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Next-generation sequencing and phylogenetic signal of complete mitochondrial genomes for resolving the evolutionary history of leaf-nosed bats (Phyllostomidae)

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ABSTRACT

Leaf-nosed bats (Phyllostomidae) are one of the most studied groups within the order Chiroptera mainly because of their outstanding species richness and diversity in morphological and ecological traits. Rapid diversification and multiple homoplasies have made the phylogeny of the family difficult to solve using morphological characters. Molecular data have contributed to shed light on the evolutionary history of phyllostomid bats, yet several relationships remain unresolved at the intra-familial level. Complete mitochondrial genomes have proven useful to deal with this kind of situation in other groups of mammals by providing access to a large number of molecular characters. At present, there are only two mitogenomes available for phyllostomid bats hinting at the need for further exploration of the mitogenomic approach in this group. We used both standard Sanger sequencing of PCR products and next-generation sequencing (NGS) of shotgun genomic DNA to obtain new complete mitochondrial genomes from 10 species of phyllostomid bats, including representatives of major subfamilies, plus one outgroup belonging to the closely-related mormoopids. We then evaluated the contribution of mitogenomics to the resolution of the phylogeny of leaf-nosed bats and compared the results to those based on mitochondrial genes and the RAG2 and VWF nuclear makers. Our results demonstrate the advantages of the Illumina NGS approach to efficiently obtain mitogenomes of phyllostomid bats. The phylogenetic signal provided by entire mitogenomes is highly comparable to the one of a concatenation of individual mitochondrial and nuclear markers, and allows increasing both resolution and statistical support for several clades. This enhanced phylogenetic signal is the result of combining markers with heterogeneous evolutionary rates representing a large number of nucleotide sites. Our results illustrate the potential of the NGS mitogenomic approach for resolving the evolutionary history of phyllostomid bats based on a denser species sampling.

1. Introduction

Among chiropteran families, the Neotropical leaf-nosed bats (family Phyllostomidae, suborder Yangochiroptera; Teeling et al., 2002) are one of the groups that have attracted the most attention from ecological and evolutionary standpoints. This focus of interest stems from their high diversity that encompasses more than 10% of all extant bat species. Moreover, among ca. 160 recognized species of phyllostomids, at least a dozen new species have only recently been described (Dávalos and Corthals, 2008; Gregorin and Ditchfield, 2005; Larsen et al., 2011; Mantilla-Meluk and Baker, 2010; McCarthy et al., 2006; Muchhala et al., 2005; Solari and Baker, 2006; Taddei and Lim, 2010; Velazco, 2005; Velazco et al., 2010; Woodman, 2007). Yet, cladistic studies based on comprehensive morphological datasets (Wetterer et al., 2000) have encountered difficulties resolving phylogenetic relationships within the family. This might be explained by the astonishing ecological diversification, mirroring the considerable species diversity of phyllostomids, and leading to a number of evolutionary convergences that makes their phylogeny difficult to resolve using morpho-anatomical characters. For instance, it has recently been shown that nectarivory evolved twice independently in distantly related subfamilies (Datzmann et al., 2010; Rojas et al., 2011).

The use of molecular data has provided a clearer picture of their evolutionary history, validating new species discoveries, and refining taxonomic assessments (Larsen et al., 2010a; Redondo et al., 2008; Velazco and Simmons, 2011). Leaf-nosed bats are currently the group of mammals with by far the most comprehensive cytochrome *c* oxidase subunit 1 (COI) gene sampling (Clare et al., 2007), with more than 8000 COI barcode sequences publicly

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available for 117 species (BOLD database as of February 2013). Moreover, molecular phylogenetic studies based on a few standard mitochondrial markers such as 12S and 16S ribosomal RNAs (rRNAs), COI, and cytochrome b (CYTB) (Baker et al., 2003; Hoofer et al., 2008a) and some nuclear exons like those of the RAG2, VWF, and BRCA1 genes, as well as the 3′-UTR of the PLCB4 gene, and the short intron of the PEPCK gene (Baker et al., 2003, 2000; Datzmann et al., 2010) have greatly improved our understanding of the phyllostomid phylogeny.

Two striking examples best illustrate the contribution of molecular data in providing a more resolved picture of the evolutionary history of leaf-nosed bats. First, for a long time the genera *Carollia* and *Rhinophylla* were considered close relatives, mainly on the basis of tooth shape similarity (Wright et al., 1999). Molecular data have shown that they actually belong to two different subfamilies (Baker and Bleier, 1971; Baker et al., 2003, 2000; Wright et al., 1999), which might have diverged more than 20 million years ago (Mya) (Datzmann et al., 2010). Second, the genus *Tonatia* has been traditionally circumscribed on the basis of ears shape in the "round-eared bats" group (Porter et al., 2003), whereas molecular data led to the distinction of *Lophostoma* from *Tonatia*. These two genera may even not be close relatives, with *Lophostoma* being phylogenetically closer to the clade including *Phyllostomus* and *Phylloderma* (Hoffmann et al., 2008; Porter et al., 2003). Similarly, divergence times may involve *ca*. 20 million years (Myr) of separated history (Datzmann et al., 2010).

At present, 56 genera classified into 11 subfamilies have been recognized within phyllostomid bats according to recent molecular phylogenetic studies and taxonomic revisions. Starting from the deepest node of the Phyllostomidae phylogeny as proposed by several authors (Baker et al., 2003; Datzmann et al., 2010; Simmons, 2005; Wetterer et al., 2000), and moving towards more recent branching points, these subfamilies are (i) Macrotinae (e.g., big-eared bats), (ii) Micronycterinae (e.g., little big-eared bats), (iii) Desmodontinae (vampire bats), (iv) Lonchorhininae (sword-nosed bats), (v) Phyllostominae (spear-nosed bats), (vi) Glossophaginae (long-tongued, long-nosed, long-tailed, single-leaf, and banana bats), (vii) Lonchophyllinae (nectar bats), (viii) Carollinae (e.g., short-tailed leaf-nosed bats), (ix) Glyphonycterinae (e.g., tricolored bats), (x) Rhinophyllinae (e.g., little fruit bats) and (xi) Stenodermatinae (e.g., neotropical fruit-eating bats). These subfamilies have followed contrasting evolutionary paths after they diverged from each other, leading to a great morphological and ecological heterogeneity among their members (Baker et al., 2012; Datzmann et al., 2010; Dávalos and Jansa, 2004; Hoffmann et al., 2008; Hoofer et al., 2008a,b; Larsen et al., 2010b; Rojas et al., 2011; Solari et al., 2009). Some phylogenetic relationships among these subfamilies and their members are strongly supported, as for example the deep branching positions of Micronycterinae and Desmodontinae, or else the paraphyly of nectarivorous phyllostomids (Datzmann et al., 2010), for which a convergence in adaptive patterns has also been recently suggested for the CYTB gene (Dávalos et al., 2012). Some other relationships are still contentious because of conflictive results obtained with different markers, as illustrated by the case of the vampyressine bats (Hoofer and Baker, 2006; Hoofer et al., 2008a; Lim, 2003; Porter and Baker, 2004). Such conflict has been shown to arise from both biological and methodological sources (Dávalos et al., 2012).

