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Research Article

Comparative genomics reveals convergent adaptations in lipid metabolism and low genetic diversity in carnivorous bats

Yang Yue[1,2], Da-Hu Zou[1,3], Shi-Lin Tian[1,4], Heng-Wu Jiao[2], and Hua-Bin Zhao[1]*

[1]Key Laboratory of Biodiversity and Environment on the Qinghai-Tibetan Plateau of the Ministry of Education, College of Life Sciences, Wuhan University, Wuhan 430072, China
[2]School of Life Sciences, Central China Normal University, Wuhan 430079, China
[3]College of Biological & Pharmaceutical Sciences, China Three Gorges University, Yichang 443002, China
[4]Novogene Bioinformatics Institute, Beijing 100015, China
*Author for correspondence. E-mail: huabinzhao@whu.edu.cn
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Abstract Dietary specialization stands as a major factor in the study of adaptive evolution and the field of conservation biology among animals. Although bats show unparalleled dietary diversification among mammals, specialized carnivory remains relatively rare within this group. Consequently, our comprehension of the genetic and conservation aspects associated with this specific dietary niche in bats has largely remained uncharted. To investigate molecular adaptations and conservation genetics in carnivorous bats, we produced a new draft genome assembly for the carnivorous bat *Vampyrum spectrum*. Furthermore, we utilized this genome alongside another distantly related carnivorous bat *Megaderma lyra*, to conduct genome-wide comparative analyses with other bat species. Our findings unveil that genes linked to lipid metabolism exhibit signatures of positive selection and convergent molecular adaptation in the two divergent lineages of carnivorous bats. Intriguingly, we have uncovered that the evolution of dietary specialization in carnivorous bats is accompanied by molecular adaptations acting on genes in the peroxisome proliferator-activated receptors pathways, which are crucial in regulating plasma lipid metabolism and sustaining lipid homeostasis. Additionally, our genomic analyses also reveal low genetic diversity in both carnivorous bat species. This pattern is attributed to their continuously declining population sizes and low levels of heterozygosity, signaling their vulnerability and emphasizing the pressing need for conservation efforts. These genomic discoveries advance our understanding of genetic underpinnings of carnivory in bats and underscore substantial conservation concerns associated with carnivorous bat species.

Key words: bats, comparative genomics, conservation, diet, molecular adaptation.

1 Introduction

Food selection carries profound evolutionary and ecological implications for organisms. Diverse dietary strategies have given rise to a multitude of dietary niches, which in turn shape the ecological niche breadth of organisms (Wilson et al., 2008; Slatyer et al., 2013). In the face of resource fluctuations and competition, foraging animals encounter a trade-off: They must decide between specializing as narrow predators or broadening their diet to encompass less-preferred prey. Specifically, when resources are abundant, foragers are expected to have more specialized diets (Pianka, 1974), but when resources become scarce, consumers may broaden their diets to include inferior foods in order to meet energy requirements (Wiens, 1993). From the Cretaceous/Paleogene (K/Pg) boundary to the present day, mammals have evolved an exceptional array of dietary strategies (Kemp, 2004; Luo, 2007; Emerling et al., 2018). In recent decades, there has been extensive research into the morphological and molecular adaptations of dietary diversification in mammals. For instance, carnivorous rodents tend to possess relatively longer and narrower incisors, along with larger temporal fossae, whereas herbivores are characterized by broader incisor blades and thicker, wider zygomatic arches (Samuels, 2009). Moreover, prior studies have revealed that the sweet receptor gene is pseudogenized or functionally lost in some mammals with a narrow dietary niche (Zhao et al., 2010; Jiang et al., 2012; Jiao et al., 2021; Li et al., 2023), and the trehalase gene (*Treh*) is believed to have experienced widespread losses in non-insectivorous mammals (Jiao et al., 2019).

As the second-largest order of mammals, comprising over 1400 species, Chiroptera (bats) stands out as a group with

the most extensive dietary diversification (Gunnell & Simmons, 2005; Simmons et al., 2008). In contrast to their more ancestral insectivorous counterparts, modern bats exhibit independent origins of carnivory, frugivory, nectarivory, omnivory, and even sanguivory—phenomena unparalleled in other mammalian clades (Altringham, 1996; Neuweiler & Covey, 2000). Specialized diets, often indicative of unique and narrow dietary niches, can lead to various physiological, biochemical, and morphological adaptations. For instance, adaptive evolution related to taste, digestion, and metabolism has been observed in two distinct frugivorous bat lineages, possibly linked to their obligate frugivory (Wang et al., 2020). Similarly, the common vampire bat has experienced the loss of certain genes associated with physiology, metabolism, and immunity due to its extreme dietary narrowness (Zhao et al., 2010; Hong & Zhao, 2014; Blumer et al., 2022). While carnivorous bats have developed traits reminiscent of traditional carnivorous animals, including larger body sizes, distinctive cranial shapes, and heightened biting forces (Santana & Cheung, 2016), there has been limited exploration of genomic basis and molecular adaptations underlying their carnivorous attributes. In the past decade, only one study has documented the evolution of an avivorous bat species (*Ia io*), revealing a set of genes associated with predation, digestion, and metabolism, thereby adapting to its avivorous habits (Gong et al., 2022). Furthermore, examinations of the genomes of other carnivorous mammals (non-bat species) have revealed evidence of shared adaptive evolution in genes related to muscle strength, sensory nerves, insulin resistance, and cholesterol homeostasis (Cho et al., 2013; Kim et al., 2016; Zhu et al., 2018). As such, we hypothesize that carnivorous bats may also display distinctive genomic signatures and molecular adaptations. It is worth noting that genuine carnivorous behavior is a rare occurrence, observed in fewer than one percent of all bat species ($n = 9$) (Gual-Suárez & Medellín, 2021). Additionally, species with specialized diets often show heightened susceptibility to environmental changes (Colles et al., 2009). In recent years, the impact of human activities related to urbanization, exacerbated by the emergence of the coronavirus disease 2019 (COVID-19) and its consequences, has posed severe threats to bat populations on a global scale (Gili et al., 2020; Zhao, 2020). This precarious situation applies equally to carnivorous bats. Despite only two out of the nine (22%) carnivorous bat species are listed in the threatened categories (Critically Endangered, Endangered, and Vulnerable), six of them (67%) are experiencing decreasing population trends (IUCN, 2022). As a result, there is a critical requirement to assess both the population trend and genetic diversity of carnivorous bats.

To explore these questions, we initiated our study by sequencing and providing a novel genome assembly for the spectral bat (*Vampyrum spectrum*). In addition to this, we utilized high-quality genome data from previously published articles for another independently evolved carnivorous lineage, the greater false vampire bat (*Megaderma lyra*), as well as for other bats representing various dietary preferences (Eckalbar et al., 2016; Dong et al., 2017; Zepeda Mendoza et al., 2018; Jebb et al., 2020; Wang et al., 2020; Tian et al., 2023). Through conducting comparative genome analyses of our newly assembled genome sequence and the existing data, our primary goal was to unravel convergent molecular adaptations that underpin their carnivorous habits in these bats. Given that carnivorous bats primarily prey on small vertebrates (*V. spectrum*: 60% birds, 25% bats, 10% terrestrial mammals, amphibians, and reptiles; *M. lyra*: 10% birds, 15% bats, 35% terrestrial mammals, amphibians, and reptiles) (Gual-Suárez & Medellín, 2021), their diet tends to be richer in fats compared to other dietary bat species, which could potentially lead to elevated plasma lipid levels. Previous researches have indicated the pivotal role of the peroxisome proliferator-activated receptors (PPARs) pathway in regulating plasma lipid and maintaining lipid homeostasis (Marx et al., 2004; Pawlak et al., 2015; Bougarne et al., 2018). Therefore, we aim to investigate whether the evolution of dietary specialization in carnivorous bats is associated with molecular adaptations in lipid metabolism and the PPARs pathway. Furthermore, the new data affords us the opportunity to infer genome-wide diversity and undertake a comparative demographic analysis for bats with diverse diets, thereby providing valuable insights into bat conservation.

