Comparative transcriptomics reveals the evidence of genetic adaptations in the macrotis group (Chiroptera: Rhinolophidae)

Lin Zhang[1], Keping Sun[1], Wentao Dai[1], Liu Tong[2], Aoqiang Li[3], and Jiang Feng[1]

[1]Jilin Provincial Key Laboratory of Animal Resource Conservation and Utilization
[2]Jilin Agricultural University
[3]Central China Normal University

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Abstract

Understanding the adaptive evolution of species has long interested evolutionary biologists. Adaptive phenotypes can result from changes in protein-coding sequences that affect protein structure and function. The Rhinolophus macrotis group as a specific group has low echolocation frequency relative to body size compared with other rhinolophids, suggesting a special evolutionary process of this group. Transcription bridges genetic information and phenotypes. Here, we sequenced transcriptomes of the brain, liver, and cochlea for five species of the macrotis group and its closely related species, R. pusillus, to explore the molecular basis of the adaptation in the macrotis group at the sequence level. Strong and significant positive selection signals for species within the macrotis group was detected in seven genes (CRYM, FOXM1, MAP6, PYCARD, SLC35A2, WRB and SPRY2) linked to hearing. Unexpectedly, we also detected five PSGs (ARRDC3, LZTFL1, RAB8A, IGFBPL1 and TRNT1) linked to vision in species with relatively low frequencies. These results suggested that natural selection has led to the positive selection of some sensory-related genes. Furthermore, PSGs identified in the macrotis group significantly enriched in GO categories related to metabolism (e.g. catalytic activity and oxidoreductase activity), which provided evidence to parse the genetic adaptations of the species with low frequencies within the macrotis group. This is the first attempt to detect genome-wide sequence evolution across the macrotis group and our study provided valuable resources for studying the genetic mechanisms of rhinolophids adaptation.

1. Introduction

Ever since the book "On the Origin of Species by Means of Natural Selection" was published by Charles Darwin 160 years ago (Darwin, 1859), adaptive evolution has become an important research question (Tian et al., 2017). Understanding the driving forces of speciation processes is a primary goal of evolutionary biology (Feldmeyer et al., 2015; Marie Curie et al., 2012). And inferring the patterns and processes that accompany the generation of phenotypic diversity and new species is a primary goal of evolutionary biology (Mason and Taylor, 2015). The development of high-throughput sequencing technology provided more genetic information of evolutionary history (Lemmon and Lemmon, 2013; Mccormack et al., 2013), adaptive traits (Ma et al., 2023; Sun et al., 2023; Yu et al., 2023), and the evolution of phenotypic diversity and speciation (Mason and Taylor, 2015). Thereinto transcriptome analysis has been widely used in the study to reveal the species evolutionary mechanisms (Dong et al., 2013; Hao et al., 2019; Koenig et al., 2013; Naumenko et al., 2017; Shao et al., 2015). Comparative evolutionary approaches can address whether interspecific differences are the result of neutral divergence over evolutionary history or whether they are the result of nonrandom processes, such as adaptation to different environmental conditions (Marra et al., 2014). Thus comparative transcriptomics was usually used to study the genetic basis of evolutionary differences among species.

Bats belong to the order Chiroptera. Due to the constant changes in external and internal factors during bat evolution, different bat species have evolved different adaptive mechanisms to adapt to the environment in the process of natural selection. Evolution is typically thought to proceed through the divergence of genes, proteins, and ultimately phenotypes (Clark et al., 2007; Hughes et al., 2010; Soskine and Tawfik, 2010). The changes in phenotype can reflect the changes in genes. The new phenotype or biological character of bats reflected the changes in the molecular level of genetic material under selective pressure fundamentally. Echolocation is an important phenotype in bats, and it is a complex phenotypic trait, which involves the production, reception, and auditory processing of ultrasonic pulses for obstacle avoidance, orientation, and hunting (Au and Simmons, 2007; Marianne et al., 2004). Rhinolophid bats possess the most sophisticated echolocation systems(Jones and Teeling, 2006), and detected the positive selection of auditory genes in the study of genome data (Dong et al., 2016). It revealed the adaptation of auditory sensory perception in the rhinolophid bat lineages (Dong et al., 2016; Wang et al., 2019; Zhao et al., 2019). Current studies into the evolution of bat acoustic have been conducted at the population level or in bats with different echolocation types, the molecular mechanisms underlying acoustic differences between closely related bat species are rare.