One way to improve the phylogenetic resolution within families of vertebrates is to combine the information from several mitochondrial and nuclear markers. Recently, complete mitochondrial genomes have been shown to provide compelling phylogenetic signal for resolving evolutionary relationships within mammalian families as, for example, Ursidae (Krause et al., 2008), Mustelidae (Yu et al., 2011a), Delphinidae (Vilstrup et al., 2011), Elephantidae (Rohland et al., 2007), and Bovidae and Cervidae (Hassanin et al., 2012; Wada et al., 2010). A recent study within the bird family Icteridae (New World blackbirds) has also demonstrated the enhanced power of complete mitochondrial genomes to resolve phylogenetic relationships, in comparison to the more traditional CYTB and ND2 genes (Powell et al., 2013). Indeed, mitochondrial genomes provide a set of unambiguously orthologous markers evolving under contrasted selective pressures and, thus, at evolutionary rates which are variable among genes (Reyes et al., 1998). Consequently, individual mitochondrial genes can provide phylogenetic signal at different taxonomic levels. Also, mitochondrial genes exhibit faster rates of molecular evolution than nuclear genes, and could thus potentially be informative enough to resolve recent divergence events, a feature reinforced by the fact that their coalescence time is shorter than for nuclear genes (Moore, 1995). However, potential shortcomings of mitochondrial DNA (mtDNA) as a phylogenetic marker are also widely acknowledged: (i) the substitutional saturation potentially reinforced by the location of the mitogenome in a metabolically active and highly oxidative environment, (ii) the detection of selection-like patterns in some taxa and genes (Castoe et al., 2008; Dávalos et al., 2012; Foote et al., 2011; Jiang et al., 2007; Tomasco and Lessa, 2011; Yu et al., 2011b), (iii) the linkage of all mitochondrial markers into a single locus (Galtier et al., 2009), and (iv) the discordance with the actual species tree in cases of introgression and/or hybridization as recently revealed in African fruit bats (Nesi et al., 2011). These advantages and shortcomings signify that mitogenomes should be supplemented by nuclear markers in a combination that has proven successful on the basis of simulations taking in account the phylogenetic resolving power of each kind of marker (Sánchez-Gracia and Castresana, 2012).

Despite the attractiveness of the mitogenome as a marker of the evolutionary history of mammals, its sequencing is not always straightforward. Extensive variability among taxa generates hypervariable regions, which hinder PCR primer design and standard amplification and sequencing procedures. Building a comparative mitogenomics framework can also become a harder task in largely diversified clades, for which having a wide taxonomic sampling is mandatory to infer reliable macroevolution and diversification patterns. To date, only 18 mitochondrial genomes of bats (as of February 2013) are available in public databases, with only two species of Phyllostomidae belonging to the single genus *Artibeus*: the Jamaican fruit bat (*Artibeus jamaicensis*) (Pumo et al., 1998) and the great fruit-eating bat (*Artibeus lituratus*) (Meganathan et al., 2012). Moreover, recent works have evidenced the need of improving data collection for leaf-nosed bats (Dávalos et al., 2012).

With the improvement of next-generation sequencing (NGS) technologies, acquiring complete mitogenomes is getting easier. The high-coverage capacities of NGS approaches based on short DNA reads appears especially suited to make use of samples from ethanol and/or DMSO preserved tissue collections and museum specimens (Mason et al., 2011; Miller et al., 2009; Rowe et al., 2011). Recent examples in mammals include ancient DNA studies using both 454 sequencing on rhinos (Willerslev et al., 2009) and Illumina technology on mammoths (Enk et al., 2011). To evaluate the contribution of a mitogenomic approach to reconstruct chiropteran phylogeny, and with a special focus on leaf-nosed bats, we sampled representatives of several major phyllostomid clades, and we sequenced the whole mitochondrial genomes of 11 species using both standard Sanger and NGS strategies. We show that the Illumina approach can be easily applied to quickly obtain complete mitochondrial genomes from fresh or frozen tissue samples using a shotgun genome sequencing approach. Using the mitogenomic dataset, we assessed the phylogenetic signal contained in individual mitochondrial genes, we compared it to the signal of two widely used nuclear exons, and we evaluated the effect of different gene combination schemes on tree inference in the context of the phyllostomid phylogeny.

2. Material and methods

2.1. Taxonomic sampling

To evaluate the phylogenetic signal provided by complete mitochondrial genomes in phyllostomid bats, we chose a set of ingroup and outgroup species satisfying the following conditions: (i) inclusion of members of several major subfamilies following the taxonomy proposed by Baker et al. (2003), which ensured covering the major clades of phyllostomid bats; (ii) availability of nuclear gene sequences (here, RAG2 and VWF) for comparative purposes. Thus, we included at least one representative of the seven ingroup families Micronycterinae, Desmodontinae, Phyllostominae, Glossophaginae, Carollinae, Rhinophyllinae, and Stenodermatinae. The four subfamilies Macrotinae, Lonchorhininae, Lonchophyllinae, and Glyphonycterinae were not sampled. Outgroup taxa for Phyllostomidae belonged to the closely-related families Mormoopidae and Mystacinidae, and to the more distantly related Vespertilionidae, Rhinolophidae, and Pteropodidae. Two species (*Bos taurus* and Canis lupus) were also used as non-chiropteran laurasiatherian outgroups. Species sampling and accession numbers of all sequences are given in Table 1.

2.2. Sequencing of complete mitochondrial genomes

We used tissue samples preserved in 95% ethanol and stored in the mammalian tissue collection of the Institut des Sciences de l'Evolution de Montpellier (Catzeflis, 1991). Tissue and voucher numbers are provided in Supplementary Table 1. Whole mitochondrial genomes for 10 phyllostomid and one mormoopid bats were sequenced following two strategies.

First, mitogenomes of three phyllostomid species — *Brachyphylla cavernarum*, *Carollia perspicillata* and *Sturnira tildae* — were PCR amplified from genomic DNA extracted with the standard phenol–chloroform protocol. To increase the likelihood of finding efficient PCR primers, we chose these three species because they were close to the only two phyllostomid bats (*Artibeus* sp.) for which mitochondrial genomes were already available. Primers were designed so that they regularly covered the whole mitogenome in overlapping fragments of at least 100 nucleotides (nt). The list of primers and the protocol of amplification are provided as Supplementary material (Supplementary Material 1 and Supplementary Table 2). The ND3–ND4 region was amplified using the primers proposed by Hoofer and Baker (2006). PCR products were then purified with magnetic beads (Agencourt AMPure XP), or, when unspecific bands were present, fragments were extracted from 1% agarose gels and then purified using the GFX™ PCR, DNA and gel band purification kit (GE Healthcare). Purified PCR amplicons were then sequenced in both directions with the BigDye® Terminator v3.1 kit (Applied Biosystems) on an Applied ABI Prism 3130 XL sequencer. Resulting sequences were manually edited and automatically assembled with Sequencher v 4.3 (Gene Code Corporation) and then aligned using the mitochondrial genome of *Artibeus jamaicensis* as a reference. Sequences of protein-coding genes were validated after translation to check for the absence of frameshifts and / or stop codons.

Secondly, to alleviate the PCR amplification difficulties associated with higher sequence divergence of more distantly related taxa, we developed an original protocol to sequence the mitogenomes of eight additional species (seven phyllostomids and one mormoopid) using a high-throughput NGS Illumina approach. For these samples, we used the QIAGEN® Blood Tissue extraction kit to extract total genomic DNA. This method was chosen because it generally results in better DNA yields and purity required for adequate NGS library construction. Indeed, when extracting DNA with the phenol–chloroform protocol, phenol traces can hamper subsequent steps in library construction by altering, for example, the measures of absorbance on which rely DNA concentration measurements. For each sample, an aliquot containing 5 μg of RNA-free total genomic DNA at a concentration of ~200 ng/μl was provided to the GATC-Biotech company (Konstanz, Germany)

Table 1
Species and accession numbers of sequences used in this work. Taxa names in bold correspond to sequences obtained in this study. In the "sequence source" column, numbers correspond to: 1 = Sanger sequencing; 2 = NGS-Illumina sequencing; 3 = Public databases. Corresponding subfamilies are indicated for phyllostomid bat species.

