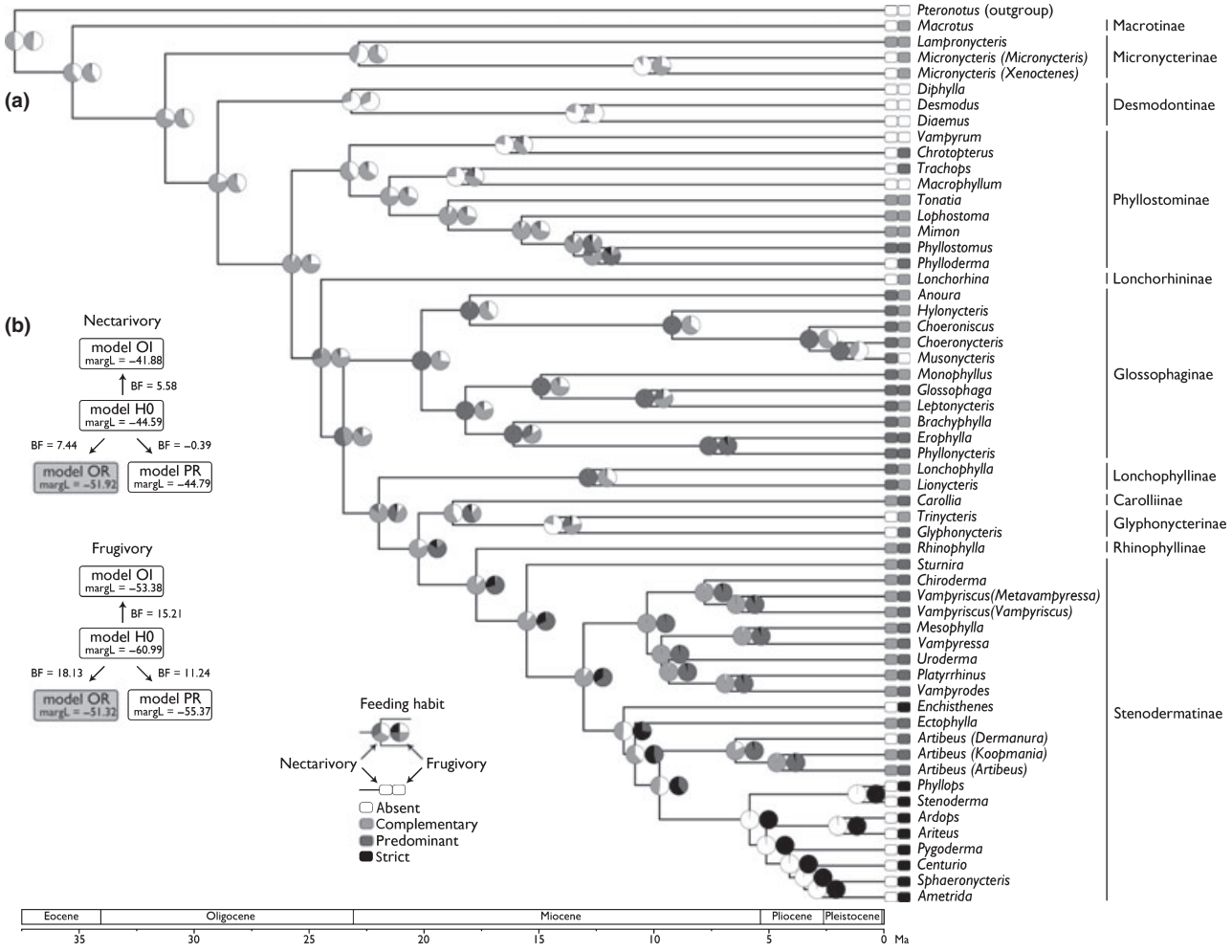

Fig. 3 Evolution of frugivory and nectarivory in phyllostomid bats. (a) Bayesian ancestral character reconstruction under the evolutionary model OR (see Methods section) for each character. (b) Support of the competing evolutionary models. margL = marginal likelihood, BF = Bayes factor.

highest value of marginal likelihood (–51.32) and a very strong support (BF = 18.13) (Fig. 3b). Under this model the highest rate of evolution (31.90) corresponded to the acquisition of frugivory as complementary habit from non-frugivory. The smallest rates corresponded to the transit from complementary to predominant (3.43) and to absent (3.13). Predominant frugivory was well supported in the most recent common ancestor of Phyllonycterini (BPP 0.89) but received a low support (BPP < 0.85) in the most recent common ancestor of *Phyllostomus* and *Phylloderma* (BPP 0.66), and in the most recent common ancestor of Nullicauda (BPP 0.71). Predominant frugivory evolved in *Chrotopterus*, *Trachops*, *Glossophaga*, *Carollia*, *Glyphonycteris*, *Rhinophylla*, *Sturnira* and *Ectophylla* from ambiguous reconstructed nodes. Strict frugivory evolved in *Enchisthenes*, and in the most recent common ancestor of Stenodermatina (5.9 Ma). Complementary frugivory were recovered in other taxa from equivocal reconstructions, including the most recent common ancestor of all phyllostomids (absent frugivory: BPP 0.42, complementary frugivory: BPP 0.55, predominant frugivory: BPP 0.03) (Fig. 3a).