2 Material and Methods

2.1 Sample collection and sequencing

Liver tissue was sourced from an adult female of the spectral bat (*Vampyrum spectrum*) loaned from the American Museum of Natural History (Catalogue number: M-267446). Genomic DNA extraction was carried out on the liver tissue using Qiagen DNeasy kits, following the manufacturer's protocols.

Subsequently, a 10× Genomics library was constructed and sequenced employing the GemCode instrument. During the GEM reaction procedure in the polymerase chain reaction (PCR), approximately 1 ng of input DNA (with a molecule length of 50 kb) was utilized, and 16-bp barcodes were introduced into the droplets. The intermediate DNA library underwent purification before droplet fragmentation and was then sheared into 500-bp fragments for library construction. Following this, sequencing was conducted on the NovaSeq instrument, and the FASTQ file containing the barcoded reads was obtained using the supernova-2.1.1 software (Weisenfeld et al., 2017). A graph-based assembly was subsequently generated to produce a FASTA file suitable for subsequent processing and analysis. In total, 78.6 Gb of qualified data was generated.

To ensure high-quality data, we filtered out sequencing errors and low-quality reads using the fastp software (Chen et al., 2018) with the following criteria: (i) reads containing ≥10% uncalled nucleotides (N); (ii) reads with more than 10 nucleotides aligned to adaptors, with a maximum allowance of ≤10% mismatches; (iii) reads with over 50% of bases having a Phred quality score below 5; and (iv) duplicated reads resulting from PCR amplification.

2.2 Estimation of genome size

We calculated the genome size through k-mer spectrum analysis. To be specific, we counted the 17-mers information using Jellyfish v2.3.0 (Marçais & Kingsford, 2011). We then eliminated the low-frequency k-mers (≤3), which could

potentially be attributed to sequencing errors. Consequently, we derived an estimated genome size for the spectral bat (~2.22 Gb) by dividing the total count of k-mers by their coverage depth.

2.3 De novo assembly for the spectral bat genome

A de novo assembly was generated using the quality-filtered 10× Genomics linked reads, employing supernova-2.1.1 (Weisenfeld et al., 2017) with default parameters. Initially, the basal graph was constructed using the de Bruijn graph and DISCOVAR algorithm. When the overlap between two edges extended beyond 200 bp, super graphs were formed by connecting them. Subsequently, we united adjacent contigs into a single scaffold based on read pairs and barcode types. The genome assembly progressed in stages after adjusting for orientation and order. Eventually, any remaining gaps within the scaffolds were addressed by aligning the 10× Genomics reads, utilizing the Gapcloser package (Luo et al., 2012).

2.4 Evaluation of genome assembly

We employed the Benchmarking Universal Single-Copy Orthologs (BUSCO) to assess the gene content of the newly sequenced carnivorous bat genome (*V. spectrum*), utilizing the mammalia_odb9 database (Simão et al., 2015; Zdobnov et al., 2017). In addition, we conducted an assessment of the genomic content distribution using quast-4.6.3 (Gurevich et al., 2013) to evaluate the completeness of the new genome assembly.

2.5 Genome annotation

We integrated three methods, homology-based prediction, ab initio prediction, and transcriptome alignment, to perform annotation of gene structure. For homology-based prediction, protein sequences from the uniform bat reference protein set (Tian et al., 2023) and three sequences [human, horse, and mouse from the National Center for Biotechnology Information (NCBI) database] were used as queries to search against the newly assembled genomes using genBlastG (She et al., 2011) with default parameters. The candidate gene regions were aligned against the query protein database to find the best match using GeneWise (Birney et al., 2004), and then final gene models were predicted. In performing transcriptome alignment, the challenge of obtaining fresh samples of *V. spectrum* made acquiring its RNA-seq data unattainable. Therefore, we addressed this limitation by downloading RNA-seq data from six other bat species (*Myotis myotis*, *Molossus molossus*, *Cynopterus sphinx*, *Phyllostomus discolor*, *Rhinolophus ferrumequinum*, and *Rousettus aegyptiacus*) available on NCBI, which were assembled into transcripts using Trinity (Grabherr et al., 2011). Then the transcripts were aligned against the newly assembled genomes using TopHat (Kim et al., 2013), and the mapped reads were used to predict gene models (Cufflinks-set) using Cufflinks (Trapnell et al., 2012). For ab initio prediction, a high-quality data set for training was generated by Program to Assemble Spliced Alignment (PASA) (Haas et al., 2003), and Augustus (Stanke & Waack, 2003), GlimmerHMM (Majoros et al., 2004), and SNAP (Korf, 2004) were used to predict genes in the repeat-masked genomes. Additionally, GeneID (Guigó, 1998) and GeneScan (Burge & Karlin, 1997) were also used to predict gene models directly. After that, all gene models that were generated by the above methods were integrated using EVidenceModeler (Haas et al., 2008).

As for annotation of gene function, we used the publicly available databases, including Swiss-Prot (UniProt Consortium, 2018), NR database from NCBI, Pfam (Finn et al., 2016), Gene Ontology (GO), and Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa et al., 2014), to search for functional motifs, domains, and possible biological processes and pathways.

2.6 Identification of orthologous genes

In addition to the newly sequenced carnivorous bat in this study (*V. spectrum*), we also examined the genomes of 11 representative Chiroptera species. This diverse set included six yinpterochiropterans (*R. aegyptiacus*, *C. sphinx*, *Megaderma lyra*, *Hipposideros armiger*, *R. ferrumequinum*, and *Rhinolophus sinicus*), along with five yangochiropterans (*Desmodus rotundus*, *Artibeus jamaicensis*, *P. discolor*, *Pipistrellus kuhlii*, and *Miniopterus natalensis*) (Fig. 1). We additionally incorporated three outgroup species (human, mouse, and horse) in our analysis, as sourced from NCBI (Fig. 1). To ensure the robustness of our analysis, we retained the longest transcripts for each gene locus and removed genes with fewer than 50 amino acids. We conducted an all-against-all BLASTP analysis for the pooled protein sequences, following the method in a previous study (Camacho et al., 2009). We utilized the filtered alignments with an *e*-value of <1e−7 as input data for OrthoMCL v2.0.9, applying the default parameters (Li et al., 2003), to predict orthologous groups. Ultimately, we generated two separate data sets, one with the inclusion of the three outgroup species and one without them.

2.7 Phylogenetic tree construction and divergence time estimation

We compiled a data set that consisted of 12 bat species and three outgroup species, yielding a total of 5989 single-copy gene families for the purpose of constructing a phylogenetic tree. To create this tree, we initiated multiple sequence alignments for each gene family using PRANK (Löytynoja, 2014) with model parameters "+F -codon -termgap." Afterward, we identified conserved alignments by employing the Gblocks program (Castresana, 2000) to remove potentially ambiguous regions. Subsequently, we isolated fourfold degenerate sites (4DTV) from these conserved alignments, utilizing FasParser with default settings (Sun, 2018). All 4DTV sites were amalgamated into a comprehensive alignment matrix, which served as the basis for constructing a phylogenetic tree using the maximum-likelihood (ML) framework. We executed this procedure in RAxML v8.2.12 (Stamatakis, 2014) with 1000 rapid bootstraps. The best-fitting model of substitutions, "GTR + GAMMA," was determined using jModelTest2 (Darriba et al., 2012) for this phylogenetic tree construction.