Family	Subfamily	Species	Sequence source	Accession number		
				Mitogenome	RAG2	VWF
Bovidae		*Bos taurus*	3	NC_006853.1	ENSBTAG00000031309	ENSBTAG00000012265
Canidae		*Canis lupus familiaris*	3	NC_002008.4	ENSCAFG00000015228	ENSCAFG00000015228
Pteropodidae		*Pteropus vampyrus*	3	Ensembl~v57 Scaffold_17814	JN398310.1	JN398278.1
		Rousettus aegyptiacus	3	NC_007393.1	EU617927.1	DQ445694.1
Rhinolophidae		*Rhinolophus monoceros*	3	NC_005433.1	AF447528.1*	AF447546.1*
Vespertilionidae		*Myotis lucifugus*	3	Ensembl~v57 Scaffold_144518	AM265673.1	JN415062.1
		Plecotus auritus	3	NC_015484.1	GU328100.1	AB079840.1
Mystacinidae		*Mystacina tuberculata*	3	NC_006925.1	AY141021.1	AY245421.1
Mormoopidae		***Pteronotus rubiginosus***	2	**HG003312**	**HG380334**	**HG380337**
Phyllostomidae	Micronycterinae	***Micronycteris megalotis***	2	**HF947304**	**HG380333**	**HG380344**
	Desmodontinae	***Desmodus rotundus***	2	**HG003310**	**HG380331**	**HG380342**
	Phyllostominae	***Lophostoma silvicolum***	2	**HG003311**	**HG380330**	**HG380340**
		Tonatia saurophila	2	**HG003315**	**HG380332**	**HG380343**
		Vampyrum spectrum	2	**HG003316**	AF316495.1	**HG380338**
	Glossophaginae	***Anoura caudifer***	2	**HG003307**	**HG380327**	**HG380339**
		Brachyphylla cavernarum	1	**HG003308**	**HG380328**	**HG380336**
	Carollinae	***Carollia perspicillata***	1	**HG003309**	**HG380329**	**HG380335**
	Rhinophyllinae	***Rhinophylla pumilio***	2	**HG003313**	**HG380326**	**HG380345**
	Stenodermatinae	*Artibeus jamaicensis*	3	NC_002009.1	FN641674.1	FN645666.1
		Sturnira tildae	1	**HG003314**	**HG380325**	**HG380341**

* Given that for *Rhinolophus monoceros* there are not any sequences available for RAG2 and VWF genes, accession numbers correspond to those available for *Rhinolophus creaghi*.

for library construction and sequencing. A library of tagged genomic DNA fragments was built for each species. Library preparation included DNA fragmentation, sizing, ligation, indexation, and pooling of samples into an equimolar mix for subsequent run on a single Illumina HiSeq2000 lane. Both library construction and sequencing were conducted under the conditions and protocols practiced by GATC-Biotech.

2.2.1. Mitochondrial genomes assembly

Mitochondrial genomes were *de novo* assembled from the Illumina reads as follows. First, raw single-ended 96-nt reads were assembled into contigs using ABySS (Simpson et al., 2009), version 1.2.0. Different assemblies were generated by varying k-mer values from 48 (half length of a read) to 64 (default maximal k-mer size allowed by ABySS). Similarity searches using BLASTN (Altschul et al., 1990) were then performed on non-redundant ABySS contigs to recover mitochondrial DNA-like matches, using the *Artibeus jamaicensis* mitochondrial genome as a query. To account for the potential high divergence of the target sequences, we set the e-value for a positive match to 1e-05. Moreover, since rapidly evolving protein-coding genes can be difficult to recover based on nucleotide similarity, we also performed TBLASTN searches using the 13 *Artibeus jamaicensis* mitochondrial proteins as a query. This strategy, taking advantage of the amino acid translation of subject contigs for all possible reading frames, also increases the probability to find the targeted mitochondrial sequences.

The final assembly of matching contigs into supercontigs was then realized with CAP3 (Huang and Madan, 1999) under default parameters. This method has proven useful for reference-free transcriptome assembly in non-model animals (Cahais et al., 2012). Visualization and minor manual editing of the resulting sequences was done with MUST (Philippe, 1993) and Seaview v4 (Gouy et al., 2010) to obtain the final mitogenomic assemblies. Site coverage was evaluated for each species from the collection of Illumina reads using custom BASH and R scripts available upon request. Read mapping and annotation of the mitogenomes were performed using GENEIOUS® Pro (Drummond et al., 2011). Reads were mapped only if 24 nucleotides consecutively matched the reference sequence, with a maximum 10% of single mismatches over the read length, a minimum of 95% similarity in overlapping regions, and a maximum 10% of indels not exceeding a gap size of 3.

2.3. Sequencing of nuclear genes

We sequenced the RAG2 and VWF nuclear markers in species for which they were not available in public databases (Table 1). The RAG2 gene was amplified using the primers RAG2F12 (fwd): 5′-TAACCATCTAAAACTGAAGC-3′ and RAG2R901 (rev) 5′-GTTTTCT GTTCTTCATTCAC-3′ with an annealing temperature of 52 °C. The VWF exon was amplified using two overlapping pairs of primers: (i) VWFF50 (fwd): 5′-CCCCGTATGTGGAAGACACC-3′ and VWFr649 (rev): 5′-AGCTGATAATCTCGTCCCTTCG-3′ with an annealing temperature of 50 °C; (ii) VWFF489 (fwd): 5′-GGGCCTGAAGAAGAA-GAAAGTC-3′ and VWFR1050 (rev): 5′-GCTGTGCTCGGACACGTAC T-3′ with an annealing temperature of 51 °C. The amplification protocol was the same as for mitochondrial fragments using the corresponding temperatures of annealing (see Supplementary material). Sequences were aligned using MUSCLE (Edgar, 2004) and verified by eye in Seaview v4 which resulted in final alignments of 1366 sites for RAG2 and 1239 sites for VWF.

2.4. Phylogenetic analyses

Mitochondrial genomes were aligned using the GENEIOUS aligner with default parameters and then corrected by eye to ensure that alignment of protein coding genes was in agreement with reading frame and to minimize the number of uninformative gaps in the rRNAs, tRNAs and control region (CR) sequences. The total mitogenome alignment comprising 17,680 sites was filtered with GBLOCKS (Castresana, 2000) under the following parameters: a minimum of 11 sequences for a conserved position, a minimum of 17 sequences for a flanking position, a maximum of 5 contiguous non-conserved positions, a minimum of 10 positions for a block, and a maximum of 50% of gaps per position. This step conserved 89% of the original alignment (15,737 unambiguously aligned sites), keeping the entire sequences of protein coding genes, but removing hypervariable regions from rRNAs, tRNAs and CR.

