Evolution of nectarivory in phyllostomid bats (Phyllostomidae Gray, 1825, Chiroptera: Mammalia)

Thomas Datzmann*1,2,3, Otto von Helversen2 and Frieder Mayer1,2

Abstract

Background: Bats of the family Phyllostomidae show a unique diversity in feeding specializations. This taxon includes species that are highly specialized on insects, blood, small vertebrates, fruits or nectar, and pollen. Feeding specialization is accompanied by morphological, physiological and behavioural adaptations. Several attempts were made to resolve the phylogenetic relationships within this family in order to reconstruct the evolutionary transitions accompanied by nutritional specialization. Nevertheless, the evolution of nectarivory remained equivocal.

Results: Phylogenetic reconstructions, based on a concatenated nuclear-and mitochondrial data set, revealed a paraphyletic relationship of nectarivorous phyllostomid bats. Our phylogenetic reconstructions indicate that the nectarivorous genera Lonchophylla and Lionycteris are closer related to mainly frugivorous phyllostomids of the subfamilies Rhinophyllinae, Stenodermatinae, Carolliinae, and the insectivorous Glyphonycterinae rather than to nectarivorous bats of the Glossophaginae. This suggests an independent origin of morphological adaptations to a nectarivorous lifestyle within Lonchophyllinae and Glossophaginae. Molecular clock analysis revealed a relatively short time frame of about ten million years for the divergence of subfamilies.

Conclusions: Our study provides strong support for diphyly of nectarivorous phyllostomids. This is remarkable, since their morphological adaptations to nutrition, like elongated rostrums and tongues, reduced teeth and the ability to use hovering flight while ingestion, closely resemble each other. However, more precise examinations of their tongues (e.g. type and structure of papillae and muscular innervation) revealed levels of difference in line with an independent evolution of nectarivory in these bats.

Background

The diversity of feeding specialization of phyllostomid bats are unique among all mammals [1-7]. They range from insect-to diverse vegetable-feeding strategies, as well as omnivory, carnivory, and even blood-feeding [8-16]. This ecological diversification is accompanied by morphological, behavioural and physiological adaptations [4,9,17-32]. A striking example is specialization for nectarivory, with several species feeding primarily on nectar. These bats have the ability to hover in front of a plant, while drinking nectar with their elongated and extensile tongues adorned with brush-like papillae and grooves for ingestion of nectar [3,26,29,30,33-37]. They digest and metabolize nectar and pollen quickly [32,38-44]. Phyllostomid bats represent the second largest chiropteran family after the vespertilionid family (Vespertilionidae Gray, 1821), with more than 150 species in at least 49 genera. Their distribution ranges from southern Arizona and the West Indies to northern Argentina [45].

Although phylogenetic analyses of morphology, chromosomes, and molecules have helped to illuminate relationships among many genera and subfamilies of phyllostomid bats, relationships among nectarivorous genera are still unclear. Many phylogenies based on morphological characters suggest a monophyletic origin for all specialized nectarivorous phyllostomids [5,46,47]. We provide a well-supported phylogenetic estimate of phyllostomid bats based on a large molecular data set, comprising 10396 bp from a total of twelve nuclear-and mitochondrial genes, and try to clarify phylogenetic relationships among nectarivorous taxa by testing whether they share a close common ancestry. Furthermore, we...
used a molecular clock approach to evaluate the evolutionary time frame of diversification in phyllostomid bats.

**Results**

**Phylogeny of the Phyllostomidae**

Figure 1 shows our maximum-likelihood reconstruction (b) compared to the latest comprehensive analysis of phyllostomid phylogeny (a) after Baker et al. (2003) [48]. Baker and colleagues used sequences of 12S and 16S rRNA, tRNA Valin and the rag2 gene for their inference. Our reconstruction shows high congruence, even though it is completely based on independent genes (see methods section: Alignment 1). Although no members of the subfamilies Lonchorhinae, Glyphonycterinae and Rhinophyllinae were included (because of incomplete data for these taxa), major branching patterns were consistently reconstructed. Our reconstruction received good bootstrap support and is in line with Baker et al.’s phylogeny of phyllostomid bats. Therefore, we combined our data with the data from Baker et al. (2003) [48].

A separate analysis of all mitochondrial and nuclear loci (Alignment 2&3) resulted in high congruent phylogenies (Figure 2). Among the frugivorous species relationships changed between the independent inferences. A sister-group relationship between Carolliinae and Glyphonycteridae could not be inferred from the mitochondrial data set. In this reconstruction glyphonycterids were found basal to all frugivores. But this relationship obtained low support (BS 50) compared to the reconstruction based on nuclear loci, where Carolliinae is sister taxon to them (BS 73).

Maximum likelihood (ML) analysis based on our super-matrix (see methods section: Alignment 5) revealed a well-resolved phylogeny for the Phyllostomidae (Figure 3), with most nodes receiving high bootstrap support (BS > 90). Monophyly of all subfamilies recognized by Baker et al. (2003) [48] was verified, and relevant nodes were highly supported by different measurements (Table 1).

Three basal lineages, comprising the taxa Macrotus (1), Micronycteris (2), and the vampire bats Desmodus and Diaemus (3), were confirmed (Figure 3). A bifurcation in more or less omnivorous bats (Phyllostominae) and predominantly vegetarian species followed. Within the frugivores a sister-group relationship between Rhinophyllinae and the Stenodermatinae was well-supported (BS 99). However, support for a sister-group relationship of Carol-
Divergence time estimation and model decision

The analysis under the lognormal relaxed clock model (UCLN) produced the smallest confidence intervals compared to the exponential-(UCED) or strict clock model (CLOC). Estimates of mean likelihood, substitution rate, and node age were most accurately inferred under the UCLN model (Table 2). The assumption of the relaxed clock, that branches differ in their substitution rates, was confirmed. A coefficient of variation of 0.405 indicated moderate rate variation [51]. Figure 4 shows the dated Bayesian tree inferred with BEAST under the UCLN model. The common ancestor of all phyllostomids was dated to the Middle Eocene (42 MYA), with a confidence interval between 49- and 37 MYA. Basal lineages within the phyllostomids arose shortly thereafter in the Late Eocene or Early Oligocene (35-32 MYA). The prominent amount of the remaining lineages emerged in a time frame of about ten million years at the transition from Oligocene to Miocene (29-20 MYA), with 21 out of 33 lineages already present in the Early Miocene (20 MYA).