The *Rhinolophus macrotis* group, belonging to the family Rhinolophidea, is a specific species group among the genus*Rhinolophus* . The *macrotis* group currently includes six species, namely *R. episcopus* ; *R. siamensis* ; *R. rex* ;*R. osgoodi* ; *R. marshalli* ; and *R. schnitzleri*(Liu et al., 2019; Zhang et al., 2018). These species are closely related species, and experienced recent and rapid diversification during the Pleistocene (Zhang et al., 2018). Compared with other*Rhinolophus* species, the echolocation frequencies for the species within the *macrotis* group exhibits differences except *R. osgoodi* , which were lower relative to their body size (Zhang et al., 2009). Although some acoustic related genes showed sequence convergence in echolocation bats (Davies et al., 2012; Li et al., 2008; Li et al., 2010; Liu et al., 2012; Liu et al., 2011), different echolocation characters among closely related species may indicated they have undergone different evolutionary processes, which may reflected at the genome level. Previous studies on morphology and behavior for this special phenomenon showed that both pinna and nasal capsule size were better predictors of echolocation call frequencies than forearm length (Wu et al., 2015). Studies using transcriptomics for *R. episcopus* and *R. siamensis* found differentially expressed genes relevant to the variation in echolocation frequency (Li et al., 2022a), and the expression variation related to acoustic signals (resting frequency) and body size (forearm length) was widely governed by natural selection (Li et al., 2022b). However, little is known about the molecular basis on gene sequences for this specific phenomenon, which will help us to understand the evolution mode for the auditory system in bats.

The species within the *macrotis* group are closely related with*R. pusillus* . Species in the *macrotis* group (except*R. osgoodi*) have low frequencies relative to body size ($\text{species}_{\text{low}}$), whereas *R. osgoodi* and *R. pusillus* emit echolocation with normal frequencies ($\text{species}_{\text{normal}}$). The difference in echolocation frequency provides the basis for understanding adaptations in their evolutionary history. The whole-genome sequence of these species is not available yet. Transcriptome analysis will help us to determine the molecular genetics for the adaptation of the *macrotis* group. In this study, we performed a comparative transcriptome analysis and obtained the sequence for multi-tissues of $\text{species}_{\text{low}}$and $\text{species}_{\text{normal}}$ for the first time, to (1) identify genes under positive selection and GO categories significantly enriched involved in the adaptation; (2) characterize the sequence differences between $\text{species}_{\text{low}}$ and $\text{species}_{\text{normal}}$; and (3) uncover the potential genetic mechanism underlying the adaptation for $\text{species}_{\text{low}}$.

2. Materials and Methods

2.1 Specimen collection and library preparation

Species sequenced in this study included five species within the*macrotis* group and one closely related species *R. pusillus*(Table 1). Samples of the brain, liver, and cochlear were biopsied quickly after euthanasia, placed in RNA later, and then stored at -80 until RNA extraction. To avoid the influence of sex, we chose mature females and ensure none were pregnant. Total RNA from these tissues was extracted using TRIzol reagent (Invitrogen Corp., Carlsbad, CA). RNA purification was performed using an RNeasy Mini Kit (Qiagen, Chatsworth, CA). Library constructions from the brain, liver, and cochlear of each species according to the

Illumina Hiseq 2500 RNA sample preparation kit (Illumina, San Diego, CA). We constructed two paired-end libraries with insert sizes of 150 base pairs (bp). All these original data have been deposited into the NCBI Sequence Read Archive database (Accession Number: SAMN35883750-SAMN35883755) (Supporting Information Table S1).

2.2 De novo assembly and annotation of transcripts

To explore genomic variation across the *macrotis* group, we generated the de novo transcriptome assembly for species sequenced. We obtained clean reads from raw data by removing reads containing adapter, reads containing ploy-N, and low-quality reads. All the downstream analyses were based on clean data. Transcriptome assembly was accomplished based on the pooled paired-end reads from three tissues using Trinity (Grabherr et al., 2011) with min_kmer_cov set to 2 and all other parameters set to default. We selected the longest transcript of a gene as the unigene and used it in the following analyses.

To obtain functional annotation for more unigenes, we used the genome data of *R. sinicus* and *H. armiger* from NCBI as references. First, the protein of each unigene was aligned to the NCBI Non-redundant (Nr) protein database using diamond v0.8.22 to produce annotation results. NCBI blast 2.2.28+ was then used to retrieve NCBI nucleotide sequences (Nt) for each unigene. Functional annotation of the unigene was undertaken based on the best match derived from the alignments to the proteins annotated in SwissProt and euKaryotic Ortholog Groups (KOG) database. And we used HMMER 3.0Package to annotate unigene in Protein family (Pfam). Descriptions of gene proteins from Gene Ontology (GO) ID were retrieved based on the results of NR and Pfam. Finally, the Kyoto Encyclopedia of Genes and Genomes (KEGG) orthology of each protein was determined with the KAAS-KEGG Automatic Annotation Server, using the bi-directional best hit (BBH) method.

2.3 Identification of one-to-one orthologous gene sets

We predicted the coding sequences (CDSs) of each unigene according to Nr and Swissprot. And we extracted the longest open reading frame (ORF) in the longest transcript per gene. Estscan 3.0.3 software(Iseli et al., 1999) was also used to determine the direction of sequences that did not have aligned results, the CDSs extracted from these unigenes were translated into amino acid sequences with the standard codon table. We used OrthoMCL v2.0.3 (Li, 2003) (e-value=1e-3) to identify orthologous genes using a Markov Cluster algorithm (MCL) (Enright et al., 2002 JEB). The longest protein sequences per gene were used as the one-to-one orthologous genes among eight species in this study and analyzed in downstream analyses.