The mitogenome phylogeny of phyllostomid bats was reconstructed using maximum likelihood (ML) and Bayesian inference (BI). Maximum likelihood analyses were performed under the general time reversible (GTR) model of nucleotide exchangeabilities, with a Gamma (Γ) distribution and a fraction of invariable (I) sites to account for the among-site heterogeneity in substitution rates. Statistical support of nodes was measured by bootstrap percentages (BS) after 100 replicates. All these ML analyses were conducted using PAUP* v4.0b10 (Swofford, 2002), with a neighbor-joining starting tree, and a tree bisection–reconnection branch swapping. Bayesian inference was performed with Phylobayes v3.3 (Lartillot et al., 2009). To account for the potential heterogeneity of the substitution pattern among the different regions of the mitogenome, the CAT site-heterogeneous mixture model was used (Lartillot and Philippe, 2004). The model of DNA sequence evolution also incorporated the GTR + Γ options (CAT-GTR + Γ. Statistical support for nodes was measured by the corresponding posterior probabilities (PP). We used a Dirichlet prior for nucleotide frequency profiles, and exponential priors for the nucleotide exchangeability, among-site rate heterogeneity, and branch length parameters. Trees were sampled every 10 cycles until reaching 10,000 trees. The convergence of three independent Markov chains Monte Carlo (MCMC) was evaluated with the *bpcomp* procedure: chains were stopped when the maximum PP difference for a given node among the three chains became less than 0.1 with a burnin of 1000 cycles.

2.5. Single-gene versus combined-gene approaches

In order to compare the phylogenetic signal carried out by each individual mitochondrial and nuclear gene to the one provided after gene concatenation, we performed a series of ML analyses with comparable taxon sampling. Using PAUP* under the GTR + Γ + I model, we inferred the phylogeny of phyllostomid bats for each of the two nuclear genes (RAG2 and VWF) as well as for a total of 17 independent mitochondrial partitions: the 13 protein-coding genes, each of the two rRNAs (12S and 16S), the concatenation of all 22 tRNAs, and the CR. These partitions were extracted from the alignment previously curated with GBLOCKS.

Then, with the aim of comparing the phylogenetic signal of the different mitochondrial and nuclear partitions within the family, we evaluated the bootstrap statistical support for several clades: Phyllostomidae (node A), Phyllostominae (B), Glossophaginae (C) and Stenodermatinae (D). Two nodes corresponding to rapid diversifications were also evaluated to assess the performance of mitochondrial genomes at resolving this kind of event: the Phyllostominae + [Glossophaginae + Carollinae + Rhinophyllinae + Stenodermatinae] clade (E) and the Glossophaginae + [Carollinae + Rhinophyllinae + Stenodermatinae] clade (F).

3. Results

3.1. Sequencing of mitochondrial genomes

Eleven new complete bat mitochondrial genomes were obtained for 10 species of phyllostomids and one species of mormoopids by

using both classical (three species) and NGS Illumina (eight species) sequencing. For the NGS approach, the total number of 96-nt reads ranged from 3,909,139 (*Anoura caudifer*) to 7,725,965 (*Desmodus rotundus*) (Table 2). From these, 0.02% to 0.35% corresponded to mitochondrial reads, and no direct correlation was identified between the total and mitochondrial number of reads. Length of assembled mitochondrial genomes varied between 16,616 (*Pteronotus rubiginosus*) and 16,671 (*Rhinophylla pumilio*) nucleotides. The total length of the three mitogenomes obtained by standard sequencing was 16,647 nt (*S. tildae*), 16,711 nt (*C. perspicillata*) and 16,785 nt (*Brachyphylla cavernarum*). Median coverage – defined as the median of the number of reads covering any given site – varied greatly from one species to another with a minimum of 6× for *Lophostoma silvicolum* and a maximum of 87× for *Micronycteris megalotis*. A drop in coverage is observed for all species — and this is more pronounced for well-covered mitogenomes — in the region corresponding to the WANCY tRNAs cluster (including the replication origin of the light strand, O_L), and at the beginning of the COI and ND4 genes (Fig. 1: arrows). A decrease in coverage is also observed in the tPhe-peripheral domain of the control region due to uncertainty in the number of satellite repeats.

Different sequencing technologies may exhibit biases in base composition (Aird et al., 2011). If not detected, artifacts in base composition can lead to misleading assemblies and potentially erroneous phylogenetic inferences. To check for these potential biases, and as a way to compare the standard and Illumina sequencing outcomes, we evaluated the base composition of the sequenced mitochondrial genomes (Table 3). A chi-square test with respect to expected base frequencies did not reject the null hypothesis of a homogeneous distribution across taxa, and this regardless of the sequencing method used (p-value <0.001).

3.2. Phylogenetic reconstructions

We compared the phylogenetic signal of mitochondrial genomes versus nuclear exons, and single-gene versus concatenation for both kinds of genes. Phylogenetic signal and statistical support of the nodes of single-gene mitochondrial and nuclear phylogenies varied greatly from one gene to another, and also as a function of the clade under focus (Table 4). A common trend for individual mitochondrial and nuclear genes is the fact that nodes defining relationships among subfamilies were either not recovered or weakly supported (Table 4: nodes E, F). The concatenation of the five mitochondrial markers used in previous works (12S and 16S rRNA, and the COI, CYTB and ND1 protein-coding genes) improved the statistical support as compared to the single-gene approach. However, clades E and F remained weakly supported, and clade B (Phyllostominae) was not recovered.

Expectedly, phylogenies inferred from concatenated mitogenomic sequences appeared better resolved and well supported with BS > 70 and PP > 0.99 for most nodes. This result is observed, with few exceptions, for both ML and BI approaches (see Table 4 and Fig. 2). Complete mitochondrial genomes provided support

Table 2
Statistics associated to the sequencing of mitogenomes using NGS-Illumina technology in 7 phyllostomid and one mormoopid bats.

Species	Total number of reads	Mitochondrial reads		Mitogenome length (nt)	Median coverage
		Number	%		
Anoura caudifer	6,499,175	5400	0.08	16,546	31×
Desmodus rotundus	7,725,965	1495	0.02	16,665	8×
Lophostoma silvicolum	5,560,092	1158	0.02	16,666	6×
Micronycteris megalotis	4,388,964	15,240	0.35	16,589	87×
Pteronotus rubiginosus	3,830,451	9779	0.26	16,616	55×
Rhinophylla pumilio	7,302,121	4,359	0.06	16,671	25×
Tonatia saurophila	4,664,854	13,867	0.30	16,628	79×
Vampyrum spectrum	3,909,179	4242	0.11	16,637	24×