Discussion

Phylogeny of phyllostomid bats

The molecular phylogeny we obtained for the family Phyllostomidae agrees with the evolutionary hypotheses of Baker *et al.* (2003) (see Supporting Information) and Datzmann *et al.* (2010), particularly in the topology at the subfamily level. A major difference is the position of *Lonchorhina*, which appears well supported in our phylogeny as the sister clade of Hirsutaglossa (BS 89, BPP 0.97). The genus is considered predominant insectivorous and complementary frugivorous (Wetterer *et al.* 2000). Jones *et al.* (2002) and Wetterer *et al.* (2000) placed *Lonchorhina* within the subfamily Phyllostominae, closely related to *Macrophyllum*. Baker *et al.* (2003) placed the genus in its own subfamily, Lonchorhininae, in the basal position within Phyllovarians. Our results provide a third hypothesis about the relationships of *Lonchorhina* with the rest of the phyllostomids. The available molecular data make difficult to determine the final position of this genus (e.g. see results of rag2 and 12S rRNA, tRNA-Val and 16S rRNA in Baker *et al.* 2003). Therefore new molecular sequences of this genus are required to elucidate the underlying pattern. Either basal to or within the subfamily Phyllostominae, we consider that the position of *Lonchorhina* should not significantly affect the reconstruction of feeding diversification. Other incongruences between the evolutionary hypothesis we proposed for Phyllostomidae and the phylogeny of Baker *et al.* (2003) refer to the position of *Vampyrum* + *Chrotopterus* (but see Datzmann *et al.* 2010), and the topology of Phyllostomini and Choeronycterina.

Including *Phyllops* in our analyses changed the topology of the subtribe Stenodermatina with regard to previous hypotheses. The members of this clade are all strict frugivores (see Supporting Information). The composition of the subtribe is well supported (BS 98) in Wetterer *et al.* (2000) not so the relationships within. We found that *Phyllops* + *Stenoderma* are basal to the subtribe (BS 99, BPP 1.00), and this result is consistent with the analyses of Dávalos (2007, 2010). The effect of different markers in the final topology of the subtribe requires further analysis. Nevertheless changes in the topology should not significantly affect the ancestral reconstruction of feeding habits as all members of the subtribe share the same feeding specialization.

Divergence times of phyllostomid lineages

The estimated age of the most recent common ancestor of the extant phyllostomid genera (35.3 Ma) is consistent with the estimates of Teeling *et al.* (2005) and Miller-Butterworth *et al.* (2007). The 95% HPD interval of this node also overlaps the estimates of other works (Eick *et al.* 2005; Jones *et al.* 2005; Datzmann *et al.* 2010).

The rate of diversification of Phyllostomidae was not constant (see Fig. 2c). During the Late Oligocene Warming mean temperature was 2–6 °C higher than mean temperature in the preceding Oligocene stages and in the following Miocene stages (Zachos *et al.* 2001). In approximately 10 Ma from this climatic event to the end of the Mid-Miocene Climatic Optimum all subfamilies started diversification and about half the genera of phyllostomid bats had arisen. Teeling *et al.* (2005) linked the global rise in temperature in Early Eocene (50–52 Ma) and the ecological related events (diversification of flowering plants and insects) to the diversification of bats. Jones *et al.* (2005) detected two significant diversification rate shifts in Phyllostomidae on the supertree of Chiroptera (Jones *et al.* 2002) and suggested a relationship between such diversification events and the diversification rate shifts in flowering plants (25–40 Ma; Davies *et al.* 2004). Therefore the Late Oligocene Warming could be a starting point to examine the role of temperature and the evolution of mutualisms in diversification rates of phyllostomid bats.