2.4 Phylogenetic analysis

Before adaptive evolution analysis, we need a phylogenetic tree to measure the relationship among these species. Protein sequences of eight species were aligned using MUSCLE (Edgar, 2004). After multiple sequence alignments and trimming by the program Gblocks, all one-to-one orthologous genes were concatenated to one sequence for each species and used to construct a phylogenetic tree. PhyML (Guindon et al., 2010) was applied to build a maximum likelihood (ML) phylogeny with 1,000 bootstrap replicates, and we used FigTree version 1.4.2 (http://tree.bio.ed.ad.uk/software/figtree/) to visualize the topology.

2.5 Calculation of evolution rates

We aligned nucleotide sequences using MUSCLE for each orthologous gene and formed a concatenated alignment for all orthologs. We used the constructed tree topology as the guide tree, and calculated pairwise rates of dS, dN, and dN/dS using the free-ratio model (Parameters: model=1, NSsites=0, fix_omega=0, omega=1) in PAML 4 (Yang, 2007) for each branch. The dN/dS value was used to measure the evolutionary rate along a lineage. The lineage-specific mean value was estimated by concatenated alignments from all orthologs.

2.6 Positive selection analyses

We indicated positive selection by the ratio of the rate of nonsynonymous to synonymous substitutions (dN/dS or ω) per gene among lineages. Using our phylogenetic tree topology as the guide tree, we ran

branch-site model (Yang, 2007) (Parameters: null hypothesis: model=2, NSsites=2, fix_omega=1, omega=1; alternative hypothesis: model=2, NSsites-2, fix_omega=0, omega=1) in the codeml module of PAML4 to detect positive selection for the one-to-one orthologous genes of five species within the *macrotis*group, respectively. For each analysis, we selected only one species as foreground branch, and regarded all other species as the background branches. A likelihood rate test (LRT) was performed to detect positive selection on the foreground branch, and the Bayes Empirical Bayes (BEB) analysis (Yang et al., 2005) was implemented to calculate posterior probabilities and to deduce positively selected sites. P -values of all positively selected genes (PSGs) were also normalized by FDR using Benjamini-Hochberg approach(Benjamini and Hochberg, 1995) and$P$ value less than 0.05 was considered significant.

2.7 Gene ontology enrichment analyses

Gene Ontology (GO) was described with GO terms. GO enrichment analysis was implemented by GOseq 1.10.0 in R packages for the PSGs orthologous groups(Young et al., 2010). We analyzed the GO enrichment for five species of the *macrotis* group, respectively. GO categories were identified at three levels (biological process, molecular function, and cellular component) that were over-represented by PSGs. Enrichment P -values were calculated and all P -values were multi-normalized FDR using the BH (best-hit) approach. Categories with an FDR of less than 0.05 were considered over-represented.

3. Result

3.1 *De novo* transcriptome assembly and functional annotation

Raw reads generated from RNA-sequencing for each of the three tissues (brain, liver, and cochlea) from *R. episcopus* , *R. rex* ,*R. marshalli* , *R. osgoodi* , and *R. pusillus* (Table 1) ranged from 44.617 Mb to 64.037 Mb (Supporting Information Table S2). After quality control, about 44.127Mb (6.62Gb) to 63.363Mb (9.5Gb) clean reads of three tissues remained for the *de novo* assembly (Supporting Information Table S2). The clean reads of three tissues were pooled for each species. We obtained the transcript for each species and extracted unigene for the following analyses. For the unigene of each species, the longest nucleotide length is 199.489Mb for *R. episcopus* , and the shortest is 138.272Mb for *R. osgoodi* . The contig N50s parameters of all samples generally ranged from 914bp to 1,173bp (Supporting Information Table S3).

GO terms for each of the annotated genes mainly covering biological GO categories at three ontologies levels (biological process, molecular function, and cellular component) were identified (Supporting Information Figure S1). The GO terms are similar for these species, and the biological processes annotated more unigene. Our analyses of GO terms generally represented the main biological GO classification and ensured the integrity of the downstream functional analyses of the candidate genes.

3.2 Orthologous genes identification and phylogenetic tree

We identified one-to-one orthologous gene pairs across 6 species sequenced (*R. episcopus* , *R. siamensis* , *R. rex* ,*R. marshalli* , *R. osgoodi* , and *R. pusillus*) and two reference genomes (*R. sinicus* and *H. armiger*). In total, 1,233 one-to-one genes were retained for the downstream analyses (Figure 1). Our final dataset, therefore, included eight species and 1,233 genes.