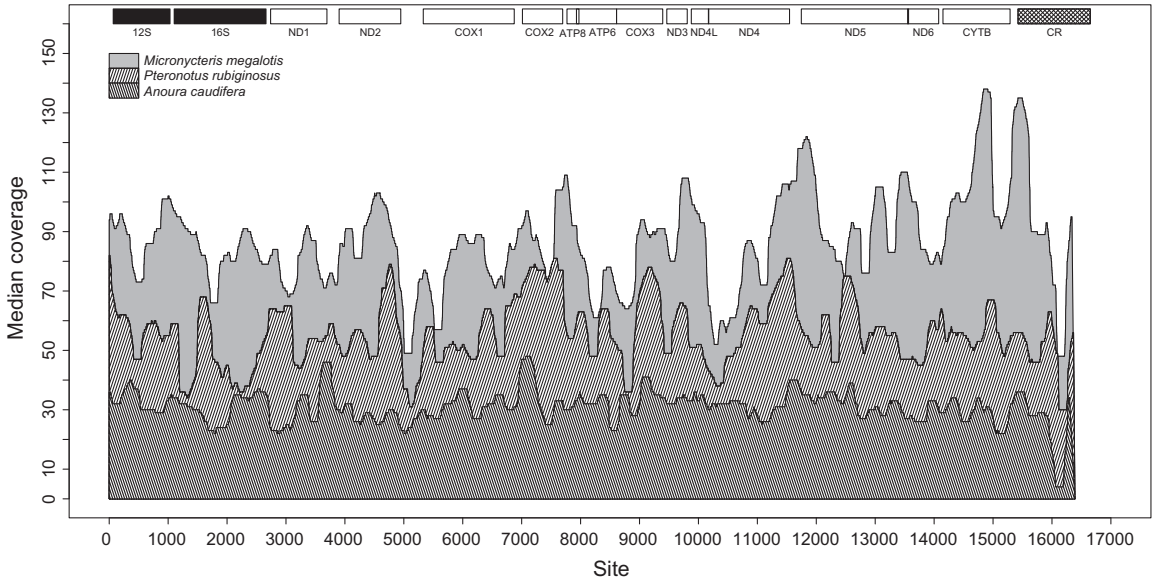

Fig. 1. Coverage for the assembled mitochondrial genomes of *Micronycteris megalotis*, *Pteronotus rubiginosus*, and *Anoura caudifer* using Illumina next-generation sequencing (NGS). The mitochondrial map is indicated on the top: rRNAs in black, protein-coding genes in white, CR in grey, with spaces corresponding to tRNAs.

Table 3
Base composition for the 11 mitochondrial genomes of phyllostomid and mormoopid bats sequenced with either Sanger or Illumina approaches.

Species	%A	%C	%G	%T	Sequencing
Pteronotus rubiginosus	33.6	27.4	13.4	25.6	Illumina
Micronycteris megalotis	32.4	29.3	13.5	24.8	Illumina
Desmodus rotundus	29.7	32.0	15.6	22.7	Illumina
Vampyrum spectrum	30.2	31.1	14.7	24.0	Illumina
Tonatia saurophila	33.5	27.5	12.6	26.4	Illumina
Lophostoma silvicolum	32.2	28.9	13.0	25.9	Illumina
Anoura caudifer	32.2	25.9	13.6	28.3	Illumina
Brachyphylla cavernarum	32.0	27.2	13.8	27.0	Sanger
Carollia perspicillata	31.5	27.7	13.7	27.1	Sanger
Rhinophylla pumilio	31.9	24.9	13.8	29.4	Illumina
Sturnira tildae	32.0	30.0	13.3	24.7	Sanger

for the close relationship between the New-Zealand family Mystacinidae and the New World bats (BS = 97; PP = 1). Within phyllostomids, nodes defining the family and some intra-familial relationships were also strongly supported (BS > 95). This was notably the case for deep divergences of the subfamilies Micronycterinae and Desmodontinae, and for the node grouping the two species of Glossophaginae (Fig. 2).

Nodes with lower support involved the monophyly of Phyllostominae (BS = 67; PP = 1), the monophyly of the two Stenodermatinae species (*A. jamaicensis* and *S. tildae*: BS = 65; PP = 0.99), the grouping of Glossophaginae with the clade Carollinae + Rhinophyllinae + Stenodermatinae (BS = 78; PP = 0.82), and the grouping of Phyllostominae with the clade Glossophaginae + Carollinae + Rhinophyllinae + Stenodermatinae (BS = 68; PP = 0.99). The major split within Chiroptera between Yinpterochiroptera and Yangochiroptera is supported by the ML tree (BS = 100), whereas *Rhinolophus* appears closer to the other echolocating bats than to Pteropodidae under the BI analysis. However, in all cases, clade supports are much higher for mitogenomic analyses than those recovered using the single-gene approach (Table 4).

Individual nuclear genes were able to solve some of the relationships in the phylogeny, but statistical support remained globally weak, especially within Phyllostomidae (BS < 70). Moreover, nuclear markers were less informative for recent divergences and for rapid diversification events. For instance, the VWF gene resolved deep divergences like those of *Micronycteris megalotis* and *Desmodus rotundus*, and more internal subfamilies like Stenodermatinae, but it was unable to define the branching pattern within Phyllostominae and Glossophaginae. The RAG2 gene, which is less variable than VWF, was able to resolve deep divergences in the chiropteran phylogeny, but failed to decipher the relationships among phyllostomid species. When comparing both topologies, some topological discrepancies can be observed, but the conflicting nodes were weakly supported and often corresponded to short internal branches.

With respect to the single-gene inference, both phylogenetic resolution and node support were improved by the concatenation of the two nuclear markers. However, mitogenomes were still more informative and provided a globally stronger statistical support than the nuclear concatenation (Fig. 3 and Table 4). Finally, when both mitogenomes and nuclear markers were concatenated, there was an increase in statistical support for the nodes that were still weakly supported by the mitogenomic inference alone (Fig. 4).

4. Discussion

4.1. The promises of next-generation mitogenomics for phylogenetics

In the last two decades, the acquisition of a large number of molecular markers has significantly contributed to mammalian phylogenetics and systematics. The development of PCR and sequencing techniques and their subsequent improvements allowed, first, the accumulation of mitochondrial sequences for a few standard markers (e.g., CYTB and CO1 genes) and second, the development and use of single-copy nuclear exons such as RBP3 (Stanhope et al., 1992). However, the efficiency of PCR-based DNA sequencing is impacted by factors such as primer specificity, which is altered by the amount of genetic divergence between taxa, and the limited size of the amplicons, which is directly correlated with

Table 4
Bootstrap support provided by individual and concatenated mitochondrial and nuclear genes for different clades within the family Phyllostomidae. Dashes (–) indicate that the clade was not recovered when using the gene under focus. Values in bold indicate significantly supported clades (i.e., BS > 70). The 12S + 16S + ND1 + COI + CYTB concatenate involve 5 mitochondrial genes classically used for chiropteran phylogenetics. Clade letters refer to Fig. 4: Phyllostomidae (A), Phyllostominae (B), Glossophaginae (C), Stenodermatinae (D), Phyllostominae + (Glossophaginae + Carollinae + Rhinophyllinae + Stenodermatinae) (E), and Glossophaginae + (Carollinae + Rhinophyllinae + Stenodermatinae) (F).

Genes/partitions	Length of the alignment (sites)	Variable sites (%)	Clades					
			A	B	C	D	E	F
12S rRNA	908	42.8	**81**	–	36	6	–	–
16S rRNA	1440	43.0	**86**	–	**78**	**88**	–	23
ND1	957	51.8	66	6	12	42	6	–
ND2	1044	63.6	51	66	**72**	16	40	26
COI	1545	41.8	26	7	49	47	–	–
CO2	684	47.8	**74**	–	**77**	–	–	26
ATP8	224	60.7	22	–	22	–	8	–
ATP6	681	52.3	–	–	16	–	–	–
CO3	785	45.9	6	14	–	8	5	11
ND3	348	58.0	37	–	–	5	–	–
ND4L	297	57.6	–	–	–	–	7	–
ND4	1378	56.5	68	15	**86**	–	12	9
ND5	1820	58.5	**77**	5	**70**	45	–	10
ND6	528	64.8	60	–	52	**79**	–	–
CYTB	1140	51.2	46	5	22	–	–	–
CR	554	63.4	**94**	–	66	41	25	–
22 tRNAs	1492	41.4	6	–	66	**87**	52	–
12S + 16S + ND1 + COI + CYTB	5990	45.6	**100**	–	**96**	**98**	34	39
Whole mitogenome	15,737	51.3	**100**	67	**100**	65	68	**78**
Nuclear VWF	1239	39.0	**95**	–	–	–	–	–
Nuclear RAG2	1363	27.2	**100**	–	**95**	**86**	–	–
Nuclear VWF + RAG2	2605	32.8	**100**	–	48	95	52	19
Mitochondrial + nuclear concatenation	18,342	48.6	**100**	**99**	**100**	**100**	**100**	**76**

