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A Rare Mitochondrial Genome of Albino *Eothenomys eleusis* Thomas 1911 (Cricetidae: Arvicolinae) from Southeastern Yunnan, China and its phylogenetic Analysis

Juan Zhang[1], Yuan Mu[2], Wanlong Zhu[1] , Xiaomi Yang[3]

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ABSTRACT

Background: Animal body color was crucial for genetics and evolution, among which albinism was the result of genetic mutations and there were multiple genetic mechanisms that impede melanin synthesis, thereby affecting animal survival. Studying the phenomenon of albinism could help deepen our understanding of the secrets of genetic and species adaptive evolution.

Methods: This study utilized high-throughput sequencing technology and bioinformatics analysis to investigate the mitochondrial genome of the albino *Eothenomys eleusis* Thomas 1911. The aim was to reveal its structural or compositional characteristics, phylogenetic locationand evolutionary rate.

Result: The characteristic results indicated that the genome sequence (16,347 bp) contained 37 genes and a control region, with a few genes in the "-" chain and most genes in the "+" chain. The base composition and skewness of the whole genome exhibited a significant AT preference. Except for *nad1* started with GTG and *nad1*, *cox3* and *nad4* ended with T (AA), most of the 13 PCGs followed standard genetic code rules. The most commonly used codons were CUA, AUCand AUA, while the relatively commonly used amino acids were Leu1, Ile and Thr. Numerous U-G mismatches were observed in the secondary structure of tRNAs. Phylogenetic analysis confirmed that albino *E. eleusis* was closely related to normal *E. eleusis*and *Eothenomys* was a monophyletic group. Evolutionary rate analysis suggested that *atp8* and *nd4l* had higher dN/dS mean values, while *cox1* and *cox2* were the opposite. In conclusion, this study not only revealed for the first time the mitochondrial genome characteristics and phylogenetic relationships of albino *E. eleusis*, but also provided a new scientific basis and reference direction for the genetic characteristics, diversity and adaptive evolution of *Eothenomys* and other albino species.

Key words: Albino *Eothenomys eleusis*, Evolutionary rate, Mitogenome, Phylogenetic trees.

INTRODUCTION

Eothenomys primarily inhabits the southeastern margin of the Qinghai-Tibet Plateau, where the unique geographical and environmental conditions in this region have facilitated rapid adaptive evolution in response radiation (Liu *et al*., 2012; Zhang *et al*., 2024). Since its establishment in 1896, the classification of this genus has been contentious due to its inherent morphological plasticity, subjective species descriptions and lack of valid molecular markers (Tang *et al*., 2021; Wang *et al*., 2022). To date, approximately 17 *Eothenomys* species have been identified in China (Wang *et al*., 2022), yet only 6 mitochondrial whole genome sequences of this genus species have been published in the NCBI GenBank database. With advancements in sequencing technology, the mitochondrial genome has become a crucial molecular tool for studying *Eothenomys*. It was widely utilized in systematic evolutionary research to elucidate the structural characteristics of the genome and its taxonomic status within the species (Liu *et al*., 2019; Wang *et al*., 2022). The mitochondrial genome was the genetic material contained within the mitochondria of a cell, typically containing of 13 PCGs, 22 tRNAs, 2 rRNAs and a longer control region, which was characterized by maternal inheritance, stable composition, multiple copies, high mutation rates, or rapid evolutionary rates (Wang *et al*.,