Using the constructed phylogenetic tree and the concatenated alignments of 4DTV, we employed the MCMCTREE program within the PAML v4.9 package (Yang, 2007) to estimate the divergence times. For this calculation, we utilized the "Independent rates" option and the "GTR + G"

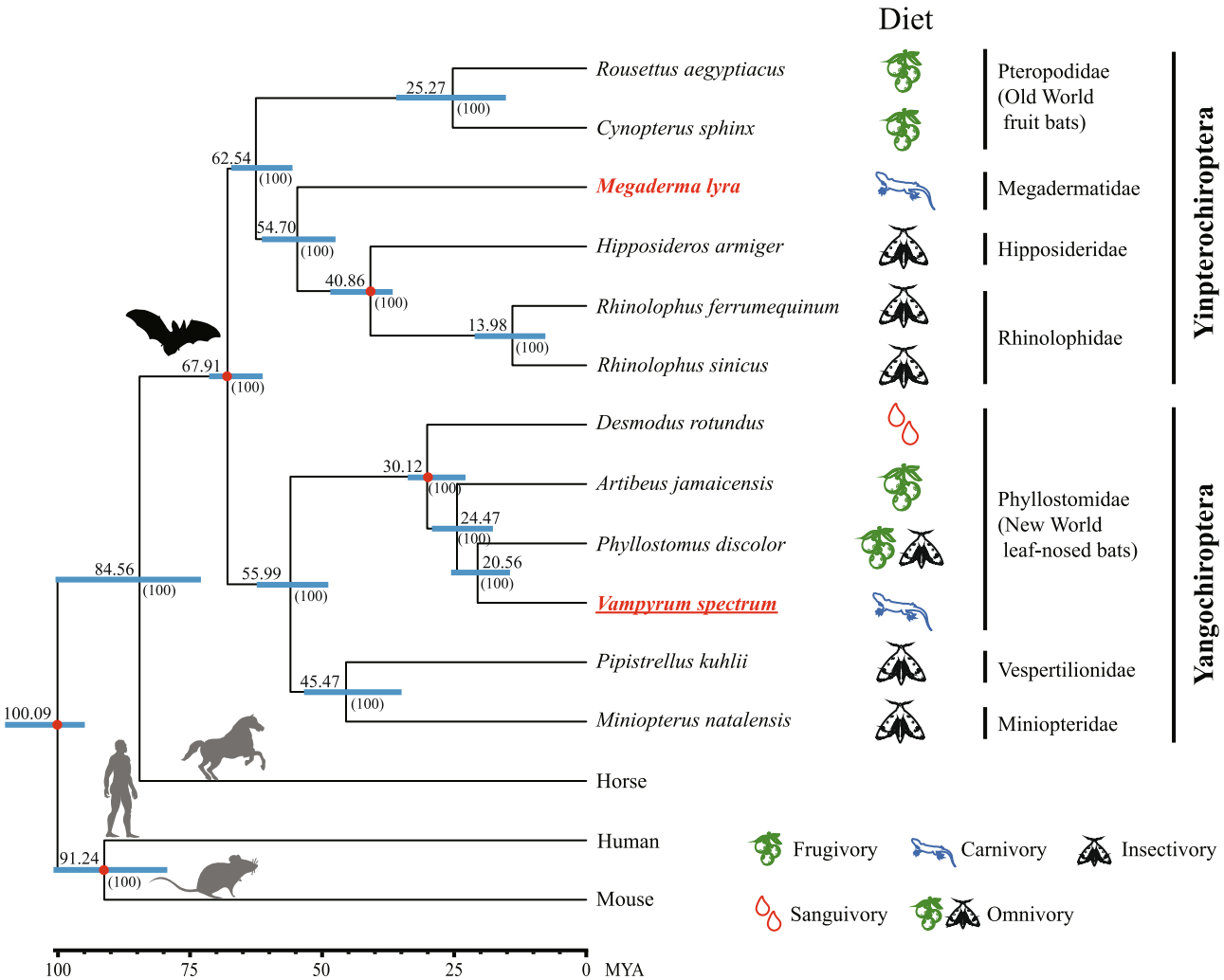

Fig. 1. Phylogenetic relationships of 12 bat species used in this study. Branch lengths of the phylogenetic tree are scaled to divergence times. Blue bars at the nodes indicate 95% credibility intervals of the posterior distributions of divergence times. Red circles at the nodes indicate the fossil calibrations used for setting upper and lower bounds of estimated divergence times. Values above branches represent divergence times, while those below in parentheses indicate maximum-likelihood bootstrap support. The two carnivorous bat species are highlighted in red, while the newly sequenced bat is additionally underlined. Diets of all bat species refer to Wilman et al. (2014) and Gual-Suárez & Medellín (2021).

model in MCMCTREE. The Markov chain Monte Carlo process was executed for 20 000 generations, following a burn-in period of 10 000 iterations. As in Wang et al. (2020), we employed the same fossil calibration points as constraints to establish the divergence times, expressed in units of millions of years (Ma).

2.8 Tests for selection

We extracted a set of single-copy orthologous genes from the OrthoMCL results, which encompassed only 12 bat species, totaling 5932 gene families, as explained earlier. These sequences underwent sequence alignments and filtering steps through PRANK (Löytynoja, 2014) and Gblocks (Castresana, 2000), respectively. To explore the potential genetic basis of carnivory in bats, we conducted selection tests considering two distinct data sets: (i) When designating the *M. lyra* lineage as the foreground branch, we excluded *V. spectrum* but retained all other species as background branches; (ii) When designating the *V. spectrum* lineage as the foreground branch, we omitted *M. lyra* but encompassed all other species as background branches. To identify positively selected genes (PSGs) and rapidly evolved genes (REGs), we employed the CODEML module within the PAML package (Yang, 2007). For PSGs, we used the branch-site model, comparing the alternative model (which includes a class of sites under positive selection on the foreground branch) with the null model containing neutral or purifying selection. For REGs, we applied the branch model, where the null model assumes that all branches evolve at the same rate, and the alternative model allows the foreground branch to evolve at a higher rate than other branches. Statistical significance was assessed through likelihood ratio tests and false discovery rate (FDR). With an FDR cutoff set at 0.05, we identified all potential PSGs and REGs. Further, we conducted

GO and KEGG enrichment analyses for both PSGs and REGs using the online tool Metascape (Zhou et al., 2019), with a significance threshold of $P < 0.05$.

2.9 Detection for molecular convergence in carnivorous bats

We employed PRANK (Löytynoja, 2014) to align the protein sequences of the single-copy orthologous gene set derived from 12 bat species, as described previously. We utilized three methods to identify signals of molecular convergence in the two carnivorous bats. (i) Zhang and Kumar′s method (Zhang & Kumar, 1997; Zou & Zhang, 2015): Reconstruction of ancestral protein sequences was conducted for 5932 single-copy orthologous genes among 12 bat species by using the Codeml program in PAML v4.9 (Yang, 2007). Convergent amino acid substitutions were defined as changes from different most recent ancestral amino acids to the same descendant amino acids in independent evolutionary lineages. In more detail, (i) the amino acid sites in both the extant *M. lyra* and *V. spectrum* lineages were identical; (ii) the amino acid sites between the extant *M. lyra* lineage and its most recent common ancestor with the ancestral node of Hipposideridae and Rhinolophidae were different; and (iii) the amino acid sites between the extant *V. spectrum* lineage and its most recent common ancestor with the *P. discolor* lineage were different. To account for noise due to chance amino acid substitutions, we compared the observed number of convergent sites for each gene with the expected number caused by random substitution under the JTT-f_{gene} amino acid substitution model. A Poisson test was conducted to assess the significance, with P-values adjusted by FDR, retaining only convergent genes with q-value < 0.1. (ii) PCOC method (Rey et al., 2018): This method took a more relaxed approach compared to the first method and focused on convergent shifts in amino acid preferences rather than convergent substitutions of the exact same amino acid. Biochemical properties of amino acids were modeled as "profiles" for each position and each branch using a vector of amino acid frequencies. PCOC relied on two models: the Profiles Change (PC) model and the OneChange (OC) model. In the PC model, a convergent site in all convergent branches preferred a specific profile different from the profile in the ancestral branch, while a nonconvergent site maintained the same profile in all branches. In the OC model, a convergent site had to display at least one substitution in each convergent branch. The PCOC convergence for each gene was detected by comparing the fit of these two models. (iii) RERconverge, a R package that considers the association between relative evolutionary rates (RER) of genetic loci and the evolution of convergent phenotypic traits over a phylogeny (Kowalczyk et al., 2019): After correcting for nonspecific factors affecting divergence on the branch, the RER of evolution on branches were calculated. These RER values were then used to estimate the correlation over the binary variable of the "carnivore" branches. Genes with a P value < 0.05 were inferred to have undergone convergence. The functional enrichment of these convergent genes in GO terms and KEGG pathways was assessed using the online tool Metascape (Zhou et al., 2019), with a significance threshold of $P < 0.05$.