High ML bootstrap values in our constructed phylogenetic tree have well-resolved phylogenetic relationships between species of the*macrotis* group and other species (Figre 2). *Hipposideros armiger* showed as an outgroup in the topological relationship, and all rhinolophids species clustered in one clade. Species of the*macrotis* group clustered together, and closely related to*R. pusillus* . Among the *macrotis* group, the *R. macrotis* complex (*R. episcopus* , *R. siamensis* and *R. osgoodi*) clustered in one highly supported clade, and formed paraphyly with the clade of *R. marshalli* and *R. rex* . The topology of our tree was congruent with previous phylogenies based on the mitochondrial genome (Zhang et al., 2021).

3.3 Evolution rate

Because outlier genes with larger dN/dS may generate deviations in evaluating the overall selective constraint for species, we filtered our dataset to remove genes with dN/dS>100. The rest of genes were used to evaluate

the overall selective constraints in the species analyzed. The dN/dS value was calculated for each species in this study (Figure 3). Higher dN/dS values in species of the *macrotis* group (*R. episcopus* : 0.2919, *R. siamensis* : 0.3723, *R. osgoodi* : 0.3355, *R. rex* : 0.2742, *R. marshalli* : 0.2683) than other species (*R. pusillus* : 0.225, *R. sinicus* : 0.2524, *H. armiger* : 0.217) demonstrated that the majority of genes experienced purifying selection on other species, suggesting that genes evolved at a higher rate in the species of the *macrotis*group.

3.4 Positive selection in the *macrotis* group lineage

A total of 141 candidate PSGs and 1,274 positively selected sites were identified with signals of positive selection along each species branch of the *macrotis* group based on the branch-site model in PAML 4 (Figure 4, Table 2). Here, 7 hearing-related genes (*CRYM* ,*FOXM1* , *PYCARD* , *SLC52A2* , *WRB* , *MAP6* , and *SPRY2*) (Table 3) showed significant evidence of positive selection in species of the *macrotis* group. And the positively selected sites tested in $\text{species}_{\text{low}}$ were species-specific and distinct from other species (Figure 4, Figure 5a - Figure 5f). This result may represent different adaptive responses of auditory development for $\text{species}_{\text{low}}$. In particular, we found one hearing-related gene, *SPRY2* , was positively selected in*R. osgoodi* , which emits normal echolocation frequency in the*macrotis* group. We found same amino acid mutation sites in four positively selected sites in *SPRY2* for *R. osgoodi* and*R. pusillus* , which were different from other species (Figure 5g). The daily activities for bats mainly rely on acoustics, but five visually related PSGs (*ARRDC3* , *LZTFL1* , *RAB8A* ,*EGFBPL1* , and *TRNT1*) were tested in $\text{species}_{\text{low}}$ (Table 4). No visual-related PSG was tested in $\text{species}_{\text{normal}}$.

3.5 Functional enrichment of PSGs

GO enrichment analyses showed that the candidate PSGs were significantly overrepresented and enriched into GO categories such as: metabolic process (GO: 0008152), protein metabolic process (GO: 0019538), single-organism process (GO: 0044699) (Table 5). These categories appeared to be biologically relevant to energy metabolism.

By comparing the GO annotations enriched in different $\text{species}_{\text{low}}$, we found some GO terms enriched more PSGs in each species. For the PSGs in *R. siamensis* , we listed six over-represented GO categories, and some of them related to catalytic activity and metabolic processes. For *R. episcopus* , three significantly enriched GO categories in terms of single-organism process, catalytic activity, and single-organism process enriched more PSGs. We found three GO categories enriched more PSGs compared with other categories in *R. marshalli* . The PSGs identified in *R. rex* were mainly enriched in GO terms of molecular function, single-organism process, catalytic activity, and protein metabolic process.

4. Discussion

Comparative transcriptomics has been widely used in the study of the phenotypic adaptability (Mason and Taylor, 2015; Mitterboeck et al., 2017; Morandin et al., 2016; Pespeni et al., 2017). Comparative analysis of auditory perception can help to elucidate the molecular basis that underpins different auditory capabilities (Dong et al., 2016). In this study, we sequenced the transcriptome of six rhinolophids, including five species in the *macrotis* group and one closely related species *R. pusillus* , and identified some important genes relevant to*Rhinolophus* species adaptation. The results showed that species within the *macrotis* group had physiological adaptations in the process of evolution.

Here, we found evidence that some hearing-related genes, visual-related genes, and energy metabolism-related genes have undergone Darwin selection associated with the evolution of special echolocation frequency. Positive selection acting on hearing-related genes in rhinolophids might result from the extreme selectivity used in auditory processing by these species.

4.1 Adaptive mechanism of hearing related genes

Rhinolophids arguably possess the most sophisticated echolocation systems, and can emit relatively long calls adapted to detect and classify the wing beats of insects. They are heavily reliant on hearing for a variety of

ecologically important roles. Previous studies have documented that hearing-related genes are predominantly evolutionarily conserved in mammals (Kirwan et al., 2013). Comparative analysis of auditory perception can help to elucidate the molecular basis that underpins different auditory capabilities (Dong et al., 2016). Dong et al. (2016) revealed some hearing-related genes undergone natural selection associated with the evolution of specialized constant frequency echolocation, probably resulting from the extreme selectivity used in the auditory processing by these bats. We performed comparative transcriptome for species with two echolocation modes, and tested some genes under selective pressure for the species within the *macrotis* group.