[1]Key Laboratory of Ecological Adaptive Evolution and Conservation on Animals-Plants in Southwest Mountain Ecosystem of Yunnan Province Higher Institutes College, School of Life Sciences, Yunnan Normal University, Kunming 650 500, China.
[2]Institute of Eastern-Himalaya Biodiversity Research, Dali University, Dali 671 003, China.
[3]Yunnan Key Laboratory of Integrated Traditional Chinese and Western Medicine for Chronic Disease in Prevention and Treatment, Yunnan University of Chinese Medicine Kunming, Kunming 650 500, China.
Corresponding Author: Wanlong Zhu, Xiaomi Yang; Key Laboratory of Ecological Adaptive Evolution and Conservation on Animals-Plants in Southwest Mountain Ecosystem of Yunnan Province Higher Institutes College, School of Life Sciences, Yunnan Normal University, Kunming 650 500, China; Yunnan Key Laboratory of Integrated Traditional Chinese and Western Medicine for Chronic Disease in Prevention and Treatment, Yunnan University of Chinese Medicine Kunming, Kunming 650 500, China.
Email: zwl_8307@163.com, 12986263@qq.com
ORCIDS:0009-0009-9643-8456, 0009-0008-6515-3100, 0000-0001-8261-4089, 0009-0000-1461-6138
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2021; Iyyappan *et al*., 2024). Therefore, it was applied to construct species evolutionary trees, infer differentiation times and geographic distributionsand investigate adaptive evolution and ecological differentiation under natural selection (Kiraz *et al*., 2024; Ghosh *et al*., 2024).

Eothenomys eleusis Thomas 1911 (Cricetidae: Arvicolinae) was an endemic species of China, primarily found in the southern regions of the country (Wei *et al*., 2021). Recent research has revealed a new distribution record for this species in Sichuan, with fossils found and confirmed in Tham Hai Cave, Vietnam, marking the first Pleistocene fossil record of *Eothenomys* in the country (Lopatin 2023; Wang *et al*., 2024). Recently, we discovered a rare natural albino *E. eleusis* in Mile City, Yunnan Province. Research on albino species has generally focused on their morphological characteristics and the molecular mechanisms of melanin synthesis. For example, studies by Ma *et al*. (2021), Sreeparna *et al*. (2022) and Yan *et al*. (2024) examined the external morphology of albino species, including skin, hairand eyes, in *Muntiacus reevesi* from Shennongjia, *Hardella thurjii* in Indiaand *Hystrix brachyura* in Henan, respectively. Additionally, Li *et al*. (2022) suggested that skin albinism in *Channa argus* was associated with decreased expression of genes involved in tyrosine metabolism and melanin deposition pathways. Shatadru *et al*. (2024), based on whole genome sequencing, found that mutations in the gene SLC45A2R lead to the loss of melanin in parrot feathers. However, despite the availability of mitochondrial genomes as molecular evidence, there has been limited in-depth exploration into the causes of albinism in various species.

Based on this, the study utilized high-throughput sequencing technology to investigate the mitochondrial genome of albino *E. eleusis*, analyzed its gene composition and structural characteristics and constructed the phylogenetic tree based on the complete mitochondrial genome sequence to explore the genetic relationships and evolutionary status of albino *E. eleusis* in *Eothenomys*. This study laid the foundation for future research on the genetic characteristics, phylogenetics and evolution of this genus, providing new directions for genetic and evolutionary investigations of other albino species.

MATERIALS AND METHODS

Animals

The albino *E. eleusis* (Fig 1) used in this one-year study was collected near farmland and shrubs in Mile City, Yunnan Province (103°24′24″E, 24°31′15″ N, 1781 m). It had a large body size, a short tailand oval-shaped ears, with a red iris, ivory white body and ear hairand a light gray tail. The maxillary M3 exhibited 4 medial and 3 lateral processes, with the third transverse lobe and its root being relatively short. The triangular processes of the mandibular M1 were connected and arranged in pairs, resulting in M1 being larger and M3 being the smallest (Liu *et al*., 2019; Zhang and Zhu, 2024). Subsequently, the collected animals were euthanized using CO_2 to extract the required liver tissue samples (0.5 g), which were stored in the Physiological Ecology Research Section of the School of Life Sciences, Yunnan Normal University.