2.10 Conserved noncoding elements (CNEs) analysis

To identify CNEs that have undergone convergence between the two carnivorous bats, we initiated the process by constructing a whole genome alignment (WGA) for the 12 bat genomes, with repeat masking, using LASTZ (Harris, 2007). This alignment was performed with *P. kuhlii* (mPipKuh1.p) as the reference genome. Subsequently, we employed the roast program in Multiz (Blanchette et al., 2004) to generate multiple alignments, aligning the species according to the tree topology from our phylogenetic analysis. To establish a nonconserved model, we utilized four-fold degenerate sites extracted from our whole-genome alignment. PhyloFit (Siepel & Haussler, 2004) was employed to estimate the value of rho, which serves as a scaling factor for the nonconserved model. We obtained an average of rho values for scaling the nonconserved model using PhyloBoot (Hubisz et al., 2011). PhastCons (Siepel et al., 2005) was then applied, utilizing both the conserved and non-conserved models, to predict evolutionarily conserved elements within the multiple alignments. To ensure accuracy, we meticulously filtered the conserved elements, particularly those that might overlap with coding regions, using the "subtract" command in the Bedtools program (https://bedtools.readthedocs.io/en/latest/index.html, last accessed May 26, 2022). As a result, we identified a total of 164 216 CNEs, each exceeding 30 base pairs in length. Our primary focus was on CNEs ($n = 119\,514$) situated within introns and the 50-kb regions both upstream and downstream of genes. For the detection of convergent evolutionary rate shifts in CNEs, we utilized the RERconverge method, as explained earlier. Trees with branch lengths for each CNE were generated by "baseml" within the PAML software suite.

2.11 Evolution of the PPAR pathways

Carnivorous bats typically have a higher dietary lipid intake compared to other bat species. In order to maintain the proper functioning of their organisms, they may exhibit differences in lipid metabolism when compared to other bats. Given that PPARs play a central role as lipid sensors, governing whole-body energy metabolism, the pathways associated with these receptors provide valuable insights into the adaptive evolution of lipid metabolism in carnivorous bats. To explore this, we specifically selected 168 genes from the pathways (R-HSA-400206; map03320) related to PPARs, sourcing the data from both the Reactome and KEGG databases. Subsequently, we retrieved gene families using the reciprocal best hit (rbh) approach (Wall et al., 2003). Using the methods described earlier, we then identified potential PSGs and convergent genes in the two carnivorous bats.

2.12 Demographic reconstruction and heterozygosity estimation

We began this section of analysis by collecting the raw, clean reads from the 12 bat species and mapped them onto the contigs of the genome assembly. Subsequently, we employed LASTZ (Harris, 2007) to align the newly sequenced genome with a reference genome, aiming to identify contigs and scaffolds belonging to the sex chromosomes. We filtered out these fragments located on the sex chromosomes and generated consensus genome sequences for all autosomes

within the filtered bam files for each of the 12 bat species, employing SAMtools (Li et al., 2009) and the script "vcfutils.pl." The PSMC model (Li & Durbin, 2011) was then utilized to infer the demographic history of each bat species. We configured the model with the following parameters: -N 30 -t 15 -r 5 -p 4 + 25*2 + 4 + 6. We conducted 100 bootstrap replicates to ensure robustness (Fig. S1). The final results were scaled using a previously established mammalian mutation rate of 2.2×10^{-9} substitutions per site per generation and an estimate of a 1-year generation time (Chattopadhyay et al., 2019).

Furthermore, to estimate genome-wide heterozygosity for each bat species, we divided the number of heterozygous sites by the effective genome size. Initially, clean reads were aligned to the reference genome using BWA software (Li, 2013) with default parameters. Subsequently, we employed the "rmdup" command in SAMtools (Li et al., 2009) to preprocess alignment reads and eliminate PCR duplicates. Raw mapping results were filtered using the "mpileup" command in SAMtools, with the -Q 30 option, and the mapped reads were then used for SNP calling via the "bcftools call" command.

3 Results

3.1 Genome sequencing, assembly, and characterization

The spectral bat (*Vampyrum spectrum*) was subjected to sequencing on the Illumina HiSeq platform, complemented by 10× Genomics (Table S1). Subsequently, we successfully generated a 2.26 Gb genome for V. spectrum, featuring a contig N50 of 112.3-kb and a scaffold N50 of 1.1-Mb (Tables S2–S5). Notably, this genome size aligns closely with that of other bat species with previously sequenced genomes (Zhang et al., 2013; Jebb et al., 2020). We employed BUSCO assessment (Simão et al., 2015), utilizing a mammal-specific gene set (Mammalia odb9), to evaluate the assembly ′s completeness. This analysis revealed the presence of 89.1% complete sequences among the 4104 conserved mammalian genes, underscoring the high level of completeness achieved in the assembly (Table S2).

3.2 Phylogenomics and genome-wide signatures of adaptive evolution

In order to elucidate the phylogenetic relationships among various bat lineages characterized by diverse diets, we generated a genome-wide phylogenetic tree employing the maximum-likelihood (ML) method. This tree was constructed based on 5,989 single-copy orthologous groups derived from the genomes of 12 bat species (Yinpterochiroptera: *Rousettus aegyptiacus*, *Cynopterus sphinx*, *Megaderma lyra*, *Hipposideros armiger*, *Rhinolophus ferrumequinum*, *Rhinolophus sinicus*; Yangochiroptera: *Desmodus rotundus*, *Artibeus jamaicensis*, *Phyllostomus discolor*, *V. spectrum*, *Pipistrellus kuhlii*, *Miniopterus natalensis*) and three outgroup species (horse, human and mouse) (Fig. 1; Table S6). All nodes on our phylogenetic tree have received 100% ML bootstrap support (Fig. 1). Our estimates for divergence times indicated that Yinpterochiroptera and Yangochiroptera diverged ~67.9 Ma, which is slightly earlier than previous studies (Teeling et al., 2005; Hao et al., 2023). Furthermore, our dating analysis also determined the divergence times for the separate origins of the two carnivorous bats, *M. lyra* and *V. spectrum*, yielding estimates of approximately 54.7 and 20.6 Ma, respectively (Fig. 1).

The initial step in understanding potential molecular adaptations for a meat-specialized diet involved the identification of all putative PSGs and REGs in the two divergent species of carnivorous bats. While conducting branch and branch-site model tests on a specific carnivorous bat as the focal branch, the other species were excluded from the background set of taxa. In the case of *M. lyra* as the foreground, we identified 60 PSGs and 72 REGs. When considering *V. spectrum* as the foreground, we obtained 218 PSGs and 124 REGs. According to the results of functional enrichment analyses, several pathways relevant to carnivory were observed, including liver development (GO:0001889) and glycerol metabolic process (GO:0006071; Tables S7, S8).