Although intervals of divergence time estimates overlap between our results and the timetrees of Datzmann *et al.* (2010) for Phyllostomidae and Hoffmann *et al.* (2008) for Phyllostominae, in many nodes mean values lie at one or outside of the limits of the intervals or the

overlapping of the intervals is only of 1 Ma. This situation could be explained by differences in the methods and in the parameters that were used. The estimates of Hoffmann *et al.* (2008) were conducted on a single tree topology and on the estimated values of Teeling *et al.* (2005) incorporated as hard bounds on calibrated nodes. In our work we applied the same method implemented by Datzmann *et al.* (2010). As the fossil record of phyllostomid bats is scarce, we relied on the fossils records that these (and other authors, e.g. Dávalos 2010) have used. For the point of divergence between Mormoopidae and Phyllostomidae differences in the hard minimum bound were not as marked as the 'soft' maximum bound of the log-normal distribution. While Datzmann *et al.* (2010) used the Cretaceous-Tertiary boundary (65 Ma) we used the upper limit of the 95% HPD interval of the estimate of Teeling *et al.* (2005). In the other shared calibration point Datzmann *et al.* (2010) also used an exponential distribution with an arbitrary lower constraint. In contrast, we used a log-normal distribution and different values for the hard minimum (fossil record of the ancestor of Phyllostomini) and soft maximum (secondary calibration value exported from Hoffmann *et al.* 2008) bounds. We implemented a log-normal distribution because it is often appropriate for summarizing paleontological information, i.e. assuming that actual divergence occurs somewhat before the oldest known fossil and the finding of an even older fossil is possible although very unlikely (Donoghue & Benton 2007; Ho 2007; Ho & Phillips 2009).

We have detected that the most ancient genera of Glossophagini, Brachyphyllini + Phyllonycterini and Stenodermatina are currently restricted to the Antilles, while the most recent genera of those clades are distributed in mainland. This provides a curious biogeographical pattern for phytophagous phyllostomid bats which is consistent with previous molecular studies in frugivorous and insectivorous clades (Dávalos 2005, 2006, 2007): some continental taxa descended from Caribbean ancestors through a series of events probably related to sea-level changes in the last 20 million years (see Miller *et al.* 2005).

Differences in the branching pattern of *Brachyphylla*, *Phyllonycteris* and *Erophylla* between our phylogeny and Datzmann *et al.*'s (2010) may explain the differences in the divergence date estimates of these endemic Antillean genera. Our results are consistent with the work of Dávalos (2010), with the morphological and molecular reconstruction of Neotropical nectar-feeding bats of Carstens *et al.* (2002), and with the hypothesis of Jones *et al.* (2002). Furthermore, Silva & Pine (1969) considered *Brachyphylla* more primitive than *Phyllonycteris* and *Erophylla* on the basis of craniodental morphology.

When did plants become important to leaf-nosed bats?

Previous studies of dietary diversification in phyllostomid bats have highlighted the role of insectivory in the family (Ferrarezi & Gimenez 1996; Wetterer *et al.* 2000). From an insectivorous ancestor (Simmons *et al.* 2008) this habit has been predominant in the order Chiroptera, even within the superfamily Noctilionoidea (*sensu* Teeling *et al.* 2005), where most shifts to other feeding habits have occurred (Baker *et al.* in press). Our reconstruction strongly supports insectivory in the common ancestor of phyllostomid bats. This habit remained as the main feeding specialization in the basal clades of the family (Macrotinae and Micronycterinae), and in the ancestors of Karyovarians, Phyllovarians, Phyllostominae, Hirsutaglossa, Dulcivarians and Nullicauda (see Fig. 2a).

Our reconstruction also supports the independent origin of the rest of feeding habits from insectivory, as proposed by Freeman (2000). But we could not determine if the insectivorous most recent common ancestor of all phyllostomids was also phytophagous. The reconstructions of nectarivory and frugivory were ambiguous for that node (see Fig. 3a). Then what could explain the evolution of phytophagy in 10 of the 11 subfamilies of phyllostomid bats?

The subfamily Phyllostominae has the widest plasticity in feeding habits within Phyllostomidae. In this subfamily carnivory is present as predominant feeding habit in three genera, predominant frugivory evolved in four genera and predominant nectarivory evolved in *Phyllostomus* (Norberg & Fenton 1988; Wetterer *et al.* 2000; Santos *et al.* 2003; Bonato *et al.* 2004; Giannini & Kalko 2004; Hice *et al.* 2004). In this group of bats carnivory represents an extreme of insectivory (Giannini & Kalko 2005), and the phenotypic modifications that allowed this shift probably represent minor changes (Freeman 2000; Popa-Lisseanu *et al.* 2007). In contrast, incorporating fruits, nectar and pollen in the diet (i.e. the evolution from insectivory to nectarivory and frugivory) should implied important changes in cranial shape and dentition (Freeman 2000), feeding behaviour (Dumont 2006) and relative brain volume (Ratcliffe 2009). According to our reconstruction these changes occurred more than once not only in the subfamily Phyllostominae but in the entire family. Baker *et al.* (in press) provide an interesting point of view: the insectivorous ancestor of all phyllostomids also feed on plants. The existence of such preadaptations (Freeman 2000) could explain the convergence on herbivorous feeding habits in the phyllostomid lineages (see Fig. 3a), irrespective of the different levels of specializations (e.g. *Musonycteris* vs. *Erophylla* or *Ametrida* vs. *Sturnira*).