We selected some specific amino acid changes in six PSGs (*CRYM* ,*FOXM1* , *MAP6* , *PYCARD* , *SLC35A2,* and *WRB*) related to hearing. *CRYM* was tested in *R. marshalli* , and the previous functional study found mutations in *CRYM* may cause hearing loss and the expression of *CRYM* is essential for maintaining cochlear cells and preserving normal hearing (Abe et al., 2003; Hosoya et al., 2016). We tested *FOXM1* , *MAP6* , and *WRB* in *R. episcopus* . Previous studies showed that *FOXM1* activities are modulated in the mouse cochlea, and *FOXM1* is associated with cell cycle control and essential for the transcriptional response during DNA damage/checkpoint signaling (Sanchez-Calderon et al., 2010). *MAP6* is involved in molecular transport, nervous system development, and function, and is related to reflect auditory fear conditioning (Hong et al., 2013). The absence of *WRB* from inner hair cells results in significantly reduced intracellular levels of otoferlin, thus causing hair cell synaptic disruption and hearing impairment (Pangrsic and Vogl, 2018). *PYCARD* and *SLC52A2* were tested in *R. siamensis* . Thereinto, *PYCARD* was related to hearing-loss and tinnitus (Wei et al., 2010). Mutation in *SLC52A2* was associated with spinocerebellar ataxia with blindness and deafness type2 (O'Callaghan et al., 2019). We tested different hearing related genes in different $species_{low}$, and this may imply these species adapted to the environment through the different evolutionary mechanisms.

Particularly, we found one PSG *SPRY2* in *R. osgoodi* , who emit echolocation with normal frequency, differ from other species within the *macrotis* group. *SPRY2* is related to hearing loss and plays an important role in the regulation of endochondral bone formation, which may influence early inner ear development (Joo et al., 2016; Wright et al., 2015; Yousaf et al., 2018). The positive selection sites detected in *SPRY2* showed similar amino acid changes with *R. pusillus* , but different with other species. This result indicated that *SPRY2* plays an important role in the echolocation development, and may experience similar acoustic evolution in *R. pusillus* , thus promote *R. osgoodi* and *R. pusillus* evolved similar acoustic characteristics.

Although the function of specific amino acid variants in these genes is still unclear, our results still indicate the potentially important function in acoustic development. These genes may regulate the neural activity or the formation process of inner ear structure, and then affect the sensitivity of specific frequency, that is, affect the echolocation signal for these bats.

4.2 Adaptive mechanism of visual related genes

We tested four visual-related PSGs in four $species_{low}$. *ARRDC3* , *LZTFL1* , and *TRNT1* reveal important roles in the retina (Sharma et al., 2017; Ying H et al., 2017; Ying et al., 2017). *RAB8A* plays a vital part in regulating outer segment protein composition and photoreceptor function (Murga-Zamalloa et al., 2010). And *IGFBPL1* associated with the growth of retinal ganglion cell (RGC) axons (Guo et al., 2018).

A previous study has suggested that the enlargement of one area of the brain might be associated with the reduction in the size of other brain areas (Harvey and Krebs, 1990). The auditory cortex and the inferior colliculus are enlarged in volume in laryngeal echolocating bats, especially in rhinolophoid bats, whereas visual brain areas are relatively enlarged in Old World fruit bats (Dechmann and Safi, 2009). Because of the extreme energetic demands imposed by neural processing, the trade-off has been proposed in investment in brain tissues. Dong et al. (2013) showed more visual perception genes have become pseudogenes in rhinolophoid bats, and speculate that some visual perception genes may have undergone relaxed natural selection in echolocating bats. They also found some hearing-related genes undergone positive selection, and such concordance suggests that some genes are impacted by natural selection, which raised

the possibility that changes in the sensory genes will have direct consequences for those genes controlling for other sensory modalities, perhaps via trade-offs. We found no visual-related genes affected by positive selection in *R. osgoodi* emit echolocation with normal frequency. This finding supports the longstanding but weakly supported assumption that bats are experiencing a trade-off between vision and audition (Dong et al., 2013).

Positive selection of both acoustic and visual genes was detected in species$_{\text{low}}$, indicating that there was no obvious inhibition of the evolution of acoustic related genes and visual related genes in these species, which were affected by natural selection and different with other species. These comparative analyses provide important evidence for the adaptation of species$_{\text{low}}$ to their special hearing mechanisms. Although laryngeal echolocation bats do not rely primarily on vision, the evolution of one sensory related gene may have influenced the evolution of other senses. So we cannot rule out the role of these genes in the evolution of bat hearing until further functional analysis is performed, especially for species in the*macrotis* group, which has a low dominant frequency of echolocation.