Reconstruction of ancestral states

Figure 3 shows the reconstruction of ancestral states by the maximum-likelihood approach under the Markov k-state model. Only relevant nodes, which will be used in the discussion chapter, are shown. Reconstructed feeding specialization of the common ancestor of all phyllostomids and of the common ancestor of important clades were mapped on the tree (Figure 3). Unambiguous character states were assigned to nodes with a probability of more than 90% for one reconstructed state. The feeding specialization of the common ancestor of all omnivorous and predominantly vegetarian phyllostomid species could not be resolved, as the reconstruction was ambiguous for this node (marked with ?). We obtained probability values of 47% for a nectarivorous-, 39% for an omnivorous-, and 12% for an insectivorous state at this node.
Discussion
Phylogeny of the Phyllostomidae

Our molecular phylogenetic reconstructions based on more than 10 kb DNA sequences obtained high bootstrap support for almost all nodes and challenges several phylogenetic relationships derived from morphological data sets. Our results partly disagree with recent classifications of phyllostomid bats [45,49] including: (1) placement of insectivorous genera *Macrotus*, *Micronycteris*, *Glyphonycteris* and *Trinicycteris* within the Phyllostomidae; (2) relationship of the fruit-eating genus *Rhinophylla* to other frugivores; and (3) relationships among nectarivorous phyllostomids.

The molecular data suggest that the genera *Macrotus* and *Micronycteris* do not belong to the subfamily Phyllostominae as proposed by Koopman (1994) [49], McKenna and Bell (1997) [47], Wetterer et al. (2000) [5], and Jones et al. (2002) [50]. Instead, they form two divergent basal lineages within phyllostomid bats (Figure 3). Our data are in line with the findings of Baker et al. (2003) [48]. The authors proposed a classification of two different subfamilies Macrotrinae and Micronycterinae. Three studies placed the genera *Glyphonycteris* and *Trinicycteris* within the subfamily Phyllostominae [5,45,49]. In contrast, our data revealed a close relationship of *Glyphonycteris* and *Trinicycteris* with frugivorous species of the subfamily Car-
olliinae. Despite low support for this sister-group relationship (BS 45), our data support a closer relationship of *Glyphonycteris* and *Trinycteris* to fruit-eating species (BS 99) than to omnivorous phyllostomids of the subfamily Phyllostominae.

The genus *Rhinophylla* does not belong to the subfamily Carolliinae, as proposed by McKenna and Bell (1997) [47], Wetterer *et al.* (2000) [5], and Jones *et al.* (2002) [50]. Our data support a sister-group relationship between *Rhinophylla* and the subfamily Stenodermatinae, as proposed by Baker *et al.* (2003) [48].

Many authors excluded the genera *Phyllonycteris*, *Erophylla* and *Brachyphylla*, all endemic to the West Indies, from other nectarivorous phyllostomids and placed them mostly into the subfamilies Phyllonycterinae and Brachyphyllinae [3,5,19,33,45,49,52-59]. In contrast, our data show that these nutritionally more generalized bats belong to the Glossophaginae (BS 100). The three genera are closely related to more specialized nectarivorous bats of the genera *Glossophaga*, *Leptonycteris* and *Monophyllus*. This is in line with previous studies of Koopman (1994) [49], Jones *et al.* (2002) [50] and Baker *et al.* (2003) [48]. The distinctness of Lonchophyllinae is also supported by fixed differences in the tongue morphology (see below) between representatives of the Lonchophyllinae and Glossophaginae [33].

In summary, our study supports the classification of phyllostomid bats after Baker *et al.* (2003) [48]. Their division into more subfamilies, compared to Koopman (1994) [49] and Simmons (2005) [45], seems justifiable, because this better reflects the remarkable ecological diversity of this family.

**Dietary diversification**

The vast majority of bats feed on insects [4]. This includes the family Mormoopidae, which represents the sister group of the Phyllostomidae. In addition, the diet of the most basal subfamilies Macrotinae and Micronycterinae consists mainly of insects (Figure 3). These findings indicate, that the common ancestor of phyllostomid bats was an insect-feeder. This supposition is also supported by the maximum-likelihood reconstruction of the ancestral state (Figure 3).

Members of the Phyllostominae have a mixed diet. The reconstruction of the ancestral state for this group revealed that their physiological pre-adaptations to omnivory could have evolved only once, and involved metabolic changes from insectivorous to an omnivorous diet. However, too little is known about the diet of these bats. A high spacial and seasonal plasticity is observed [60]. A few members of the Phyllostominae are carnivorous.
Figure 4 Bayesian dating of phyllostomid diversification. Maximum clade credibility tree under the UCLN model in BEAST built on 48,003 sampled trees. The Geological Time Scale (2004) of The International Commission on Stratigraphy (ICS) was used as a timetable. Node ages (bold) in million years ago (Mya) with their 95% HPD interval (in parenthesis) are shown, rounded to nearest integer. 95% HPD ranges can be seen as confidence intervals. Nodes marked with an asterisk are calibrated with fossils. Absolute species numbers within each subfamily, according to the actual species list [45], are given in the broad vertical bars.
### Table 2: Model comparison.