Subsequently, we undertook a comparison of protein sequences to identify convergent amino acid substitutions shared between the two species of carnivorous bats. We sought to determine if the observed number of convergent sites could be attributed to random substitutions and were able to identify 12 convergent genes using the JTT-f_{gene} model (Zhang & Kumar, 1997; Zou & Zhang, 2015). Functional enrichment analysis of these genes revealed significant terms related to the regulation of innate immune response (GO:0045088) and the lipid biosynthetic process (GO:0008610), which might be attributed to their carnivorous diet (Tables S9, S10). This observation aligns with the enrichment results of PSGs and REGs. Importantly, we found that the two key convergent genes, *ARV1* and *MED1*, encoded identical substitutions in the two species of carnivorous bats (F176L in ARV1; A264T in MED1; Fig. 2). The convergent substitution in ARV1 is located within the transmembrane domain, while the convergent substitution in MED1 lies within the functional region that interacts with the mediator complex (Tong et al., 2010; Zhou et al., 2021). This suggests that these two convergent substitutions likely modify protein binding capabilities, enabling them to play pivotal roles in cholesterol transport and lipid metabolism. In a comparable manner, we observed that 251 convergent genes identified through the PCOC method (Rey et al., 2018) were also enriched in categories related to lipid metabolism (steroid metabolic process, GO:0008202; Tables S11, S12). Furthermore, utilizing the RERconverge method (Kowalczyk et al., 2019), which focuses on RER rather than amino acid substitutions or preferences, we uncovered evidence of convergent evolution in the two carnivorous bats for two key genes involved in the formation and metabolism of lipoproteins: *APOC2* and *APOE* (Table S13). In addition to investigating protein-coding genes, we also predicted a comprehensive data set of 164 216 CNEs by using genomes of the 12 bat species. Notably, one CNE located in intron 2 of *ABCA1* and another situated in the untranslated region of *APOC3* displayed more pronounced convergent acceleration in both carnivorous bats according to the RERconverge method (Fig. 3A). Furthermore, the genes regulated by these two convergent CNEs are involved in the same pathway associated with lipoprotein and lipid metabolism along with the key convergent genes identified from coding regions (Fig. 3B).

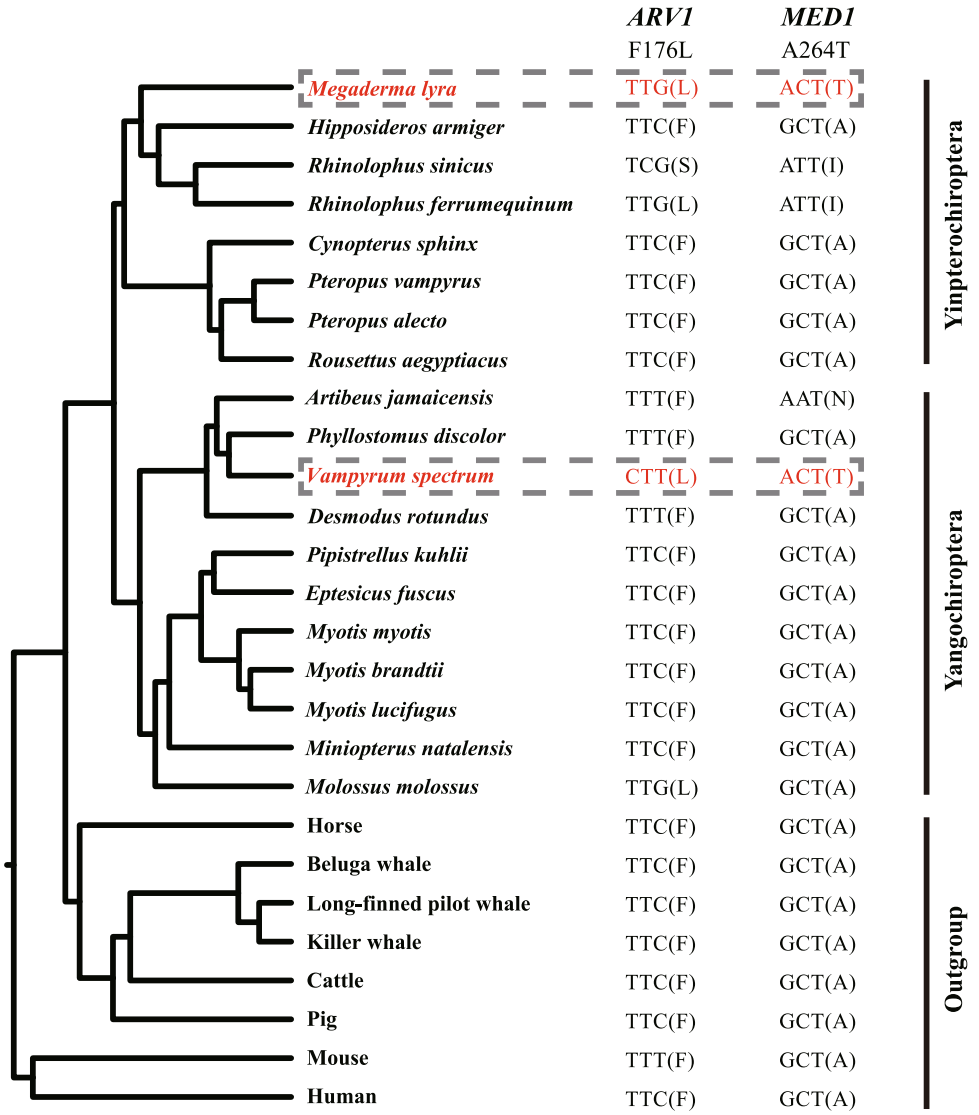

Fig. 2. Convergent amino acid substitutions of ARV1 and MED1 in the two carnivorous bats (*Megaderma lyra* and *Vampyrum spectrum*). Both proteins contain a single parallel amino acid change (shown in red) in two carnivorous bats compared with other species (ARV1: F176L; MED1: A264T).

3.3 Adaptation in the PPAR pathways linked to lipid metabolism

Given the intimate association of the PPAR pathways with lipid metabolism, our aim was to explore deeper into understanding how carnivorous bats have adaptively evolved their carnivorous diets with regard to lipid homeostasis. We focused on 168 genes within the PPAR-related pathways, encompassing the regulation of lipid metabolism by PPARα (Reactome pathway: R-HSA-400206) and the PPAR signaling pathway (KEGG pathway: map03320). Among the 12 bats illustrated in Fig. 1, we identified a total of 156 orthologous genes. Subsequently, we uncovered that 10 genes were subject to positive selection, and 30 genes exhibited convergence in the two carnivorous bats. Notably, four genes (*ABCA1*, *MED1*, *SREBF2*, and *SORBS1*) were simultaneously under positive selection and convergent evolution (Fig. 4). *ABCA1* encodes a membrane-associated protein that belongs to the superfamily of ATP-binding cassette (ABC) transporters. This protein plays a pivotal role not only in the formation of nascent high-density lipoprotein but also in the regulation of intracellular cholesterol efflux after activation of PPARs (Chinetti et al., 2001). *MED1* and *SREBF2*, serving as the coactivator and transcription factor recruited and bound by the PPARα/RXR heterodimer, exert influence over the expression of multiple target genes involved in lipid metabolism and adipocyte differentiation by controlling transcriptional activity (König et al., 2007; Viswakarma et al., 2010). *SORBS1*, on the other hand, emerges as a target gene bound by PPARs upon activation, primarily regulating adipocyte differentiation and insulin signaling (Sasaki et al., 2006). Together, these findings appear to provide more detailed evidence of the adaptive evolution of lipid metabolism in carnivorous bats when compared to our genome-wide analysis.