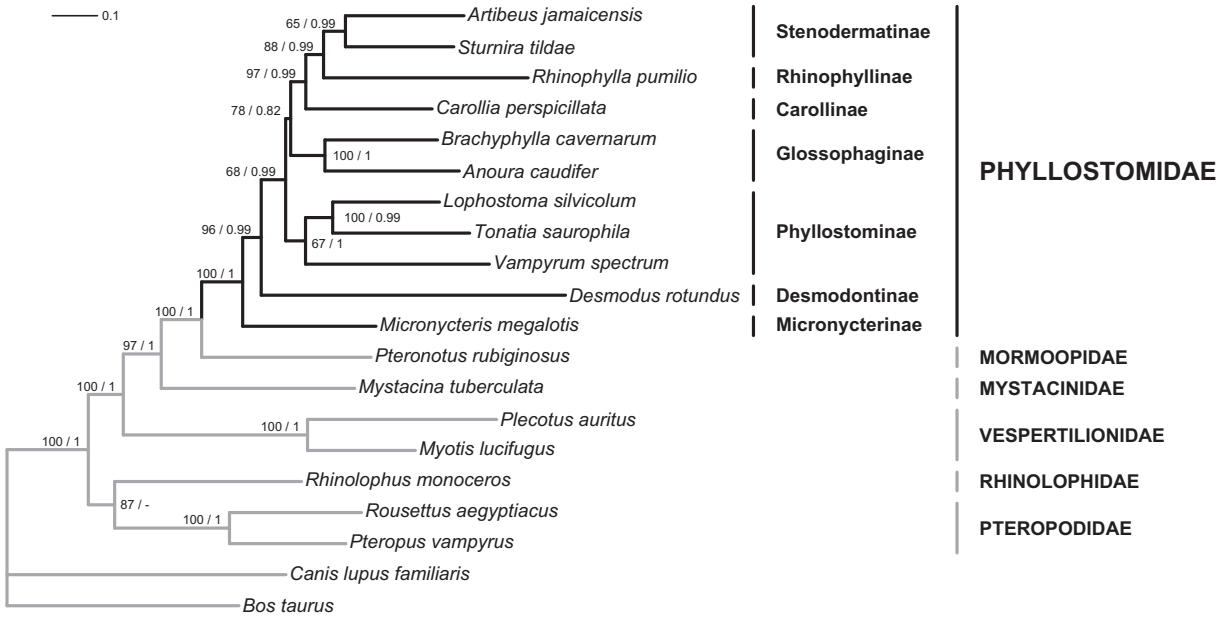

Fig. 2. Phylogeny of phyllostomid bats inferred from complete mitogenomes using maximum likelihood (ML) (*a*) and Bayesian inference (BI) (*b*) approaches. Values on nodes correspond to bootstrap support (BS) and posterior probability (PP) values, respectively. Black branches connect the ingroup taxa (Phyllostomidae) and gray branches connect outgroup taxa. For the Phyllostomidae family, subfamilies are indicated. Branch lengths are expressed as a number of nucleotide substitutions per site.

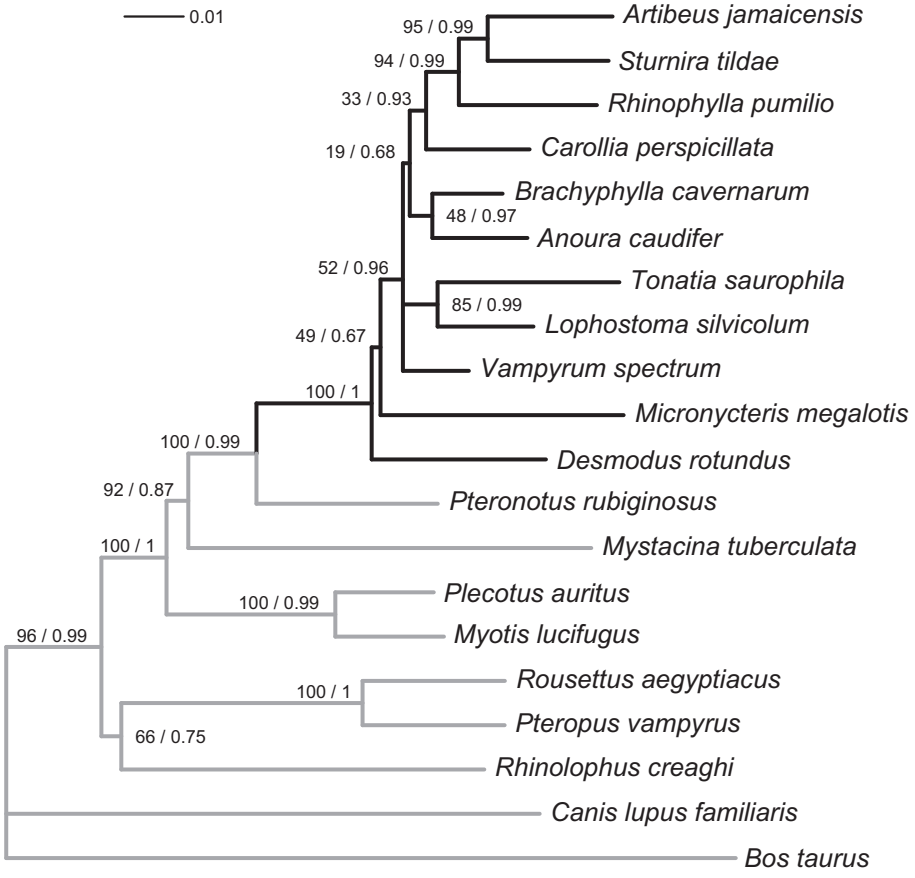

Fig. 3. Maximum likelihood molecular phylogeny of phyllostomid bats inferred from a concatenation of RAG2 and VWF gene sequences. Values on nodes correspond to bootstrap support (BS) and posterior probability (PP) values, respectively.

the quality of the DNA template especially for museum specimens. Besides, several complications can be encountered for the different kinds of targeted markers. The need of specific primers and custom amplification conditions for each of the taxa under focus, especially when the evolutionary rate of targeted genes and / or their flanking regions is high, renders PCR amplification tedious at a large scale.