| Molecular Clock Model | CLOC - 3 priors |  | CLOC (without data) |  |
|-----------------------|----------------|---|---------------------|---|
|                       | mean           | < 95% HPD | > 95% HPD | mean           | < 95% HPD | > 95% HPD |
| Likelihood            | -8.42E+004     | -8.42E+004 | -8.42E+004 | - | - | - |
| rate [**]             | 1.40E-003      | 1.28E-003  | 1.52E-003  | 50.3 | 4.82 | 99.61 |
| rootHeight [*]        | 61.04          | 56.42      | 66.05      | 60.75 | 40.81 | 86.73 |
| Molossidae ×          | 39.32          | 37.49      | 41.64      | 41.15 | 37.64 | 46.01 |
| Vespertilionidae      |              |            |            |      |      |      |
| p1 [*]                | 56.4           | 51.3       | 61.86      | 38.39 | 34.09 | 44.75 |
| Mormoopidae ×         | 47.99          | 43.64      | 52.57      | 35.71 | 34    | 39.1  |
| Phyllostomidae        |              |            |            |      |      |      |
| p2 [*]                | 56.4           | 51.3       | 61.86      | 38.39 | 34.09 | 44.75 |
| Macrotus × rest of   | 41.5           | 37.71      | 46.88      | 42.43 | 37.71 | 49.8  |
| Phyllostomidae        |              |            |            |      |      |      |
| p3 [*]                | 35.49          | 34         | 38.5       | 67.49 | 37.86 | 100.44 |
| Molecular Clock Model | UCED - 3 priors |  | UCLN - prior p1 |  |
|                       | mean           | < 95% HPD | > 95% HPD | mean           | < 95% HPD | > 95% HPD |
| Likelihood            | -7.68E+004     | -7.69E+004 | -7.68E+004 | -7.68E+004     | -7.68E+004 | -7.68E+004 |
| rate [**]             | 3.96E-003      | 3.04E-003  | 4.81E-003  | 1.90E-003      | 1.15E-003  | 2.73E-003  |
| rootHeight [*]        | 62.9           | 42.24      | 90.14      | 81.84 | 51.92 | 116.2 |
| Molossidae ×          | 41.5           | 37.71      | 46.88      | 42.43 | 37.71 | 49.8  |
| Vespertilionidae      |              |            |            |      |      |      |
| p1 [*]                | 39.24          | 34.77      | 46.91      | 77.49 | 54.67 | 104.63 |
| Mormoopidae ×         | 35.49          | 34         | 38.5       | 67.49 | 37.86 | 100.44 |
| Phyllostomidae        |              |            |            |      |      |      |
| p2 [*]                | 56.4           | 51.3       | 61.86      | 38.39 | 34.09 | 44.75 |
| Macrotus × rest of   | 29.07          | 23.35      | 37.01      | 35.81 | 34    | 39.43 |
| Phyllostomidae        |              |            |            |      |      |      |
| p3 [*]                | 22.89          | 12.51      | 34.16      | 22.39 | 12.97 | 33.52 |
| Molecular Clock Model | UCLN - prior p2 |  | UCLN - prior p3 |  |
|                       | mean           | < 95% HPD | > 95% HPD | mean           | < 95% HPD | > 95% HPD |
| Likelihood            | -7.68E+004     | -7.68E+004 | -7.68E+004 | -7.68E+004     | -7.68E+004 | -7.68E+004 |
| rate [**]             | 4.09E-003      | 3.09E-003  | 4.95E-003  | 3.50E-003      | 2.94E-003  | 4.07E-003  |
| rootHeight [*]        | 38.32          | 30.94      | 49.32      | 42.73 | 34.99 | 51.79 |
| Molossidae ×          | 22.89          | 12.51      | 34.16      | 22.39 | 12.97 | 33.52 |
| Vespertilionidae      |              |            |            |      |      |      |
| p1 [*]                | 34.99          | 30.37      | 42.93      | 37.1  | 28.73 | 46.06 |
| Mormoopidae ×         | 29.07          | 23.35      | 37.01      | 35.81 | 34    | 39.43 |
| Phyllostomidae        |              |            |            |      |      |      |
| p2 [*]                | 34.99          | 30.37      | 42.93      | 37.1  | 28.73 | 46.06 |
| Macrotus × rest of   | 29.07          | 23.35      | 37.01      | 35.81 | 34    | 39.43 |
| Phyllostomidae        |              |            |            |      |      |      |
| p3 [*]                | 22.89          | 12.51      | 34.16      | 22.39 | 12.97 | 33.52 |
| Molecular Clock Model | UCLN - 2 priors p1+p3 |  | UCLN - 3 priors |  |
|                       | mean           | < 95% HPD | > 95% HPD | mean           | < 95% HPD | > 95% HPD |
| Likelihood            | -7.68E+004     | -7.68E+004 | -7.68E+004 | -7.68E+004     | -7.68E+004 | -7.68E+004 |
| rate [**]             | 3.04E-003      | 2.58E-003  | 3.43E-003  | 3.13E-003      | 2.76E-003  | 3.48E-003  |
| rootHeight [*]        | 53.85          | 45.94      | 63.48      | 52.26 | 45.42 | 61.28 |
| Molossidae ×          | 40.99          | 37.7       | 45.85      | 40.89 | 37.64 | 45.39 |
| Vespertilionidae      |              |            |            |      |      |      |
| p1 [*]                | 22.89          | 12.51      | 34.16      | 22.39 | 12.97 | 33.52 |
Table 2: Model comparison. (Continued)

| Astrophyllidae × Astrophyyllidae | 44.56  | 37.34  | 53.21  | 42.16  | 37.13  | 48.61  |
| Phyllostomidae × Phyllostomidae | 36.96  | 34     | 42.72  | 35.82  | 34     | 39.58  |

Divergence time estimations of specific nodes under different molecular clock models and different calibration settings are shown. Strict- (CLOC), relaxed exponential- (UCED) and relaxed lognormal- (UCLN) clock models are compared. Likelihood value, mean mutation rate, root age and time to the most recent common ancestor (tMRCA) of taxa subsets are given. [*] Estimated age of taxon subset in million years ago (Mya). [**] Estimate of the evolutionary rate across the whole tree in units of substitutions per site per million years (Myr). < > Lower and upper bound of the 95% highest posterior density (HPD) interval. 95% HPD is the shortest interval, that contains 95% of the sampled values and is equivalent to a confidence interval.

Rous and feed on small vertebrates [61]. For example, Trachops cirrhosus is specialized on tungara frogs [62,63]. Such a unique specialization likely evolved in a formerly insectivorous/omnivorous species. It was shown, for the seasonally carnivorous Greater Noctule bat (Nyctalus lasiopterus, Vespertilionidae), that only minor changes are needed to switch from insectivory to carnivory (inclusion of small vertebrates in the diet) [64,65]. The transition from large-bodied insects to small vertebrates as prey does not need any major adaptations and occurred several times independently in different bats and is correlated with an increase in body size [61].