Acquisition, sequencing and analysis of mitochondrial genome

To analyze the structural characteristics and composition of the mitochondrial genome of albino *E. eleusis*, samples were sent to Shanghai Personalbio Technology Co. for sequence acquisition and de novo sequencing. The steps were as follows: Firstly, the total DNA was extracted by the improved 2×CTAB method and then the quality and concentration were detected by Thermo Scientific Nano Drop 2000, Agilent 2100 Bioanalyzer and agarose gel electrophoresis to build a DNA library of 400 bp of different insertions segment. Secondly, Paired-end sequencing was performed on these libraries using the Illumina NovaSeq platform, followed by quality control employing Fastp (Chen *et al*., 2018) and assembled and identified of the final mitochondrial sequence utilizing SPAdes (Andrey *et al*., 2020), GetOrganelle (Jin *et al*., 2020) and Pilon v1.18 (Walker *et al*., 2017). Thirdly, submitted the sequences to NCBI (GenBank accession number: PP475794), annotated it with MITOS (Matthias *et al*., 2012) and drawn a circle graph with CGView (Paul *et al*., 2019). Finally, PhyloSuite (Zhang *et al*., 2020) was used to analyze the base and amino acid content of each component in the mitochondrial genome, including A+T content, AT_skew, GC_skew, *etc*

Fig 1: The image of albino *E. eleusis*. This image was taken by Wei Zhang and Juan Zhang.

and calculated the relative synonymous codon usage rate (RSCU).

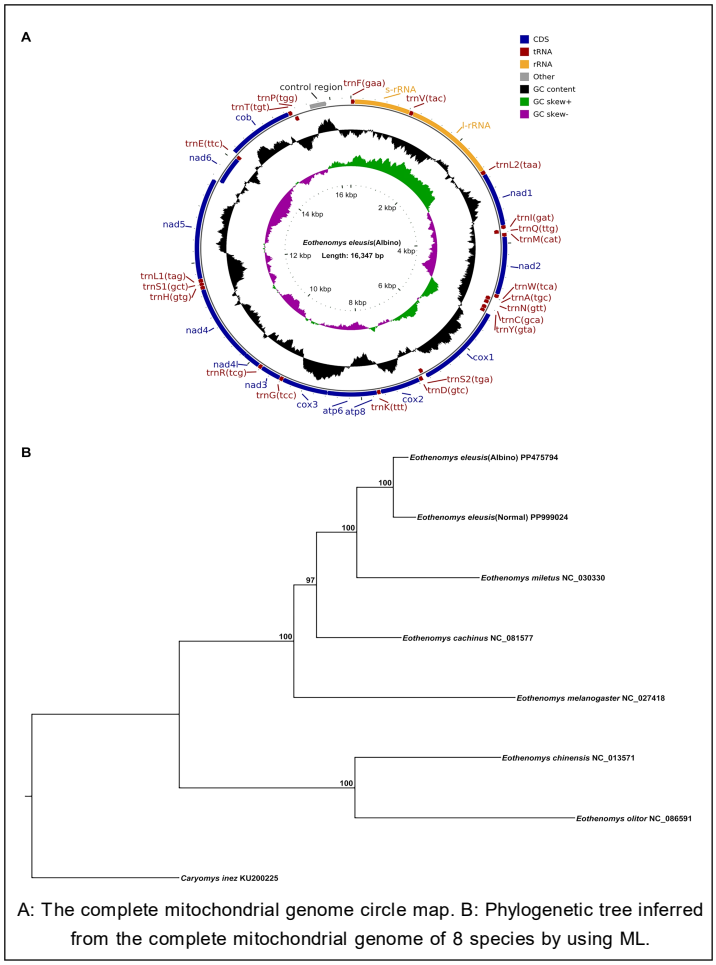
Analysis of phylogenetic and evolutionary rates