4.3 Adaptive mechanism of metabolism related genes

Through the analysis of GO terms enriched by positive selection genes, we found that positive selection genes were mainly significantly enriched in GO terms related to metabolism, and involved in various metabolic processes, for example: oxidation-reduction process, lipid metabolism process, catabolic process, and biosynthesis process. The abundance of these metabolism-related positive selection genes in species$_{\text{low}}$ indicates that these species have the stronger positive selection in energy metabolism or biosynthesis than other species.

Adaptation of energy metabolism plays a very important role in the evolutionary process of species. Bats can fly, which requires huge energy consumption (Shen et al., 2010), so genes related to energy metabolism play an important role in the evolutionary process of bats. Due to the high efficiency of aerobic respiration, which plays a major role in the energy supply process, species$_{\text{low}}$ can obtain more energy by improving mitochondria and oxidation-reduction pathways. Some positive selection genes and positive selection sites were detected in mitochondria (Zhang et al., 2021), the main energy supply organelles, which is consistent with the results of positive selection detected in genes related to energy metabolism in this study. These results suggested that energy-related genes have undergone adaptive evolution in species$_{\text{low}}$, and these species have a stronger selective role in energy metabolism and may improve some aspects of adaptability by regulating energy metabolism in the process of evolution.

Although GO terms enriched by positive selection genes are mostly related to metabolism, there are differences in GO terms enriched among different species, indicating that these species may experience different evolutionary processes, and corresponding genes may play potentially important roles in the evolutionary process of different species.

5. Conclusion

In summary, our comparative transcriptome analyses of the brain, liver, and cochlear in the *macrotis* group provided the theoretical basis for the adaptive mechanism in the evolution of species$_{\text{low}}$. The evidence of genetic adaptation provides insights into hearing related genetic adaptation, visual related genetic adaptation and energy metabolism related genetic adaptation. And GO enrichment analysis revealed that PSGs detected in the species$_{\text{low}}$ mainly enriched in GO terms related to energy metabolism. The important genes and mutations selected may be of great significance for the bat adaptability. While more detailed functional studies of all the selected candidate genes will be necessary to confirm the roles of individual genes. Studying the function and potential pathways of these genes will contribute to a better understanding of phenotypic evolution in bats, espically for the evolution of bat acoustic. Further study of species$_{\text{low}}$ will play an important role in understanding of their adaptive ev olution mechanisms.

Author Contributions

Lin Zhang : Conceptualization (equal); Data curation (equal); Formal analysis (lead); Funding acquisition (equal); Investigation (equal); Methodology (lead); Visualization (lead); Writing-original draft (lead);

Writing-rewiew and editing (equal). **Keping Sun** : Conceptualization (equal); Data curation (equal); Funding acquisition (equal); Project administration (lead); Supervision (lead); Writing-review & editing (equal). **Wentao Dai** : Investigation (equal). **Tong Liu** : Investigation (equal). **Aoqiang Li** : Investigation (equal). **Jiang Feng** : Funding acquisition (supporting); Resources (lead).

Ethical Approval

Bat capture methods and experiments in this study conformed to the Northeast Normal University Guidelines for Animal Research. All experimental procedures adhered to the ASAB/ABS Guidelines for the Use of Animals in Research.

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Conflict of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Data used in this study are available in NCBI under BioProject SAMN35883750-SAMN35883755.

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Figure titles and legends

Figure 1. Venn diagram of orthologs genes for 8 species.

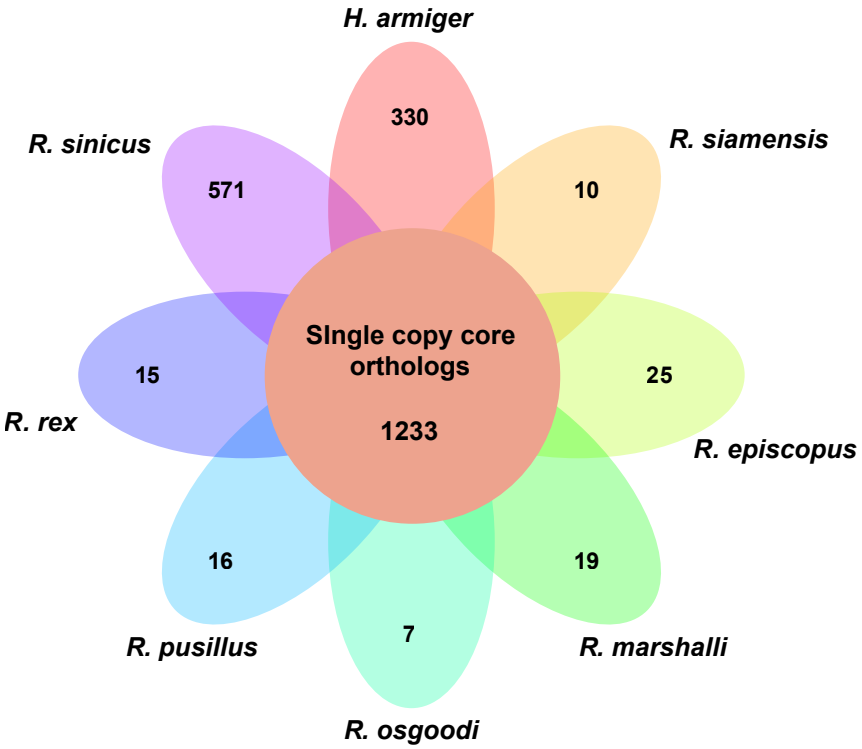
Figure 2. Maximum-likelihood tree constructed based on 1,233 one to one single copy orthologs genes of 8 species. Colored branches indicate bat species within the *macrotis* group. Orange and blue lines represent species with low and normal echolocation frequency relative to body size respectively.