A large number of phyllostomid species have a vegetarian diet. They form a monophyletic clade (BS 99), comprising the subfamilies Rhinophyllinae, Stenodermatinae, Carollininae, Glossophaginae, Lonchophyllinae, and surprisingly the Glyphonycterinae. The last subfamily includes several strict insectivorous species; thus, a shift from a vegetarian diet back to insectivory seems to be the most plausible scenario. Alternatively, the Glyphonycterinae retained the ancestral insectivorous lifestyle. This assumption would require that the frugivorous subfamilies Rhinophyllinae, Stenodermatinae and Carollininae have evolved their feeding specialization independently from each other. However, the relevant node is weakly supported in our phylogenetic reconstruction. It is also possible (see Figure 2 based on mitochondrial data) that the Glyphonycterinae represents a basal lineage to all frugivores and therefore possess the plesiomorphic state for this group. The common ancestry of all frugivorous bats was also postulated by previous studies [5,46,48,50,59]. However, there is a high dietary plasticity in this group. The common ancestry of all frugivore species and insectivorous/omnivorous species. It was shown, for the frugivorous Lonchophyllinae and Glossophaginae, that only minor changes are needed to switch from insectivory to carnivory (inclusion of small vertebrates in the diet) [64,65]. The transition from large-bodied insects to small vertebrates as prey does not need any major adaptations and occurred several times independently in different bats and is correlated with an increase in body size [61].

The diphyly of the nectarivorous Lonchophyllinae and Glossophaginae is surprising, since they resemble each other in many morphological, behavioural, ecological, and physiological traits (e.g. skull elongation, reduction of dentition, hovering flight, forest foraging behaviour and ability to metabolize pollen). Accordingly, these similarities have evolved independently by natural selection during the adaptation to a nectar-feeding lifestyle. This hypothesis is supported by some obvious differences in these adaptive traits [33]: The lonchophyllines have a deep longitudinal groove in their tongue, lined dorsal and ventral with hairlike papillae. This groove is missing in the glossophagines and hairlike papillae are distributed anterodorsal, forming a brush tip. Furthermore, the lonchophyllines lost most types of papillae found on the tongues of other phyllostomids, including the glossophagines. Also, the internal tongue structure is very different. The lonchophyllines have complex, omnidirectional bundles of muscles within the tongue, while glossophagines have predominantly horizontal skeletal muscle bundles. The complex orientated muscles in the lonchophyllines are supposed to control the shape of the groove during nectar feeding [33]. Drinking behaviour varies widely between both subfamilies (Marco Tschapka, pers. comm., [30]). Other characters show similar apomorphic states in lonchophyllines and some glossophagines (e.g. posterior shift of sternohyoid origin, xiphoid origin of sternohyoid, elongated hyoglossus and loss of connection to hyoid bone, double insertion of geniohyoid, posterior shift of genioglossus insertion [33]), however, there are no consistent patterns. The endemic West Indian genera, Brachyphylla, Erophylla, and Phyllonycteris, show many plesiomorphic characters. It seems that functional constraints on the muscular innervation of the tongue curtain the evolutionary signals of these characters. Hence, it is possible that lonchophyllines and glossophagines may have evolved these adaptations for nectar-feeding independently (but see also [67,68]).

The large number of species within the clade of frugivorous and nectarivorous bats (Figure 2) suggests, that a shift to a vegetarian diet accelerated the diversification rates in this group. The majority of phyllostomid bats, 117 out of 158 listed species [45], i.e. 74%, belong to this clade. Possibly the presence of numerous vacant ecological niches in tropical and subtropical regions of America (see also [69]) resulted in allopatric speciation.
Time frame of evolution
Our analysis revealed a time frame of ten million years (29-20 MYA) from Oligocene to Early Miocene, in which all prominent lineages evolved (Figure 4). Most of the species diversification occurred subsequent to the Oligocene epoch (since 23 MYA). During the Miocene substantial changes of the landscape occurred in Tropical America due to massive plate tectonics [70-75]. Global climate cooled and resulted in an increase in aridity [76-78]. Frequent isolation events could have resulted in allopatric populations and thus promoted speciation [79]. Interestingly, the radiation of extant hummingbirds (Trochilidae), another alimentary competitor, shows a similar pattern of diversification or insufficient amount of sequence data. In order to test whether these factors have also promoted speciation in bats, and to infer other underlying evolutionary mechanisms, a much denser taxon sampling is required.

Conclusions
Our analysis of more than 10.000 base pairs of concatenated DNA sequences reveals a strongly supported phyllostomid phylogeny, thus allowing for clear predictions about the evolution of feeding specialization of these bats. Several morphological and even molecular studies were unable to resolve the specific branches with sufficient support, either due to the convergent nature of the analyzed characters or insufficient amount of sequence data. Our multi-gene approach, combined with a relaxed clock analysis, detected and dated major splitting events within this family. This study gives support for the classification of phyllostomid bats after Baker et al. (2003) [48]. All prominent lineages with diverse feeding strategies evolved within a relatively short time frame of about ten million years from Oligocene to Early Miocene. Geologic and climate changes as well as the shift to a vegetarian diet may have promoted the radiation into diverse lineages. In this context, the diphyl of the nectarivorous Lonchophyllinae and Glossophaginae is remarkable. Despite many similarities between both groups, it seems plausible, that they evolved their adaptations to nectarivory independently from each other. This would represent an example of convergent evolution within bats that led to very similar features, which play a major role in food acquisition.

Methods
Taxon sampling
Thirty-seven phyllostomid species of 29 genera were analyzed. Our sampling comprises members of all extant subfamilies [45,49], except bats of the subfamily Lonchophyllinae. We used species and subfamily assignments according to Baker et al. (2003) [48]. One representative each of the families Mormoopidae, Furipteridae, Noctilionidae, Molossidae and Vespertilionidae were used as outgroup taxa. Two closely related specimens were used for the family Rhinolophidae, because we were not able to analyze all loci entirely for one taxon. GenBank accession numbers are given in additional file 1. Tissue samples were provided by cooperation partners (see acknowledgements). The name of the body which gave approval and corresponding reference numbers could be obtained from them.