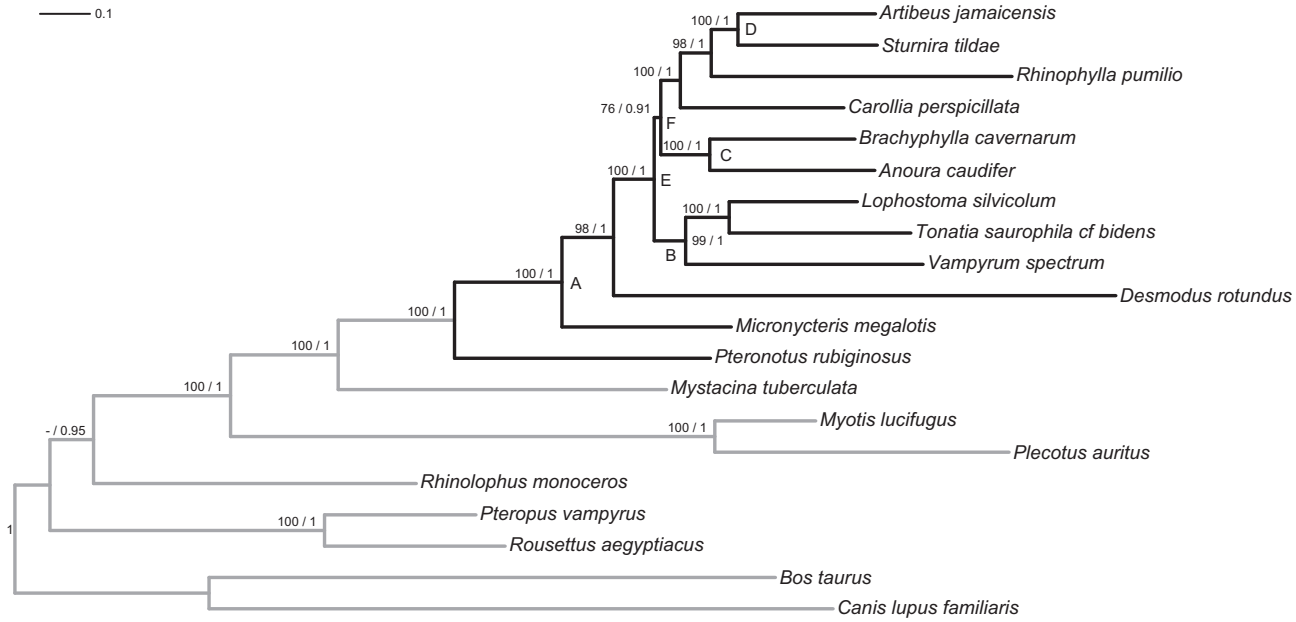

Fig. 4. Maximum likelihood molecular phylogeny of phyllostomid bats inferred from a concatenation of the sequences of complete mitochondrial genomes and the nuclear markers VWF and RAG2. Values on nodes correspond to BS and PP, respectively. Capital letters label the clades used for comparison of the different partitions (see Table 4).

Also, the development of nuclear markers is complicated by the mosaic structure of mammalian genes, which require targeting exons of reasonable length while avoiding the occurrence of large introns (Ranwez et al., 2007). Finally, in the case of mitochondrial markers, a recurrent problem is the co-amplification of nuclear copies of mitochondrial fragments (Hassanin et al., 2010; Richly and Leister, 2004).

Another critical aspect of standard PCR-based sequencing is the amount of DNA required to complete, for instance, a whole mitochondrial genome sequencing, which is highly dependent upon the source of genetic material (tissues, hairs, bones, skin, blood, or saliva). The DNA yielded by samples from museum specimens is often degraded and allowed only short amplicons to be produced (Cooper et al., 1992; Haddrath and Baker, 2001; Rowe et al., 2011; Thomas et al., 1990). To circumvent this problem, procedures of sequence capture and enrichment in mitochondrial fragments have been developed (Enk et al., 2011; Mason et al., 2011; Willerslev et al., 2009). Our results show that a shotgun approach with high-coverage sequencing can also be useful to quickly obtain complete mitogenomes. Based on the sequencing of short DNA fragments, Illumina NGS is especially suited for the analysis of small amount of tissues or samples from museum specimen in which DNA is naturally scarce and / or fragmented. Moreover, the possibility of using tags to label individual sample libraries allows sequencing several taxa simultaneously, offering thus a powerful approach for comparative mitogenomics of highly diversified groups such as phyllostomid bats. Note, however, that alternative approaches without tagging have been explored to assemble highly divergent sequences (Rubinstein et al., 2013; Timmermans et al., 2010).

4.2. The ratio of mitochondrial over nuclear DNA

Given the overrepresentation of mitochondrial DNA compared to nuclear DNA in most tissues (Robin and Wong, 1988; Veltri et al., 1990), the shotgun Illumina NGS approach allows assembling whole mitochondrial genomes with adequate coverage from less than 10 millions reads in most cases. This means that, in theory, 200 millions of reads (i.e., the yield of a single HiSeq lane) can lead to at least 20 complete mammalian mitochondrial genomes. Taking *Micronycteris megalotis* as an example, we assume that each cell of the initial tissue contained two copies of 2 Gb of nuclear DNA (a typical nuclear genome size in bats: see e.g., *Myotis lucifugus* in Ensembl v.70) versus a number of mitochondria each containing on average two copies of the mitogenome (Robin and Wong, 1988). Under the assumption that the DNA amplification steps during the construction of the Illumina libraries have not biased the initial ratio of mitochondrial to nuclear sequences, and with a mitogenome length of 16,589 bp and 0.35% of mitochondrial reads (Table 2), this yields a putative total of $0.35 \times 2 \times 2.10^9/(2 \times 16{,}589 \times 100)$, i.e., ca. 420 mitochondria per cell. This value falls in the range of the estimates available for mammalian cells from the literature (Robin and Wong, 1988). However, the variation of the mitogenome coverage among species (*cf.* Table 2) can be explained by a variable number of mtDNA circles per mitochondrion and of mitochondria per cell and tissue (Fuke et al., 2011; Robin and Wong, 1988; Veltri et al., 1990).

Given this excess of mitochondria versus nuclei number in each cell, and as a consequence of their relative per-cell number of copies, nuclear elements of mitochondrial origin (numts) are expected to be in a smaller proportion than those corresponding to functional mitochondrial genes (Maricic et al., 2010). In other words, the probability of getting one numt read among millions of other nuclear ones will still be smaller than that of sequencing a true mitochondrial read, except in the case of a massive amplification of numts after their transfer to the nucleus. If numt reads are less abundant and if they display some level of sequence divergence as compared to their mitochondrial counterpart, they are less likely to be assembled in the final mitogenome.

4.3. The assembly and coverage of the mitogenomes

A valuable advantage of NGS comes from the resulting high coverage, which significantly eases mitogenome assembly. With more reads overlapping on a given genomic region, *de novo* assembly strategies are facilitated, even for divergent taxa. Moreover, higher coverage ensures higher sequence reliability as each position is supported by several independent reads with associated quality

score. This allows the identification of reads exhibiting minority polymorphisms likely corresponding to sequencing errors, mitochondrial heteroplasmy and / or numts. In case of limited NGS read coverage, we however identified two factors that can play an important role in the performance and efficiency of the assembly and mapping steps: the phylogenetic position of the closest reference mitogenome, and the evolutionary rate of the targeted taxa.

A striking example of these situations is illustrated by the case of the common vampire bat (*Desmodus rotundus*), whose mitogenome was weakly covered despite the high number reads produced. Indeed, this species not only diverged early within phyllostomid bats (Baker et al., 2003; Datzmann et al., 2010), but its mitogenome has accumulated a higher number of nucleotide substitutions as compared to other phyllostomid bats, as attested by the longer branch observed in inferred phylogenies (Fig. 2). Our initial reference mitogenome was the Jamaican fruit-eating bat (*A. jamaicensis*) since only two *Artibeus* species were available among phyllostomid bats. These species belong to the more recently diverged subfamily of frugivorous bats, the Stenodermatinae. Because of the high sequence divergence between *Desmodus* and *Artibeus*, some regions of the mitogenome — e.g., variable parts of the 12S rRNA, 16S rRNA, and ND5 protein-coding gene — were much more difficult to recover by nucleotide-based similarity searches. The missing regions corresponding to protein coding genes were only identified through the TBLASTN strategy. Otherwise, faster-evolving regions corresponding to rRNAs, tRNAs, and control region can be more tricky to recover using nucleotide BLAST and mapping tools. When coverage is adequate, *de novo* assembly should provide contigs containing these regions. When the sequencing coverage is low, denser taxonomic sampling may provide closer relatives of the group under focus, thus increasing the efficiency of the mapping approach.