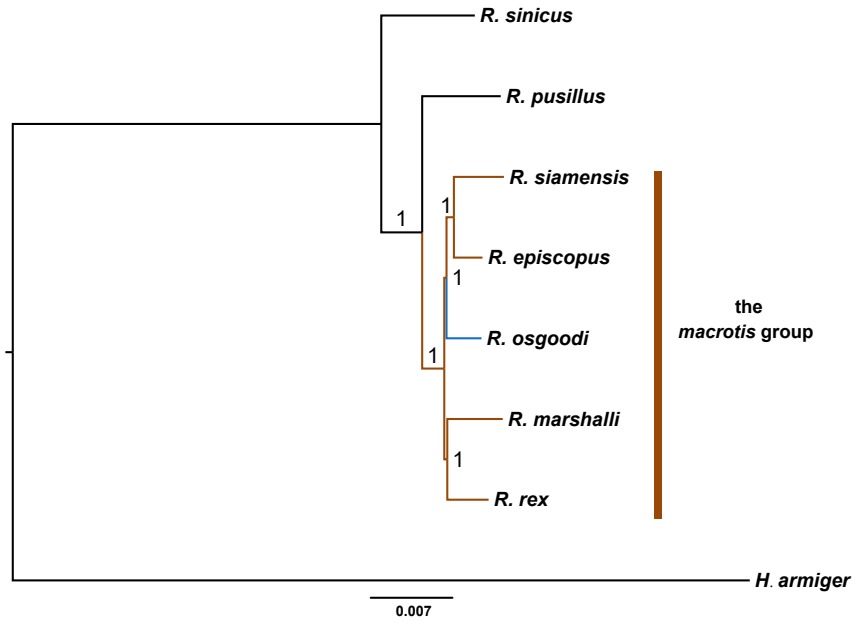
Figure 3. dN/dS values for each species calculated from sequence of concatenated homologous genes.

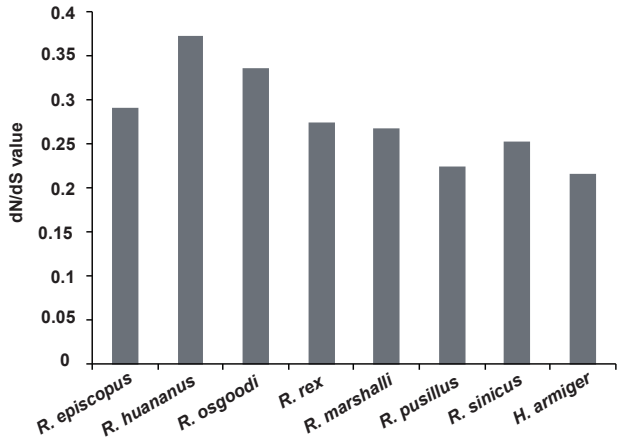
Figure 4. Venn diagram of positively selected genes selected from branches of species within the *macrotis* group.