Genetic analyses
Extraction of total genomic DNA was done by Chloroform-Isomyl-Phenol precipitation. A 1.3 kb fragment of the exon 28 of the von Willebrand factor gene (**vwf**) was amplified with the primers vWF-A and vWF-B [81], or with vWF-A and vWF-B2 [81] within a Nested PCR. Primer vWF-B2 anneals 139 bp upstream from vWF-B. An approximately 1.4 kb fragment of the recombination activating gene 2 (**rag2**) was amplified with the primers RAG2-F1 and RAG2-R2 [59], or with RAG2-F1B and RAG2-R2 [59]. The PCR Mastermix (25 µl final reaction volume) included 2 µl of total genomic DNA extract, 1.25 µl of each primer (10 µM), 1 µl of MgCl₂ (25 mM), 1 µl of a dNTP-Mix (10 mM) and 1 unit of Peglab Taq polymerase. Nested PCR was performed using 2 µl from a 1:40 delution of the first PCR reaction. The fragments were amplified following a Two-Step protocol. Thermocycling consisted of a 3 min initial denaturation at 95°C, followed by 5 cycles of 30s at 95°C, 50s at 65°C (for the **vwf**), or 30s at 60°C (for the **rag2**), and 90s at 72°C. 35 cycles with 50s annealing at 62°C (for the **vwf**) and 30s at 57°C (for the **rag2**) were performed, followed by a final extension of 6 min at 72°C. A fragment of exon 11 of the breast cancer susceptibility gene (**brca1**) was amplified with the primers BRCA1-F126 [82] and a newly designed primer ER 65 and ER 66 [85]. Published primer sequences of five additional mitochondrial loci (COI, **nd1**), the tRNA Leu phospholipase C beta 4 gene (**plcb4**) [83] and short intron of the phosphoenolpyruvate carboxykinase gene (**pepck**) [84];

We amplified a mitochondrial fragment of the NADH dehydrogenase subunit 1 gene (**nd1**) and the tRNA Leucin, using the primers ER 65 and ER 66 [85]. Published sequences of five additional mitochondrial loci (COI,
Cytb, 12S rRNA, 16S rRNA and tRNA Valin) were incorporated. For all analyses, the ribosomal RNAs and the tRNA Valin were combined (12SrRNA16S). Accession numbers are given in Additional file 1. It also includes an overview of all incorporated taxa, loci and sequences and the percentage of missing data per species, as well the geographic origin of our samples. The overall amount of missing data is about 30%.

Alignments and model selection

All alignments were done with Sequencher v4.7 [86] and Bioedit v7.0.9 [87,88] and checked manually by eye. We performed bootstrap analyses of each individual loci to check for compatibility of their individual phylogenetic signal. Because none of the strongly supported clades based on individual loci were mutually incompatible, we concatenated all loci except the ribosomal RNAs, tRNA Valin and the rag2 gene. These loci were already used by Baker and colleagues to infer a molecular phylogeny of phylllostomid bats [48]. We avoided in a first step the inclusion of them to get an independent data set [Alignment 1]. In a second step we concatenated all mitochondrial loci (this time with the inclusion of the ribosomal RNAs and the tRNA Valin) [Alignment 2] and also all nuclear loci (with rag2) [Alignment 3]. We concatenated all loci into one supermatrix for the final analyses. The supermatrix contained three nuclear protein-coding genes (rag2, vwf and brca1), two non-coding nuclear markers (pepck, plcβ4), three mitochondrial protein-coding genes (col1, cytβ and nd1), two tRNAs (Valin, Leucin) and two mitochondrial rRNAs (12S, 16S). For the Bayesian analyses, we excluded all 3rd codon positions in the mitochondrial protein-coding genes because they showed a high degree of homoplasy (homoplasy index, HI = 0.75 - parsimony analysis of the 3rd codon positions in PAUP 4.0 beta [89]). Such high homoplasy characters give a misleading phylogenetic signal and lead especially to an underestimation of real branch lengths. Therefore, we excluded them from the analyses. This resulted in a final length of 10396bp, including 2761 parsimony informative characters [Alignment 4 - Additional file 2]. For the maximum-likelihood analyses, we used a second alignment, in which the mitochondrial protein-coding sequences were translated in amino acids and combined with the remaining DNA sequences [Alignment 5 - Additional file 3]. The best fitting evolutionary model for the protein data was inferred with Prottest v1.4 [90]. The MTMAM model, designed for the evolution of mitochondrial proteins of mammals [91], showed the highest fit. We ran jModelTest [92] for the remaining DNA sequences separate for the alignments 1-5. Except for alignment 3, GTR+Γ [93] was proposed to be the best fitting evolutionary model according to Akaike- (AIC) and Bayesian (BIC) information criterion [94,95]. The slightly simpler Symmetrical Model SYM+Γ [96] was proposed for alignment 3 by jModelTest. However, we also used the GTR+Γ model for this dataset for general compatibility among the inferences. Genes could have a different sequence evolution. Therefore, we generated five partitioning schemes [97] for alignment 5 to decide, which is the best adjustment for our analysis: (1) no partitioning; (2) mitochondrial- and nuclear loci separately; (3) three partitions; (4) eight partitions; and (5) 14 partitions with partitioning into codon positions for all nuclear genes. According to AIC and BIC, scheme 5 was preferred.

Maximum-Parsimony analysis

Equal weighted maximum-parsimony (MP) analyses were performed with PAUP 4.0 beta [89] with a heuristic search using the TBR (tree-bisection-reconnection) algorithm for branch swapping. Bootstrap inferences were conducted separately for each loci with 500 pseudoreplicates.

Maximum-Likelihood analysis

Maximum-likelihood (ML) inferences were performed with RAxML v7.0.4 [98-100]. ML searches were conducted with the rapid hill-climbing algorithm [101,102] under GTR+Γ with four rate categories as model of evolution. Multiple independent runs were started to get an impression of the robustness of the phylogenetic reconstruction. Support values were obtained through a full non-parametric bootstrap- or rapid bootstrap inference (stated for each analysis).