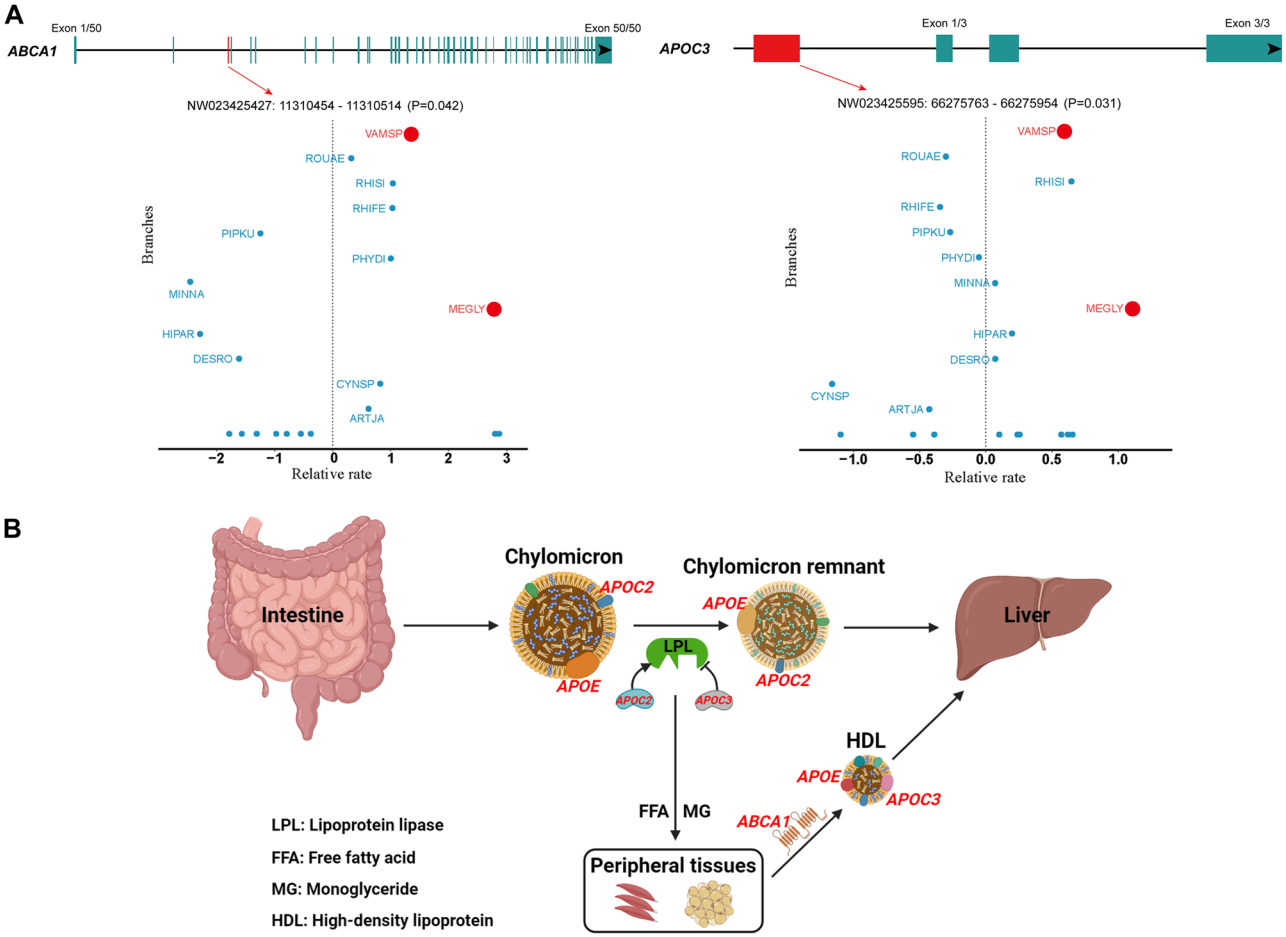

Fig. 3. Convergently accelerated conserved noncoding elements (CNEs) and convergent genes in two carnivorous bats. **A**, Both *ABCA1* and *APOC3* exhibit a single convergent CNE displaying an elevated rate of acceleration in the two carnivorous bats. The information regarding CNEs, including the scaffold, start, and end positions, is incorporated into their respective names. The *P*-values reflect the results of the Wilcoxon-rank sum test for rate acceleration on carnivore branches when compared to other bat branches. ARTJA, *Artibeus jamaicensis*; CYNSP, *Cynopterus sphinx*; DESRO, *Desmodus rotundus*; HIPAR, *Hipposideros armiger*; MINNA, *Miniopterus natalensis*; PHYDI, *Phyllostomus discolor*; PIPKU, *Pipistrellus kuhlii*; MEGLY, *Megaderma lyra*; RHIFE, *Rhinolophus ferrumequinum*; RHISI, *Rhinolophus sinicus*; ROUAE, *Rousettus aegyptiacus*; VAMSP, *Vampyrum spectrum*. **B**, Four convergent genes (*APOC2*, *APOE*, *APOC3*, and *ABCA1*) are involved in the formation and metabolism of lipoproteins, which play important roles in maintaining plasma cholesterol homeostasis; Images were created with BioRender.com.

3.4 Demographic history and genetic diversity of carnivorous bats

To uncover the historical fluctuations in effective population size (*Ne*) for the 12 bats with distinct dietary preferences, we employed the pairwise sequentially Markovian coalescent (PSMC) model (Li & Durbin, 2011). For *M. lyra* and *V. spectrum*, we identified two periods of population declines, with the former experiencing a more substantial decline than the latter (Fig. 5A). The first major decline was estimated to have occurred between 1.6×10^6 years ago and 5×10^5 years ago, followed by a gradual decline before the onset of the Last Glacial Maximum, approximately 21 000 years ago (Fig. 5A). It is worth noting that both carnivorous bats exhibited a similar trend of declining *Ne*, characterized by an overall decrease from the early Pleistocene (PI) to the present day, remaining consistently at the lowest level among all bats examined in this study (Fig. 5A). In contrast, the *Ne* for the common vampire bat (*D. rotundus*) displayed the highest peak among all 12 bats, around 65 000 years ago, followed by a severe bottleneck at 40 000 years ago (Fig. 5A).

We also calculated genome-wide heterozygosity for each carnivorous bat by aligning their short reads back to their respective reference genomes. We then compared these heterozygosity values to those of other bats with varying dietary habits. The carnivorous bats exhibited an average genomic heterozygosity of 0.0018 (*M. lyra*: 0.0017; *V. spectrum*: 0.0019), which was notably lower in genetic diversity compared to the other 10 bats we examined, except for *M. natalensis* (Fig. 5B). In contrast, the frugivorous bat *C. sphinx* displayed the highest heterozygosity at 0.0084, followed by *P. discolor*, *A. jamaicensis*, *D. rotundus*, *P. kuhlii*, *R. sinicus*, *H. armiger*, *R. aegyptiacus*, and *R. ferrumequinum*, with heterozygosity values ranging from 0.0022 to 0.0071 (Fig. 5B).

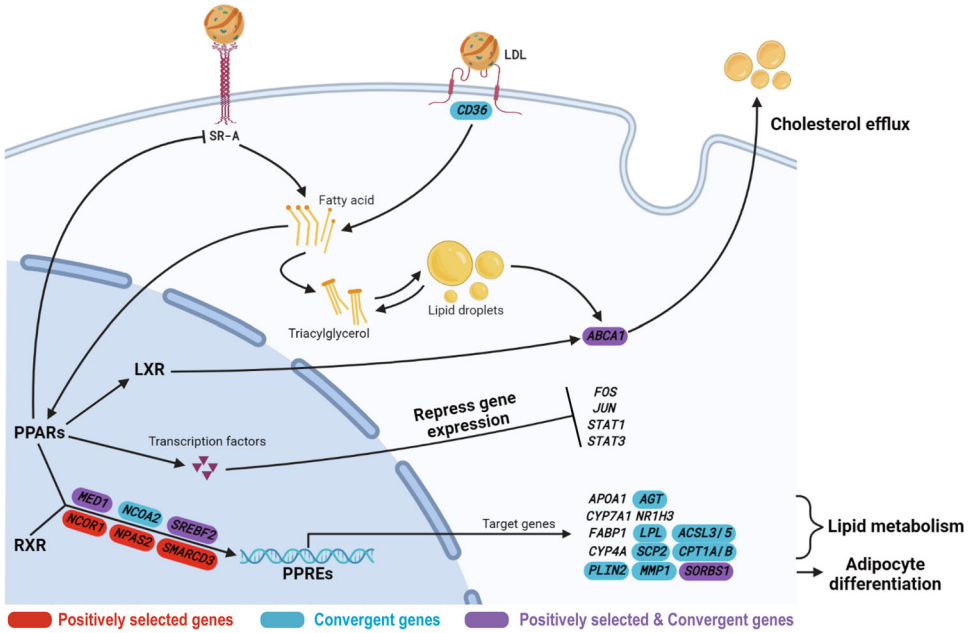

Fig. 4. Diagram of PPAR (Peroxisome proliferator-activated receptors) signaling pathways in the carnivorous bats. Genes in red are under positive selection, genes in blue are under convergent evolution, and genes in purple are under both positive selection and convergent evolution. LDL, low-density lipoprotein; LXR, liver X receptor; PPREs, PPAR response elements; RXR, retinoid X receptor; SR-A, scavenger receptor class A. Images were created with BioRender.com.