About targeted sequences of mitochondrial genes, we expected that on average each fragment would be sequenced in a similar number of copies and that the coverage would be uniform. However, our results show that the Illumina reads coverage is not uniform along the mitochondrial genome (Fig. 1). Some drops in median coverage are observed in the region corresponding to the WANCY tRNA cluster preceding the COI gene, and in the ND4 gene. These observations have also been reported in previous mitogenomic studies of mammals using NGS sequencing (Mason et al., 2011; Rowe et al., 2011). These decreases in read coverage are likely due to the presence of secondary structures of the DNA impairing their efficient sequencing by NGS techniques. For instance, GC content (especially GGC motifs) and inverted repetitions can inhibit single-base elongation (Nakamura et al., 2011). In the nuclear genome, it has also been found that CpG islands and promoters are often less covered than the rest of the genome (Wang et al., 2011).

4.4. The phylogenetic signal of mitogenomes in bats

Single mitochondrial and nuclear genes contribute to resolve relationships at high taxonomic level in bats such as the branching pattern among subfamilies. However, their phylogenetic signal is not sufficient when it comes to resolve more recent nodes like intergeneric relationships within phyllostomid subfamilies (Table 3). Although there are some differences in branching order among the topologies resulting from single-gene analyses, none of them is strongly supported statistically. The conflicting nodes often involved short internal branches therefore reflecting the lack of information rather than true conflicting signals. In the case of the two nuclear markers, RAG2 exhibits a lower proportion of variable sites as compared to VWF. Also, this marker seems to be sensitive to taxon sampling as previous studies considering a larger number of species obtained better topological resolution and statistical support (Baker et al., 2000).

It has been shown that the concatenation of individual genes into a single supermatrix provides more phylogenetic signal by increasing the amount of informative sites (de Queiroz and Gatesy, 2007; Gadagkar et al., 2005). Recently, it has also been suggested that a combination of nuclear and mitochondrial markers should provide strong phylogenetic signal thanks to the combination of sites with heterogeneous evolutionary rates and, thus, complementary phylogenetic signal (Sánchez-Gracia and Castresana, 2012). Mitochondrial genomes intrinsically offer a concatenation of markers including rRNAs, tRNAs, protein-coding genes and a non-coding control region, which results in a sum of alignments characterized by heterogeneous gene-specific substitution patterns (Reyes et al., 1998). In the context of the phyllostomid phylogeny, Datzmann et al. (2010) built a mitochondrial dataset that combined the 12S and 16S rRNAs, and the protein-coding COI, CYTB, and ND1 genes. We inferred the phylogeny of phyllostomid bats using the same five genes to evaluate the signal of such a concatenation with respect to our taxon sampling. We obtained a fairly well resolved phylogeny with high bootstrap values for several nodes. However, the phylogenetic signal provided by this set of genes was not strong enough to recover several clades, including Phyllostominae (node B, BS = 62), and nodes E and F were only weakly supported (BS = 34 and 39, respectively) (see Table 3). The same nodes remained either unresolved or weakly supported even after correcting for saturation and with increased taxonomic sampling (Baker et al., 2012; Dávalos et al., 2012).

Complete mitogenomes contribute to resolve difficult nodes. Indeed, bootstrap values for nodes such as E and F, which were not well supported by single genes, increased significantly when complete mitogenomic sequences were used. Besides, most nodes of the phylogeny were strongly supported (Fig. 2), even those only supported by nuclear markers. Similarly, a concatenation of the two nuclear genes resulted in an improvement of bootstrap support compared to single-gene trees. Moreover, phylogenetic signal of whole mitochondrial genomes is comparable to that of a concatenation of nuclear and mitochondrial markers, and this holds true not only for topological resolution, but also for associated statistical support values. With respect to chiropterans, the ML analysis, but not the Bayesian one, recovered the major split between Yinpterochiroptera (Rhinolophidae + Pteropodidae) and Yangochiroptera, thus breaking the monophyly of the former Microchiroptera (echolocating bats) (Teeling et al., 2002). Because a single taxon was included as a representative of each family for non-phyllostomid bats, the parameter-rich Bayesian CAT mixture model may lack information to retrieve this result. We also recovered the close relationship of the New Zealand short-tailed bat (*Mystacina tuberculata*) and South American bats (Mormoopidae + Phyllostomidae) (Hoofer et al., 2003; Teeling et al., 2003, 2005). Within Phyllostomidae, we retrieved the deep divergences of *Micronycteris megalotis* and *Desmodus rotundus* (members of the Micronycterinae and Desmodontinae subfamilies, respectively) (Baker et al., 2003; Datzmann et al., 2010). Similarly, the monophyly of the subfamilies Phyllostominae and Stenodermatinae is strongly supported, but this would need to be confirmed by expanding the taxon sampling. It is however noticeable that the relationships among phyllostomid bats inferred from comparative mitogenomics are in agreement with those based on individual mitochondrial and nuclear genes coupled with denser taxon sampling (Baker et al., 2012, 2003, 2000; Datzmann et al., 2010).

Conflicting branching and the weak support observed for some nodes are problems frequently encountered when working with highly diversified groups, which often include divergence events followed by rapid diversifications. This diversification pattern generates short branches in the phylogeny and the few accumulated

substitutions are not informative enough to clarify the evolutionary history at these nodes. This effect can be clearly observed in topologies resulting from single-gene analyses. Within the phylogeny of phyllostomid bats, this seems to have occurred twice: within the subfamily Phyllostominae and after the divergence of the clade formed by Glossophaginae, Carollinae, Rhinophyllinae and Stenodermatinae (see nodes E and F in Figs. 3 and 4).

Although all mitochondrial genes are linked by the same organismal history, they can efficiently recover the species tree. One advantage of mtDNA lies in its reduced effective population size compared to nuclear genes which makes it more likely to retrace the species evolutionary history (Moore, 1995). Moreover, in our case, a concatenation of mitochondrial genes provided much more variable sites than the two studied nuclear genes. Conversely, a problem of mitogenomics markers is their saturation with respect to multiple substitutions. Their usefulness is therefore restricted to rather low taxonomic scales such as intra-familial relationships in mammals. For instance, some saturated sites have been identified in protein-coding COI and CYTB, and in the loops of the 16S rRNA genes of phyllostomid bats (Dávalos et al., 2012). This underlines the importance of improving taxon and site sampling as well as the models of sequence evolution to better take into account the heterogeneities in the substitution process.

5. Conclusions

We have shown the advantages of coupling a high-throughput sequencing technology like Illumina with a comparative mitogenomics approach as a first step towards resolving the phylogeny of a highly diversified family of bats. This combination allowed assembling a large and informative dataset, which can easily be extended at moderate cost for including a representative sample of phyllostomid species. Moreover, we demonstrated that mitogenomes assembled from NGS data can provide adequate phylogenetic signal for resolving intra-familial relationships in mammals. Finally, the use of mitogenomic sequences is not restricted to barcoding and phylogenetic purposes. Complete mitogenomes can also be used to investigate other challenging questions about their molecular evolution such as the determinants of evolutionary rate variations among taxa (Nabholz et al., 2008) and among genes (Nabholz et al., 2013).

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2013.07.003.

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