Reconstruction of ancestral states

Ancestral character states were reconstructed in Mesquite v2.71 [103]. Observed character states (insectivore, sanguinivore, omnivore, frugivore and nectarivore) of the main diet were mapped on the original maximum-likelihood tree (Figure 3). We used the "Trace Character History" analysis with a symmetric, one-parameter Markov k-state model [104,105], which computes likelihoods for categorical characters, and reconstructs ancestral states by the maximum marginal probability (MLE) criterion.

Bayesian analysis

Bayesian inferences were performed with BEAST v1.4.8 [106]. The searches were conducted under Hasegawa-Kishino-Yano HKY+Γ [107] with four rate categories as model of evolution. We chose a simpler model of sequence evolution for the Bayesian analyses as proposed by jModelTest because there was a trade-off between computational power and model complexity. It was not possible to get a consistent phylogenetic reconstruction between different runs under the GTR+Γ model in reasonable time. Bayes factor analysis between these runs under the GTR+Γ model resulted always in values far above 20 and sampling efficiency was drastically reduced.
compared to the HKY+Γ model. A high Bayes factor is a sign for incompatibility and poor convergence among the trees gathered in independent runs.

Calibration of the molecular clock

We incorporated three different calibration points including: (1) divergence between Vespertilionidae and Molossidae set at 37 million years ago (MYA) in the Middle Eocene [47]; (2) age of the Mormoopidae oldest fossils from Whitneyan (30-32 MYA) land deposits in Florida [108]; and (3) age of the oldest crown group fossils of the phyllostomids in the Laventan about 11.8 to 13.8 MYA [109] and age of the oldest stem group fossils in the Whitneyan within the Early Oligocene [110]. We used the proposed age of the fossils and lognormal distributions to model minimum age constraints for the specific nodes (1,2). Maximum age constraints were set to the Cretaceous-Tertiary boundary at 65 MYA (1,2). Additional, a maximum age constraint for the phyllostomids (3) was set with an exponential distribution to 34 MYA with an arbitrarily lower limit of 11.5 MYA.

Model- and prior decision

We performed several Bayesian inferences under one strict (CLOC) and two relaxed (UCLN, UCED) molecular clock models [111,112]. Always 10 million steps were performed. We examined the joint influence of the calibrations on the divergence time estimates by running a strict clock model with fixed topology, but with no sequence data. Further, we examined the influence of each individual calibration by running several inferences under an uncorrelated lognormal relaxed clock model (UCLN) with all possible combinations of the three calibrations. A precise examination and comparison of the results were performed in Tracer v1.4 [113]. An overview of important parameters for model comparison is given in Table 2. Important parameters, such as mean likelihood value, substitution rate, and node age, were calculated for every inference and compared with each other. Confidence intervals measured as 95% highest posterior density interval (HPD) were also computed. The clock model that produced the smallest confidence intervals altogether was considered most appropriate for the data [112].

Estimation of divergence times

We conducted three independent runs for the final divergence time estimates under the UCLN model with 20 million inferences and a sample frequency of 1000 steps. We used always the same parsimony tree as starting point. We compared the results and calculated pairwise Bayes factors for the difference in their marginal likelihoods. The first 4 million steps were cut off as burnin for each comparison. Low Bayes factors are a sign for high convergence of the values and compatibility of the inferences, while high Bayes factors indicate incompatibility. Individual runs were combined with LogCombiner, TreeAnnotator and analyzed with Tracer v1.4 and FigTree v1.1.2 [114]. TreeAnnotator and LogCombiner are provided as part of the BEAST package.

Additional material

Additional file 1 Incorporates sequences GenBank accession numbers of all incorporated sequences are shown. Dotted lines indicate missing data. Percentage of overall missing base pairs per lineage are given (completeness). Sample origins of our analyzed individuals are coded with two-letter abbreviations according to the International Organization of Standardization: RU Russia, CU Cuba, CR Costa Rica, JM Jamaica, MX Mexico, p.e. GenBank sequences published earlier. Question marks (?) are used for samples with unknown origin. (*) Asterisks indicate sequences published within this paper and were submitted to EMBL-EBI database hosted by the European Molecular Biology Laboratory.

Additional file 2 Input file for the Bayesian analysis in BEAST (see methods section: Alignment 4) XML formatted input file for the Bayesian analysis in BEAST. Can be opened within a browser, or executed with the software BEAST.

Additional file 3 Concatenated alignment for the maximum-likelihood inference with RAxML (see methods section: Alignment 5) Alignment file is in PHYLIP format and can be viewed with every text editor, or used directly in most phylogenetic software packages.

Authors’ contributions

OvH was the initiator of this study. FM supervised the whole project, gave many ideas, helped to evaluate the results and to draw up the manuscript. TD made all the lab work, performed the phylogenetic analyses and wrote the manuscript.

Acknowledgements

We thank Marco Tschapka, Martina Nagy, Mirjam Knornschl, and Ralph Simon for fruitful discussions and for providing tissue samples. Wolfram Schulze, Jana Ustinova, Dagmar Dachlauer, Andrea Ross, Claudius Kerth, Corinna Koch von Helversen, Sebastian Ebert, Heiko Stuckas, Kai Drilling, and Dirk Berger supported the study at different stages: We would like to thank Christian Vogt, Simon Ghanem, Markus Zweier, Inge Müller, Conni Schoebel, Ulrich Marckmann, Volker Runkel, Dina Dechmann, Kirsten Jung, Stefan Prost, Sebastian Lippoll, Michael Knapp, and Maria Helbig for their willingness to discuss diverse aspects and giving advice. We thank the CBSU Web Computing Resources (BIOHPC) for their free web-based service for phylogenetic analysis with BEAST. Fruitful discussions occurred with Alexandros Stamatakis, Ignacio Gonzalez Bravo, and Olaf Bininda-Emonds during the Carolinensiel summer school in 2008. Finally we thank two anonymous reviewers for their excellent comments and help to improve this manuscript. The study was financed by the Luse-Prell-Stiftung and the Schmauser-Stiftung at the University of Erlangen-Nürnberg.