4 Discussion

Recent advancements in whole genome sequencing have opened up unprecedented avenues for investigating the genetic underpinnings of dietary diversification and specialization. Within the scope of this research, we have generated a new genome assembly for the spectral bat (*Vampyrum spectrum*), a carnivorous species. Through our comparative evolutionary analyses, we have unveiled substantial genetic changes in genes associated with adaptations to carnivorous diets. Building upon the model of independently evolved carnivores from two divergent families, our findings not only shed light on molecular adaptations responsible for lipid metabolism in carnivorous species but also establish a crucial genomic resource for the conservation of carnivorous bats.

Our phylogenomic tree included 12 bat species, each with diverse dietary preferences including frugivory, carnivory, insectivory, sanguivory, and omnivory, effectively covering nearly all known bat dietary niches (Fig. 1). Of particular note are the two carnivorous bats (*Megaderma lyra* and *V. spectrum*) found in two phylogenetically distant families, Megadermatidae and Phyllostomidae (New World leaf-nosed bats), demonstrating their independent evolution of carnivorous diet. Our genome-wide selective pressure analyses provided insights into PSGs and REGs associated with the absorption and metabolism of fat, protein catabolic processes, and liver development. These molecular adaptations appear to be responses to the carnivorous bats' specialization in a fat-rich diet. Among these genes, *DGAT1* (Diacylglycerol *O*-acyltransferase 1) emerges as a key player, uniquely responsible for facilitating the absorption of dietary fats and synthesizing fat for storage to safeguard organisms from the detrimental effects of high-fat diets, as previously demonstrated (Orland et al., 2005; Cheng et al., 2008; Chitraju et al., 2019). *APOB* (Apolipoprotein B), a crucial component of various lipoproteins, is well-known for its influence on the regulation of plasma cholesterol and lipid metabolism (Olofsson & Borèn, 2005; Behbodikhah et al., 2021). Besides, previous studies have already suggested that carnivorous bats tend to exhibit larger body sizes and stronger bite forces than their counterparts with alternative dietary preferences, as observed through morphological comparisons (Santana & Cheung, 2016). Considering the substantial differences between insects and vertebrates in terms of strength, size, and athletic ability, predation on vertebrates represents a novel challenge for bats when compared to their more ancestral insectivorous habits.

Similar selective pressures can drive distantly related species to undergo convergent phenotypic evolution. Convergence is often observed from various molecular perspectives, such as amino acid substitutions, shifts in amino acid preferences, and changes in the RER of genes (Hao et al., 2019). In our study, we identified convergent amino acid substitutions in two crucial genes, *ARV1* and *MED1*, within the two carnivorous lineages. *ARV1* encodes a fundamental component involved in the transport of sterols from the endoplasmic reticulum and plays a crucial role in regulating hepatic cholesterol and bile acid homeostasis. Meanwhile, *MED1* plays a pivotal role in promoting hepatic autophagy and regulating lipid metabolic homeostasis (Tong et al., 2010; Zhou et al., 2021). The presence of single convergent amino acid substitutions in these two genes suggests that this edge in lipid metabolism may be particularly advantageous during the evolution toward a carnivorous diet. Due to variations in the genetic background and evolutionary history of dietary changes across different orders, the genetic basis determining

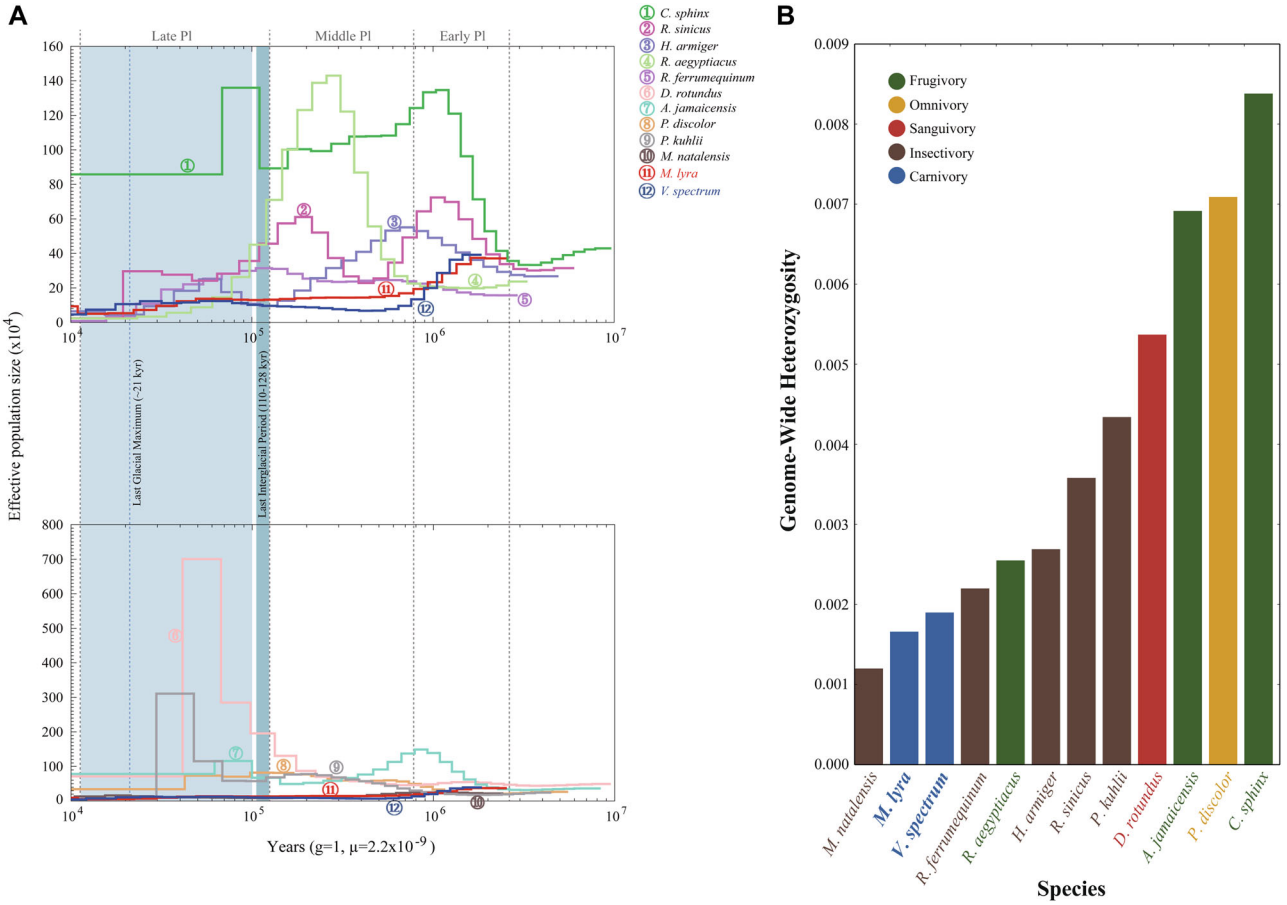

Fig. 5. Population demographic history and genetic diversity in bats. **A**, Depiction of effective population size fluctuations in the two carnivorous bats compared to five other bats (above) and an additional five bats (below), with scaling applied using a mutation rate of 2.2×10^{-9} substitutions per site per generation and a generation time of 1 year. The Pleistocene (Pl) is divided into Early, Middle, and Late subepochs. The alternating blue shadings denote the periods of the last interglaciation and the last glaciation. **B**, A comparison of genome-wide heterozygosity across the 12 bat species under investigation.

carnivorous traits in cetaceans may involve other sites in these two genes and possibly even other genes (Fig. 2). Furthermore, we also detected convergent signatures of accelerated rates in genes associated with lipid metabolism. Specifically, *APOE* contributes to cholesterol transport from peripheral tissues to the liver through plasma and interstitial fluids, while *APOC2* encodes the protein activated by lipoprotein lipase, which is responsible for hydrolyzing plasma triglycerides and plays a critical role in the metabolism of triglyceride-rich lipoproteins. Our findings underscore the importance of noncoding and regulatory elements in driving these convergent traits. In fact, CNEs, as regulatory regions, are more likely to underlie convergent changes than protein-coding sequences, especially when shared pathways are involved (Sackton et al., 2019). Our study identified two convergently accelerated CNEs located at *ABCA1* and *APOC3*. These two genes are involved in the same pathway as the key convergent genes identified from coding regions, highlighting the significant role of lipoproteins and lipid metabolism in the convergent adaptation to a carnivorous diet in bats (Fig. 3B).