To go deep into the systematic evolution of albino *E. eleusis* and its *Eothenomys*, this study constructed the phylogenetic tree based on the mitochondrial genome sequence of albino *E. eleusis* acquired by sequencing and 6 *Eothenomys* species sequences in the NCBI database, with *Caryomys inez* as the outgroup. The process mainly involves extracting, aligningand concatenating sequences of 8 mitochondrial genomes employing PhyloSuite software. Using the ML method for phylogenetic analysisand Modelfinder to determine the optimal model (Kalyaanamoorthy *et al*., 2017; Zhang *et al*., 2020). Perform 1000 repetitions of the ultrafast bootstrap algorithm in IQ-TREE (Nguyen *et al*., 2015) and then beautified the tree graph utilizing FigTree v.1.4.0 (http://tree.bio.ed.ac.uk/software/Figtree/). In addition, to gain a deeper understanding of the survival status of albino *E. eleusis* in the wild, the CodeML (Alvarez-Carretero *et al*., 2023) program in PAML was used to analyze the evolutionary rate of 13 PCGs in 8 species. Namely, the dN/dS values of each branch in the phylogenetic tree were calculated utilizing the free-ratio model. Afterwards, collected and organized the dN/dS dataand performed statistical analysis using R (https://www.r-project.org/) to examine the differences in the evolutionary rates of different PCGs.

Results and Discussion

Structure and compositional characteristics of mitochondrial genome

The full-length mitochondrial genome sequence of albino *E. eleusis* determined in this study was 16,347 bp, containing 37 coding genes and a control region (OH, OL) (Fig 2A), which was similar to the genome length of other normal *Eothenomys* species that have been published. Moreover, the structure, composition and distribution pattern of the genome also showed a high degree of consistency, further confirmed its high conservation in the evolutionary process (Yang *et al*., 2012; Chen *et al*., 2015; Mu *et al*., 2019; Zhu *et al*., 2023). Specifically, among the 37 genes, the "-" chain contains 9 genes, with 1 gene responsible for encoding proteins and the remaining 8



A: The complete mitochondrial genome circle map. B: Phylogenetic tree inferred from the complete mitochondrial genome of 8 species by using ML.

Fig 2: The map of the complete mitochondrial genome of albino *E. eleusis* and phylogenetic analysis.

genes involved in the synthesis of tRNA, while the "+" chain contains 28 genes, with 12 PCGs, 14 tRNAs and 2 rRNAs. It's worth noting that there were intervals or overlaps phenomenon between different genes and control regions in the genome, namely 16 gene intergenic regions (1-416 bp), the longest between *trnP* and OH and 8 gene overlap regions (1-43 bp), the longest between *trnK* and *atp8* (Table 1). In addition, among the base compositions and skewness of all genes, the A + T content of the whole genome was 59.19%, much higher than the G + C content of 40.8% and the AT_skew was 0.115, indicating a significant preference for AT. Moreover, all genes except for OH had a positive AT_skewand all genes except for OL had a negative GC_skew (Fig 2A, Table 2). This preference was similarly present in other species of *Eothenomys*, with only the specific AT content and skewness ratios varying slightly due to differences among species (Mu *et al*., 2019; Zhu *et al*., 2023). However, compared to the impact of codon preference on translation efficiency, the differences in base composition of the mitochondrial genome were more closely related to the specificity of the coding chains (Niu *et al*., 2024).

Table 1: The mitochondrial genome characteristics and location of albino *E. eleusis*.