Author Details

1. Museum für Naturkunde, Leibniz Institute for Research on Evolution and Biodiversity at the Humboldt University Berlin, Invalidenstr. 43, 10115 Berlin, Germany, 1. Department of Zoology, Animal Physiology, University of Erlangen-Nürnberg, Staudtstrasse 5, Erlangen, Germany and 1. Senckenberg Natural History Collections Dresden, Museum of Zoology, Königsbrucker Landstrasse 159, 01109 Dresden, Germany

References

1. Gardner AL: Chromosomal variation in Vampyressa and a review of chromosomal evolution in the Phyllostomidae (Chiroptera). Syst Zool 1977, 26:300-318.
2. Findley JS. Bats: a community perspective Cambridge, Cambridge Univ Press; 1993.
3. Gimenez EDA, Ferrari Rezzi H, Taddei VA: Linguistic morphology and cladistic analysis of the New World nectar-feeding bats (Chiroptera: Phyllostomidae). J Comp Biol 1996, 1-64.

4. Freeman PW: Morphology in microchiroptera: recoupling morphology and ecology with phylogeny. Eco Evol Res 2000, 3(254):317-335.

5. Wetterer AL, Rockman MV, Simmons NB: Phylogeny of phyllostomid bats (Mammalia: Chiroptera): Data from diverse morphological systems, sex chromosomes, and restriction sites. Bull Amer Mus Nat Hist 2000, 248(1):201-229.

6. Gaammi NP, Kalko KV: Tropheic structure in a large assemblage of phyllostomid bats in Panama. Oikos 2004, 105:209-220.

7. Rex K, Kelm DH, Wiesner K, Matt FG, Kunz TH, Voigt CC: Species richness and structure of three Neotropical bat assemblages. Biol J Linn Soc 2008, 89(5):617-629.

8. Humphrey SR, Bonaccorso FJ, Zinn TL: Guild structure of surface-feeding bats in Panama. Ecology 1983, 64:284-294.

9. Dobat K, Peikert-Holte T: Blüten und Fledermäuse: Bestäubung durch Fledermäuse und Flughunde (Chiroptera) Frankfurt am Main, Germany: Waldemar Kramar, 1985.

10. Fleming TH: The short-tailed fruit bat: A study in plant-animal interactions Chicago Univ Press; 1988:1-380. [Wildlife Behavior and Ecology series].

11. Freeman PW: Frugivorous and ammalivorous bats (Microchiroptera): Dental and cranial adaptations. Biol J Linn Soc 1988, 33:249-272.

12. Medellin RA: Prey of Chrotoperus auritus, with notes on feeding behavior. J Mammal 1988, 69:841-844.

13. Handle DJ, Wilson JP, Gardner AL: Demography and natural history of the common fruit bat, Artibeus jamaicensis, on Barro Colorado Island, Panama. Smithsonian Contrib Zool 1991, 51:1-173.

14. von Helversen O: Adaptations of flowers to the pollination by glossohagine bats. In Animal-plant interactions in tropical environments. Edited by: Barthlott W, Naumann CM, Schuchmann SLK, Schuchmann KL. Bonn, Germany, Museum Koenig; 1993:41-59.

15. Freeman PW: Nectar-feeding mechanisms in bats. Biol J Linn Soc 1995, 56:439-463.

16. Proctor M, Yeo P, Lack A: The natural history of pollination Portland, Oregon, Timber Press, 1996.

17. Vogel S: Chiropterophile in der neotropischen Flora. Neue Mitteilungen III. Flora, B 1969, 158:289-323.

18. Phillips CJ: The dentition of glossohagine bats: development, morphological characteristics, variation, pathology, and evolution. In Miscellaneous Publications of the Museum of Natural History Volume 54. University of Kansas; 1971:1-138.

19. Smith JD: Chiropteran evolution. In Biology of the Bats of New World Phyllostomidae. Part I: Edited by: Baker RJ, Jones JK, Carter DC. Spec Publ Muse Tex Tech Univ; 1976:49-69.

20. Baker RJ, Bass RA: Evolutionary relationship of the Brachyphyllinae to Liponycterinae. Bull Amer Mus Nat Hist 2005, 209:219-220.

21. Hopkins HC: Floral biology and pollination ecology of the Neotropical species of Parkia. Ecology 1984, 72:1-21.

22. Egurute L, Burrey R: Reproductive ecology of Manfreda braschiasthya, an iteroparous species of Agavaceae. Southwest Nat 1987, 32:169-178.

23. Gribel R, Hay JD: Pollination ecology of Caryocar brasiliense (Caryocaraceae) in Central Brazil cerrado vegetation. J Trop Ecol 1993, 9:199-211.

24. von Helversen O: Blumenfledermäuse und Fledermausblumen - Wechselbeziehungen zwischen Blüte und Bestäuber und energetische Grenzbedingungen. In Rundgespräche der Kommission für Ökologie. Band 10, Tropenforschung Bayr Akad Wiss, 1995:217-229.

25. Luckow M, Hopkins H: A cladistic analysis of Parkia (Leguminosae: Mimosoideae), Am J Bot 1995, 82:1300-1320.

26. Winter Y, Voigt CC, von Helversen O: Gas exchange during hovering flight in nectar-feeding bat Glossophaera soricina. J Exp Biol 1998, 201:237-244.

27. Dumont ER: The effect of food hardness on feeding behaviour in frugivorous bats (Phyllostomidae): an experimental study. J Zool 1999, 248:219-229.

28. Carstens BC, Lunding BL, Myers P: A Phylogeny of the Neotropical nectar-feeding bats (Chiroptera: Phyllostomidae) based on morphological and molecular data. J Mamm Evol 2002, 9:23-39.

29. von Helversen O, Winter Y: Glossophagine bats and their flowers: costs and benefits for plants and pollinators. In Bat Ecology Volume 2003. 2nd edition. Edited by: Kunz TH, Fenton MB. Chicago and London, Chicago Univ Press: 346-397.

30. Winter Y, von Helversen O: Operational tongue length in phyllostomid nectar-feeding bats. J Mammal 2003, 84:886-896.

31. Kelm DH, von Helversen O: How to budget metabolic energy - torpor in a small Neotropical mammal. J Comp Physiol B 2007, 177(6):667-677.

32. Voigt CC, Speakman JR: Nectar-feeding bats fuel their high metabolism directly with exogenous carbohydrates. Funct Ecol 2007, 21:913-921.