Previous studies have demonstrated that PPARs not only serve as major regulators in modulating lipid and plasma lipoprotein metabolism but also influence the cardiovascular system by exerting anti-inflammatory activities (Chinetti et al., 2000; Pawlak et al., 2015; Bougarne et al., 2018). As carnivores, *M. lyra* and *V. spectrum* must consume a diet richer in dietary fat compared to other bats. To prevent cardiovascular diseases stemming from excessive fat intake, we have strong reasons to believe that PPAR transcription factors and their target genes may have undergone adaptive evolution in these two bats. Through our analysis of molecular evolution of a gene set related to PPAR signaling pathways, we have indeed identified numerous genes displaying genetic signatures of positive selection and convergence. For instance, the gene *CD36*, responsible for encoding the scavenger receptor, which is implicated in binding to oxidized low-density lipoprotein (LDL) and regulating lipid uptake, shows distinct convergent features. Furthermore, *ABCA1*, a gene activated by PPAR ligands and collaborating with the liver X receptor (LXR) to facilitate cholesterol efflux, provides compelling evidence of both positive selection and convergent evolution. This ABCA1-mediated reverse-cholesterol-transport pathway, in combination with PPARs' trans-repression activity, likely forms the

foundational mechanism underpinning the anti-inflammatory properties of PPARs. This mechanism contributes significantly to the reduction of vascular atherosclerosis and, consequently, mitigating cardiovascular risk (Chawla et al., 2001; Marx et al., 2004; Montaigne et al., 2021). Furthermore, we have also identified several PSGs and convergent genes that play roles in the binding and initiation of transcription of target genes responsible for regulating lipid metabolism, adipocyte differentiation, and cholesterol homeostasis within the PPARs signaling pathway. These findings potentially constitute a genetic basis for the adaptive evolution of lipid metabolism in carnivorous bats. However, further experimentation is needed to validate the potential mechanisms underlying the selection of these genes within the PPAR signaling pathways of carnivorous bats.

Each individual genome typically contains a record of a species' response to climatic fluctuations (Li & Durbin, 2011; Nadachowska-Brzyska et al., 2015; Mays et al., 2018). The Quaternary, the most recent period of the Cenozoic era, marked by alternating glacial and interglacial cycles, has played a significant role in driving biotic diversification and speciation across diverse biomes and taxonomic groups (Hewitt, 2000). Our study reveals historical population trends across the 12 bat species. Two carnivorous species in particular exhibited a consistent, slow decline in population size, remaining at notably low levels. This pattern aligns with the general trend observed among species with higher trophic levels, characterized by smaller population sizes (Fig. 5A). Their relatively stable PSMC curves (Fig. 5A) further suggest that these populations face challenges in recovering, even when environmental conditions become favorable again after prolonged declines resulting from climatic and environmental changes. Notably, most bats exhibited reduced effective population sizes (*Ne*) as they entered the Holocene, suggesting that this period might not have been characterized by population genetic stability for many bat species. Furthermore, our demographic reconstruction focused on a time period exceeding 10 000 years ago, as PSMC inferences from single genomes have limited power to detect recent demographic changes (Li & Durbin, 2011). In addition to *Ne*, a species' genetic diversity is a vital indicator that defines its evolutionary potential and long-term survival (Reed & Frankham, 2003). The carnivorous bats, in comparison to bats with other dietary preferences, exhibited relatively lower levels of genetic diversity, which corresponds with the PSMC results. Only one species, *M. natalensis*, a small-sized insectivore widely distributed in southern and East Africa, displayed exceptionally low genome-wide heterozygosity (Fig. 5B). This low genetic diversity may stem from a bottleneck in its isolated peripheral population. Overall, the genetic diversity observed in most bat species within our study surpasses that of carnivorous bats and is comparable to the levels found in many bird species (Li et al., 2014; Kim et al., 2016). While this comparison is based on estimates from a limited number of individuals and may not provide a comprehensive view of genetic diversity, it does offer valuable insights for supporting bat conservation efforts. Bats are currently facing unprecedented threats due to widespread habitat loss and fragmentation caused by human activities and urbanization, as well as negative associations with diseases such as coronavirus disease 2019 (COVID-19) (Russo & Ancillotto, 2015; Zhao, 2020; Zhou et al., 2020). It is essential for conservation management to prioritize bats and minimize habitat destruction to ensure the enduring survival of these remarkable mammals, especially the carnivorous bats. Future analyses should encompass genomic data from a broader range of bat species and individuals to obtain more accurate evolutionary trajectories across a wider taxonomic and temporal scale, ultimately reducing the risk of their extinction. The preservation of these extraordinary mammals warrants our unwavering commitment and attention.

5 Conclusions

To sum up, this study has produced the draft genome assembly of the spectral bat (*Vampyrum spectrum*), a representative of one of the most strictly carnivorous bat lineages in the neotropical region. Our extensive evolutionary and comparative genome analyses have illuminated critical signatures of selection within genes specific to carnivorous bats, particularly those linked to high-fat diets. It is worth noting that we have observed convergent evolution in lipid metabolism across phylogenetically distant carnivorous bat lineages, underscoring the pivotal role of lipid metabolism in their evolutionary adaptation. Furthermore, it is possible that more intricate signals of adaptive evolution in carnivorous bats have arisen by acting on candidate genes associated with lipid metabolism in the PPAR signaling pathways. These findings not only demonstrate that carnivorous bats exploit a distinctive dietary niche when confronted with ecological dietary opportunities, but they also provide valuable insights into the molecular adaptations necessary for their obligate carnivorous lifestyle. Additionally, our study highlights that both carnivorous bat species have relatively small population sizes and limited genetic diversity. This finding underscores the need for a systematic framework to develop innovative conservation strategies, monitor the populations and habitats of the most vulnerable bat species, and implement clear and achievable conservation actions.

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Declaration of Competing Interest

The authors declare that they have no competing financial interests or personal relationships that could influence the work reported in this paper.

Data Availability Statement

All genome sequencing raw reads have been deposited into the NCBI Sequence Read Archive and GenBank under the BioProject accession number PRJNA1025361.

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Supplementary Material

The following supplementary material is available online for this article at http://onlinelibrary.wiley.com/doi/10.1111/jse.13060/suppinfo:

Table S1. Data production.

Table S2. Statistics of the *Vampyrum spectrum* genome assembly sequenced in this study.

Table S3. Distribution of genomic contents.

Table S4. Statistics of gene structure of the *Vampyrum spectrum* genome.

Table S5. Summary of the repetitive sequences in *Vampyrum spectrum* genome.

Table S6. Information of bat genomes used in this study.

Table S7. Functional enrichment analysis of 218 positively selected genes in *Vampyrum spectrum*. The corrected *P*-value cutoff was set to 0.05.

Table S8. Functional enrichment analysis of 124 rapidly evolved genes in *Vampyrum spectrum*. The corrected *P*-value cutoff was set to 0.05.

Table S9. Convergent genes between the two carnivorous bats under the JTT-F_{gene} model.

Table S10. Functional enrichment analysis of 12 convergent genes in the two carnivorous bats under the JTT-F_{gene} model. The corrected *P*-value cutoff was set to 0.05.

Table S11. Convergent genes between the two carnivorous bats under the PCOC method.

Table S12. Functional enrichment analysis of 251 convergent genes in the two carnivorous bats detected by the PCOC method. The corrected *P*-value cutoff was set to 0.05.

Table S13. Convergent genes between the two carnivorous bats under the RERconverge method.

Fig. S1. Demographic history of the two carnivorous bats (*Megaderma lyra*; *Vampyrum spectrum*) with 100 rounds of bootstrapping.