Feature	Strand	Position (start-end)	Length (bp)	Initiation codon	Stop codon	Anticodon	Intergenic nucleotide
trnF	+	1-66	66			GAA	2
rrnS	+	69-1,016	948				
trnV	+	1,017-1,085	69			TAC	
rrnL	+	1,086-2,650	1,565				1
trnL2	+	2,652-2,726	75			TAA	
nad1	+	2,727-3,681	955	GTG	T(AA)		
trnI	+	3,682-3,750	69			GAT	-3
trnQ	-	3,748-3,819	72			TTG	
trnM	+	3,820-3,888	69			CAT	
nad2	+	3,889-4,923	1,035	ATT	TAA		2
trnW	+	4,926-4,992	67			TCA	2
trnA	-	4,995-5,063	69			TGC	2
trnN	-	5,066-5,135	70			GTT	2
OL	+	5,138-5,167	30				-1
trnC	-	5,167-5,234	68			GCA	
trnY	-	5,235-5,301	67			GTA	1
cox1	+	5,303-6,847	1,545	ATG	TAA		-3
trnS2	-	6,845-6,913	69			TGA	3
trnD	+	6,917-6,984	68			GTC	1
cox2	+	6,986-7,669	684	ATG	TAA		3
trnK	+	7,673-7,736	64			TTT	
atp8	+	7,737-7,940	204	ATG	TAA		-43
atp6	+	7,898-8,578	681	ATG	TAA		-1
cox3	+	8,578-9,361	784	ATG	T(AA)		
trnG	+	9,362-9,430	69			TCC	
nad3	+	9,431-9,778	348	ATT	TAA		1
trnR	+	9,780-9,847	68			TCG	2
nad4l	+	9,850-10,146	297	ATG	TAA		-7
nad4	+	10,140-11,517	1,378	ATG	T(AA)		
trnH	+	11,518-11,585	68			GTG	
trnS1	+	11,586-11,644	59			GCT	-1
trnL1	+	11,644-11,713	70			TAG	
nad5	+	11,714-13,525	1,812	ATA	TAG		-4
nad6	-	13,522-14,046	525	ATG	TAA		
trnE	-	14,047-14,115	69			TTC	5
cytb	+	14,121-15,263	1,143	ATG	TAA		2
trnT	+	15,266-15,332	67			TGT	
trnP	-	15,333-15,400	68			TGG	247
OH	+	15,648-15,930	283				416

PCGs, RSCU and RNAs

The 13 PCGs of albino *E. eleusis* typically followed standard genetic code rules when encoding proteins, using ATN (including ATT, ATG, ATA) as the start codon and TAN (including TAA, TAG) as the stop codon. However, there were also some exceptions, such as *nad1* gene employed an atypical GTG as the start codon, while *nad1*, *cox3* and *nad4* genes end their coding sequences with T (*i.e.* AA) as the incomplete stop codon (Table 1). Further analysis of the codon preference, or RSCU, of these PCGs discovered that 25 codons had RSCU values > 1, of which 14 ended in A/U, indicating that the third position of the codon tended to preferentially use A/U bases. Among these codons, the most commonly used codons were CUA, AUC and AUA, with RSCU values of 2.95, 1.16 and 1.61, respectively (Fig 3A). Among all encoded amino acids, Leu1 (12.68%), Ile (9.96%) and Thr (8.08%) had relatively high contents, while Arg (1.72%), Ser1 (1.29%) and Cys (0.82%) had relatively low contents (Fig 3B). These commonly used codons and amino acids further reinforce the clear AT preference of this genome. Specifically, the stop codons UAA and UAG didn't correspond to the translation of any amino acid and the codons AGA and AGG weren't used in the translation of Arg in albino *E. eleusis* (Fig 3A). In addition, the gene lengths

Table 2: The base content and skewness in the mitochondrial genome of albino *E. eleusis*.