33. Griffins TA: Systematics of the New World nectar-feeding bats (Mammalia: Phyllostomidae) based on the morphology of the hyoid and lingual regions. Am Mus Novit 1982, 274(2):1-43.

34. Heithaus ER, Stasko E, Anderson PK: Cumulative effects of plant-animal interactions on seed production by Bauhinia unguifolia, a Neotropical legume. Ecology 1982, 63:1294-1302.

35. Winter Y: Energetic cost of hovering flight in a nectar-feeding bat measured with fast-response respirometry. J Comp Physiol B 1998, 168:434-444.

36. Norberg UM, Kunz TH, Steffenssen JF, Winter Y, von Helversen O: The cost of hovering and forward flight in a nectar-feeding bat, Glossophaga soricina, estimated from aerodynamic theory. J Exp Biol 1993, 182:207-227.

37. Hedenström A, Johansson LC, Wolf M, Busse R, Winter Y, Spedding GR: Bat flight generates complex aerodynamic tracks. Science 2007, 316(5826):894-897.

38. Howell D: Bats and pollen: physiological aspects of the syndrome of chiropterophily. Comp Biochem Physiol 1974, 48:263-276.

39. Stanley RG, Linskens HG: Pollen biology, biochemistry, management Berlin, Heidelberg, New York, Springer-Verlag, 1974.

40. Law BS: Physiological factors affecting pollen use by Queensland blossom bats, Syconycteris australis. Funct Ecol 1992, 65:634-648.

41. Fleming TH: The use of stable isotopes to study the diets of plant-visiting bats. In: Bats: Ecology, Behavior, and Evolution Edited by: Racey PA, McDonald U, Swift S. Oxford, Oxford Univ Press, 1995:99-110.

42. Herrera LGM, del Rio CM: Pollen digestion by New World Bats: Effects of processing time and feeding habits. Ecology 1998, 79:2828-2838.

43. Schondube JE, Herrera LGM, del Rio CM: Diet and the evolution of digestion and renal function in phyllostomid bats. Zoology 2001, 104:59-73.

44. Mirón LLM, Herrera LGM, Ramírez PN, Hobson KA: Effect of diet quality on carbon and nitrogen turnover and isotopic discrimination in blood of a New World nectarivorous bat. J Exp Biol 2006, 209:541-548.

45. Simmons NB: Order Chiroptera. In Mammalian Species of the World: A Taxonomic and Geographic Reference 3rd edition. Edited by: Wilson DE, Reeder DM. Baltimore. Maryland: Johns Hopkins Univ Press, 2005:312-529.

46. Baker RJ, Hood CS, Honeycutt RL, van Den Busche RA: Diversification among New World leaf-nosed bats: An evolutionary hypothesis and classification inferred from digenomic congruence of DNA sequence. Occ Pap Mus Tex Tech Univ 2003, 230:1-32.

47. Koopman KF: Chiroptera: Systematics. Handbook of Zoology, Vol 8, Part 60 Berlin, Germany: Walter de Gruyter, 1994.

48. Jones KE, Purvis A, Malmann A, Brinda-Emmons ORP, Simmons NB: A phylogenetic supertree of the bats (Mammalia: Chiroptera), Bull Rev Biol 2002, 72:233-259.

49. Drummond AJ, Ho SYW, Rawlence N, Rambaut A: A rough guide to BEAST 1.4. 2007.

50. Miller GSJ: The families and genera of bats. Bull US Nat Mus 1907, 1:282.

51. Simpson GS: The principles of classification and a classification of the mammals. Bull Am Mus Nat Hist 1945, 85:1-350.

52. de la Torre L: The evolution, variation, and systematics of the Neotropical bats of the genus Sturnira. In Ph.D Thesis Univ Illinois, Urbana, 1961.

53. Silva-Taborda GS, Pine RH: Morphological and behavioral evidence for the relationship between the bats genera Brachyphyllle and the Phyllostomines. Biotropica 1969, 1:10-19.
110. Czaplewski NJ, Morgan GS, McLeod SA: Evolution of Tertiary mammals of North America, Vol 2, small mammals, Xenarthrans, and marine mammals. Cambridge, Cambridge Univ Press; 2008.
111. Drummond AJ, Nicholls G, Rodrigo AG, Solomon W: Estimating mutation parameters, population history and genealogy simultaneously from temporally spaced sequence data. *Genetics* 2002, 161:1307-1320.
112. Drummond AJ, Ho SYW, Phillips MJ, Rambaut A: Relaxed phylogenetics and dating with confidence. *RasS Biology* 2006, 4(5):e88.
113. Molecular evolution, phylogenetics and epidemiology [http://tree.bio.ed.ac.uk/software/tracer/]
114. Molecular evolution, phylogenetics and epidemiology [http://tree.bio.ed.ac.uk/software/figtree/]
115. Ferrarezzi H, Gimenez EDA: Systematic patterns and the evolution of feeding habits in Chiroptera (Archonta: Mammalia). *J Comp Biol* 1996, 1:75-94.
116. Dumont ER: Feeding mechanisms in bats: Variation within the constraints of flight. *Integr Comp Biol* 2007, 47:137-146.
117. The Sorenson lab Boston University [http://people.bu.edu/msoren/TreeRot.html]
118. Pons J, Barradough TG, Gomez-Zurita J, Cardoso A, Duran DP, Hazell S, Kamoun S, Sumlin WD, Vogler AP. Sequence-based species delimitation for the DNA taxonomy of undescribed insects. *Syst Biol* 2006, 55:595-609.
119. Fontaneto DE, Herniou C, Boschetti M, Caprioli G, Melone C, Ricci C, Barradough TG. Independently evolving species in asexual bdelloid rotifers. *PLoS Biology* 2007, 5(6):e87.
120. Monaghan MT, Wild R, Elliot M, Fujisawa T, Balle M, Inward DJG, Lees DC, Ranaivosolo R, Eggleton P, Barradough TG, Vogler AP. Accelerated species inventory on Madagascar using coalescent-based models of species delination. *Syst Biol* 2009, 58(3):298-311.

Cite this article as: Datzmann et al., Evolution of nectarivory in phyllostomid bats (Phyllostomidae Gray, 1825, Chiroptera: Mammalia) *BMC Evolutionary Biology* 2010, 10:165