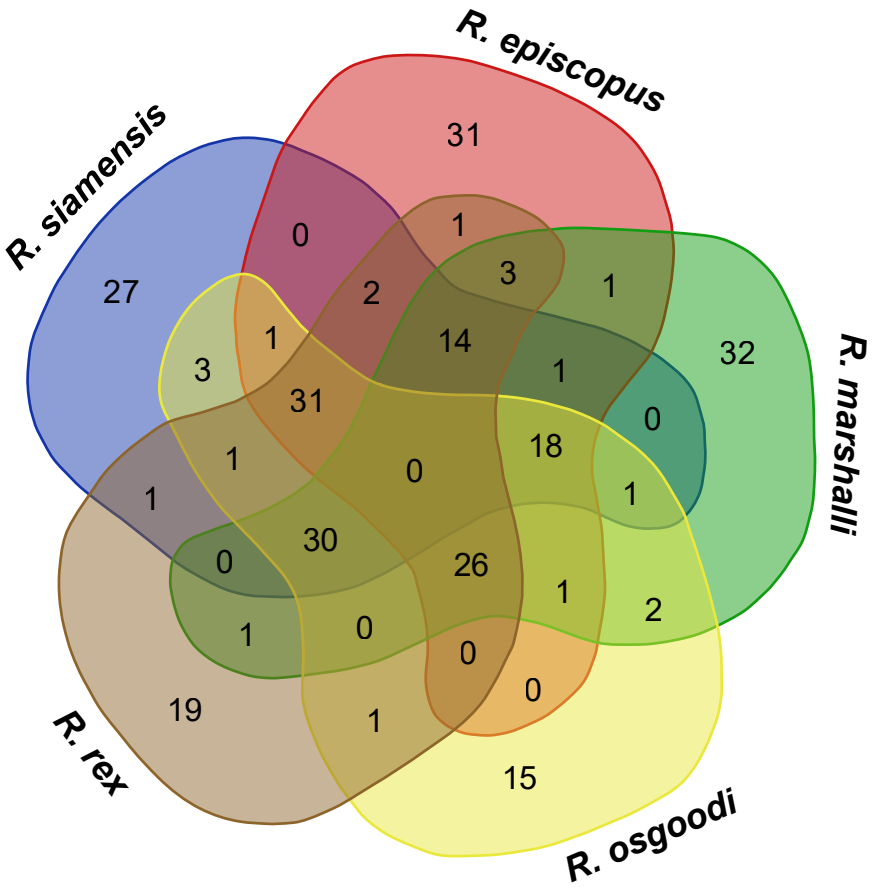
Figure 5. A part of amino acid sequence alignments of the genes related to hearing. (a)-(f) In these sequences, the first and the last positive selected sites were labeled, and the posterior probability of corresponding sites were represented. The species used to test the amino acid change were indicated by a line. (g) The positive

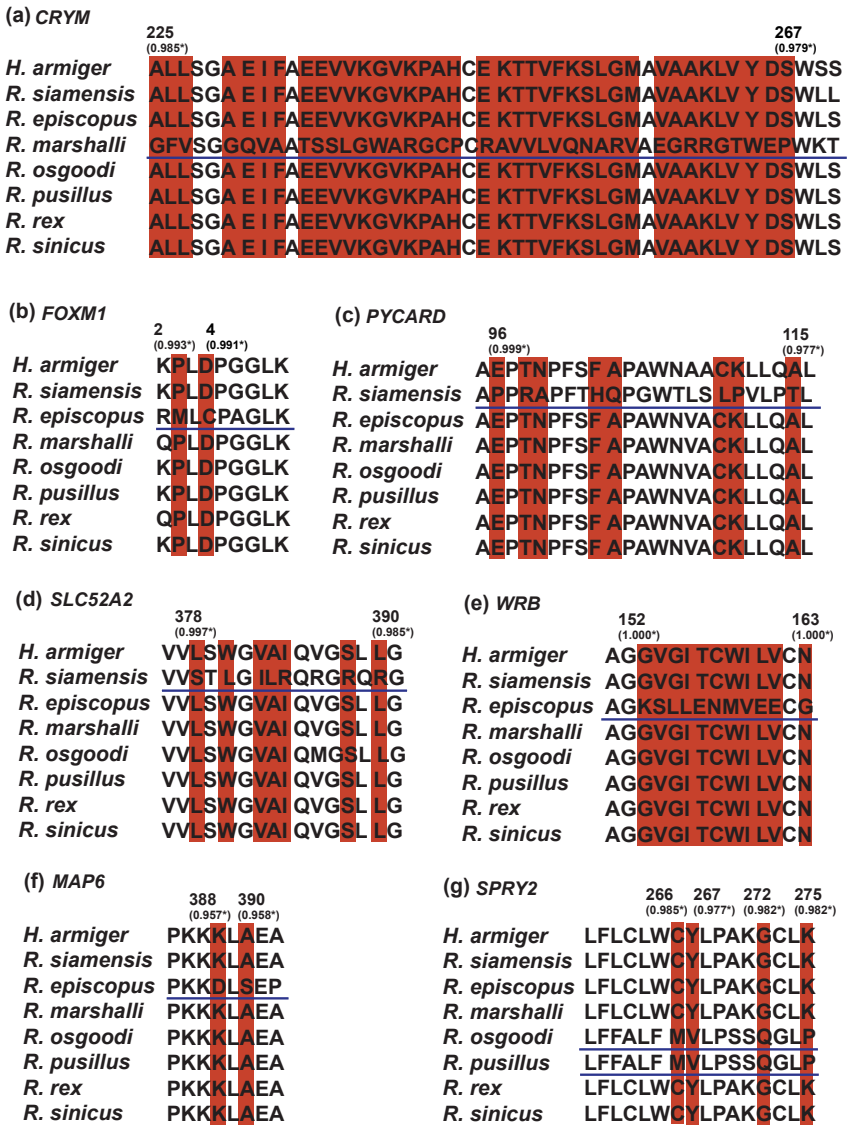
selected sites were labeled and the posterior probability of corresponding sites were represented. The same amino acid change occurred in *R. osgoodi*and *R. pusillus* were indicated by the line.











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