Region	A%	C%	G%	T%	A+T%	G+C%	AT_skew	GC_skew
Whole genome	32.98	27.38	13.43	26.21	59.19	40.81	0.115	-0.342
rrnS	36.81	22.15	17.72	23.31	60.13	39.87	0.225	-0.111
rrnL	36.93	20.77	18.27	24.03	60.96	39.04	0.212	-0.064
nad1	31.1	30.79	12.36	25.76	56.86	43.14	0.094	-0.427
nad2	35.65	29.76	8.7	25.89	61.55	38.45	0.159	-0.548
OL	23.33	26.67	36.67	13.33	36.67	63.33	0.273	0.158
cox1	28.22	27.44	16.76	27.57	55.79	44.21	0.012	-0.242
cox2	32.02	29.68	13.6	24.71	56.73	43.27	0.129	-0.372
atp8	38.73	27.94	6.37	26.96	65.69	34.31	0.179	-0.629
atp6	30.69	29.81	11.31	28.19	58.88	41.12	0.042	-0.45
cox3	28.57	30.61	14.41	26.4	54.97	45.03	0.039	-0.36
nad3	31.61	28.16	11.21	29.02	60.63	39.37	0.043	-0.431
nad4l	29.63	30.98	10.77	28.62	58.25	41.75	0.017	-0.484
nad4	34.11	28.74	10.89	26.27	60.38	39.62	0.13	-0.451
nad5	32.95	29.8	10.98	26.27	59.22	40.78	0.113	-0.461
nad6	18.86	8.76	31.62	40.76	59.62	40.38	0.367	0.566
cytb	30.36	30.97	12.77	25.9	56.26	43.74	0.079	-0.416
OH	22.97	27.56	19.79	29.68	52.65	47.35	-0.128	-0.164

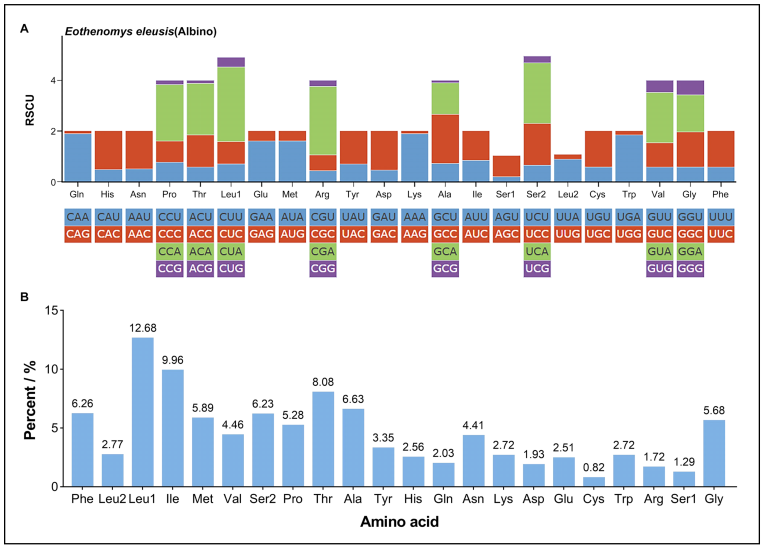


Fig 3: A: Relative synonymous codon usage (RSCU) information of 13 PCGs in albino *E. eleusis*. B: Amino acid composition information of 13 PCGs.

of *rrnS* and *rrnL* were 948 bp and 1,565 bp, respectively. The gene length of 22 tRNAs ranged from 59 bp (*trnS1*) to 75 bp (*trnL2*) (Table 1) and except for the *trnS1* gene, which lacked DHU arm, all the other genes exhibited a standard clover structure. Meanwhile, many U-G mismatches were also observed in the secondary structure of tRNAs (Fig 4), but these mismatches didn't impact subsequent transcriptional functions. On the contrary, they may play a crucial role in maintaining the secondary structural stability of tRNA (Varani and McClain, 2000; Chen *et al*., 2024).

Phylogenetic and evolutionary rates analyses

Based on the constructed maximum likelihood tree, the results indicated that albino *E. eleusis* was a sister branch