Parallel evolution of characters associated to nectarivory in Glossophaginae and Lonchophyllinae and

differences in the adaptations to nectarivory in specialized and non-specialized phyllostomid nectarivorous bats have been analysed elsewhere (Freeman 2000; Nicolay & Winter 2006; Datzmann *et al.* 2010). Similarities and variation in dietary adaptations of frugivorous bats have also been assessed (Dumont 2003; Swartz *et al.* 2003). This parallel evolution is plausible on the basis of the plasticity in the diet that can be found in Phyllovarians. Lineages that are considered specialized nectarivores or frugivores apparently perform also as opportunistic generalists (Rex *et al.* 2010). Particularly, in some genera of independent lineages that are mainly frugivorous but also nectar-consumers (e.g. *Carollia*, *Sturnira*, *Chiroderma*, *Uroderma*, *Mesophylla*, *Vampyressa*, *Ectophylla* and *Artibeus*) insectivory seems to be more important than previously considered (Rex *et al.* 2010). Also insect-consumption has been reported in high frequency in nectarivorous bats of the subfamily Glossophaginae (Soto-Centeno & Kurta 2006; Mancina & Herrera 2010). In fact, insectivory has been present across the evolutionary diversification of the family and it has been lost in *Diphylla*, *Diaemus*, *Vampyriscus* and the core of strict frugivorous of the subfamily Stenodermatinae (see Supporting Information File S1). On one side such evidences support the evolutionary model of a direct dietary diversification in phyllostomids from insectivory (see Fig. 2a,b). On the other side, if the specializations to the effective exploitation of nectar, pollen and fruits did not imply the loss of the adaptations to feed on arthropods then it should be expected that during the evolution of phyllostomids switches to new feeding mechanisms of abundant and/or underexploited resources provided selective advantages that favoured the appearance of ecological innovations independently in different lineages of the family. Under this framework the role of the spatio-temporal predictability of fruit (Fleming *et al.* 1987) and flower (Fleming & Muchhala 2008) resources in shaping diversity in phytophagous phyllostomid bats is an interesting subject that requires further attention and analysis.

In conclusion, in the family Phyllostomidae nectarivory and frugivory evolved as main feeding habits in three and five subfamilies, respectively. Therefore the hypothesis of a single origin of frugivory and nectarivory in phyllostomid bats (Ferrarezi & Gimenez 1996) can be rejected, the hypothesis of the independent evolution of adaptations to nectar and pollen consumption should be extended from two lineages (Datzmann *et al.* 2010) to three, and the hypothesis of the independent evolution of adaptations to frugivory could be assumed in five lineages of the family. The incidence of complementary nectarivory and frugivory in all phyllostomid subfamilies but Desmodontinae suggests that the adaptations to phytophagy were already present in the most recent common ancestor of phyllostomid bats (Freeman 2000; Baker *et al.* in press). But this hypothesis was not supported with the ancestral reconstruction of nectarivory and frugivory, and should be contrasted with fossil evidence. Our results do support the hypothesis that the evolution of frugivory in phyllostomid bats occurred from a group of genera dispersing soft fruits of species of secondary growth to more specialized bats that feed on harder or more fiber-rich fruits of plants of primary growth and canopy (Muscarella & Fleming 2007). The ecological and phenotypic specialization of phytophagous phyllostomids needs to be addressed in a phylogenetic framework with a combination of ecomorphological approaches and analyses of seasonal and spatial variation of diet in order to provide new insights into the evolutionary diversification and the adaptative radiation of one of the most attractive families of living mammals.

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Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 Data matrix for feeding habits of Phyllostomidae.

Table S2 Dietary information of the Phyllostomidae at the species level.

Table S3 GenBank accession numbers for the 57 taxa included in this research.

Table S4 Divergence date estimates in million years and 95% Highest Posterior Densities limits (HPD) of Phyllostomidae.

Table S5 Classification of leaf-nosed bats (Chiroptera: Phyllostomidae).

File S1 Coding dietary data.

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