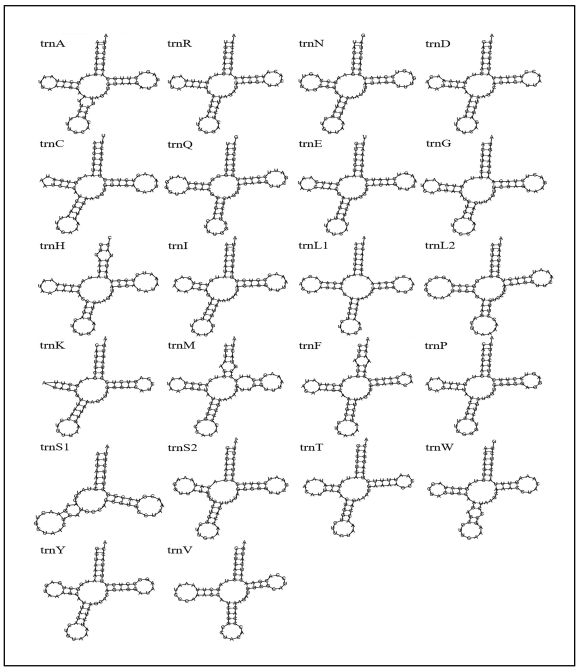


Fig 4: Secondary structure characteristics of tRNAs of albino *E. eleusis*.

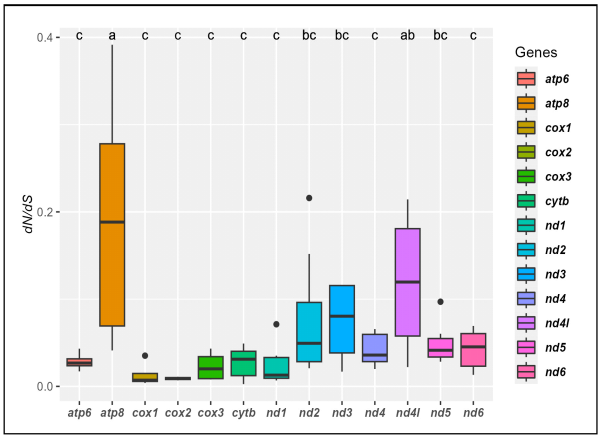


Fig 5: The dN/dS ratio and differential analysis of 13 PCGs from 8 species.

of normal *E. eleusis*and was grouped with *E. miletus*, *E. cachinus* and *E. melanogaster*. This phenomenon has been validated through high bootstrap values (Fig 2B), which was consistent with the latest research results of Abramson *et al*. (2021), Wang *et al*. (2022) and Zhu *et al*. (2023) on *Eothenomys*. In addition, mitochondrial genomes often exhibit adaptive evolution under multiple selection pressures, providing necessary energy for organisms to adapt to changing environments (Liu *et al*., 2023). So, for the analysis of the evolutionary rates of the 13 PCGs in *Eothenomys*, *atp8* and *nd4l* genes had higher dN/dS mean values, suggesting that they may had undergone rapid evolution or some changes in function. On the contrary, *cox1* and *cox2* genes had lower dN/dS mean values, indicating that their evolution was relatively slow and their functions were relatively conserved. This was basically consistent with previous studies by Sun *et al*. (2023), Chen *et al*. (2024) and Ghosh *et al*. (2024). Subsequently, further statistical analysis showed that there were also significant differences in the evolutionary rate between different genes ($P<0.05$), among which the differences between *atp8* and *nd4l* genes were not significant, but significant differences between *atp8* and other genes (Fig 5). Therefore, *cox1* and *cox2* genes can be used as molecular markers for phylogenetic reconstruction and species identification of *Eothenomys*.

CONCLUSION

This study utilized high-throughput sequencing technology and a variety of bioinformatics software to sequence and analyze the mitochondrial genome of albino *E. eleusis*, revealing for the first time the structural and compositional characteristics of the genome. Moreover, the phylogenetic tree constructed based on the complete mitochondrial genome sequenceand further analysis of the evolutionary rate of 13PCGs, clarified the taxonomic status, evolutionand survival situation of albino *E. eleusis*. This provided valuable evidence for the phylogeny and evolution of *Eothenomys*, as well as important references and scientific foundations for the study of mitochondrial genomes of other albino species.

ACKNOWLEDGEMENT

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Ethical approval

All animal procedures were within the rules of Animals Care and Use Committee of School of Life Sciences, Yunnan Normal University. This study was approved by the committee (13-0901-011).

Conflict of interest

All authors declare that they have no conflicts of interest.

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