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Multidimensional Characterization and Adaptive Differentiation of Leptin Gene in *Eothenomys miletus*: Based on Bioinformatics and System Evolution Analysis

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ABSTRACT

Background: To investigate the differences between leptin gene in *Eothenomys Miletus* and other 55 mammal species, this study conducted a comparative analysis of multidimensional characteristics and adaptive differentiation of leptin genes.

Methods: In this study, molecular biology experimental strategies have been used to extract and reverse transcription, gene cloning and sequencing of *E. miletus* and bioinformatics evaluation techniques have been used to analyze the sequence and structure, multi-sequence alignment and evolutionary evaluation of *E. miletus* and ecological and distribution statistical strategies had been used to analyze the leptin genes of 56 mammalian species such as *E. miletus*.

Result: Leptin gene in *E. miletus* contained a Sec/SPI signal peptide, with α-helices dominating its structure. A model was constructed using leptin-LepR trimer (8 × 80.1. C leptin) as template. It encodes 127 amino acids with high proportions of leucine and serine; its molecular formula is $C_{824}H_{1353}N_{221}O_{252}S_8$ (2658 atoms) with molecular weight of 18644.62 and theoretical isoelectric point of 5.86. The protein is unstable and hydrophilic, yet more hydrophobic than Tupaia chinensis and Homo sapiens. *E. miletus* was genetically close to Microtus, distant from Lagomorpha and clusters with temperate Rodentia. Its codon preference differed from Lagomorpha, with moderate GC content, GC3 content and ENC values. Motifs 1-5 were conserved in its leptin. Functional annotation shows regions related to signal peptide, leptin and extracellular domains. The ω value of ~0.5661 suggests evolutionary conservation. These results provide basis for exploring leptin gene evolution in *E. miletus* and mammals, offering perspective on mammalian adaptation to extreme environments and high-altitude protection.

Key words: Adaptation, Dimensional effect, *Eothenomys miletus*, Leptin gene, Mammals.

INTRODUCTION

Leptin, a conserved protein hormone encoded by the obesity gene and primarily secreted by white adipose tissue (Priyadarshini *et al*., 2015), was identified in humans and mice in 1994 and has since become a key research focus. As a core regulator of energy metabolism, it modulates energy balance and suppresses food intake (Zhang *et al*., 2009; Sun, 2019), while also participating in immune responses (Wei *et al*., 2013), infectious diseases and reproduction (Jin *et al*., 2021; Zhang, 2015). Molecular and genetic studies have explored leptin genes in animals (Pang *et al*., 2016; Li *et al*., 2021; Meena *et al*., 2017), their associations with diseases (Yan *et al*., 2023; Ma, 2012) and leptin receptor genes (Yan *et al*., 2011; Li *et al*., 2011). Its physiological roles, linked to gene structural and functional variations, are critical for thermoregulation and metabolic heat production under environmental temperature stress.

Eothenomys miletus, a Cricetidae rodent, which was endemic to the Hengduan Mountains of Yunnan (Han *et al*., 2021), it has been studied by our team for adaptive strategies, including physiological ecology, such as correlations between serum leptin levels and physiological parameters under varying conditions (Hou *et al*., 2020; Yang *et al*., 2013; Yang *et al*., 2016) and leptin-mediated regulation of body mass and energy metabolism via hypothalamic neuropeptides (Zhu, 2015). However, research on the molecular evolutionary mechanisms of its leptin gene remains limited. To clarify the leptin gene's bioinformatics characteristics and evolutionary selection pressure, this study integrated leptin sequences from 55 mammals across five orders (Rodentia, Dermoptera, Lagomorpha, Scandentia, Primates) - taxa with wide distributions from equatorial tropics to cold zones, subject to strong temperature selection pressure. Recent studies note cold-dwelling *Vulpes lagopus* exhibits leptin receptor sensitivity,

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while tropical *Tupaia belangeri* has unique leptin expression regulation (Zhu *et al*., 2014), indicating temperature-driven adaptive evolution of leptin. However, the universality of such latitude- and temperature-related adaptations across taxa and cross-order evolutionary patterns of leptin genes, remain unclear.

This study analyzed the leptin gene's protein structure and physicochemical properties in *E. miletus* and performed cross-order comparative analyses of leptin sequences from 56 mammals (including *E. miletus*) to reveal the gene's molecular characteristics, adaptive evolutionary signals and cross-order evolutionary patterns in mammals.

MATERIALS AND METHODS

Obtaining the complete sequence of leptin gene from *E. miletus*

From 2024 to June 2025, experiments and follow-up data analysis is conducted at the School of Life Sciences of Yunnan Normal University.

Primers were designed according to the sequence of leptin gene in *E. miletus*. The upstream primer F was 5′-ATGTGCTGGGAGACTCCTGTG-3′ and the downstream primer R was 3′- CAGCATCAGCTAAGGTCC-5′. Primers were synthesized by the company. The size of the amplified product was 503bp and the temperature of cleavage was 89.8°C. The steps and operations of extracting and purifying total RNA from hypothalamus in *E. miletus* was details in Zhu's (2015). The whole sequence is transmitted to NCBI (GenBank No: pv838002).

Sequence analysis of leptin gene in *E. miletus*

Signal peptide prediction and protein secondary and tertiary structure analysis

The sign peptide of leptin protein was once estimated by using SignalP-6.0 on-line tool, the secondary shape of leptin protein was once envisioned through SOPMA on line software and the tertiary shape of leptin protein used to be analyzed by means of SWISS-MODEL on line software.

Analysis of physicochemical properties of leptin protein from *E. miletus*

The hydrophilicity and hydrophobicity of leptin protein in *E. miletus*, *Ochotona princeps*, *Tupaia belangeri* and *Homo sapiens* were predicted and compared by using the ProtScale Program in the online software of ExPASY. The physical and chemical properties of rat leptin protein were analyzed by using the ProtParam program of ExPASY online software.

Cross order comparison of leptin gene sequences in 56 species

Collation sequence

Leptin gene sequences from 55 mammalian species available on the NCBI platform, along with the newly obtained sequence from *E. miletus*, were organized (Table 1). These sequences are sorted out by means of techniques such as (Mao *et al*., 2022; Yang, 1997).

Construction of phylogenetic tree

The systematic tree was constructed by the neighbor-joining (NJ) method and then the optimal model and the maximum likelihood method (ML) were used to construct the systematic tree and the self-expansion test (Bootstrap) was set to set 1000 iterations. Compare tree structures from different methods to ensure accuracy. Beautify the phylogenetic tree using Chiplot online software.

Distribution analysis of 56 species in five temperature zones

From the perspective of ecological and biological distribution, the main distribution of species is stable survival and reproduction, with large population, high density and wide distribution range. If the species are mainly distributed in a certain temperature zone, it is recorded as 1 and other distributions are recorded as 0. With the support of animal geographical distribution, ecological habits, professional literature and database, the main distribution of 56 species in 5 temperature zones were counted by Excel. Chiplot was used to analyze the clustering of 56 species in each temperature zone.

Codon preference and determination of GC content

The number of effective codons (ENC), GC content and third GC content (GC3s) of the sequence were calculated using the online tool EMBOSS Explorer.

Conservative region determination and functional annotation analysis of multiple sequences

MEME online software is used to predict the conservative motifs of multiple sequences, compare the predicted conservative motifs with Pfam, PROSITE and other databases, annotate the functional domains corresponding to the motifs and visualize the conservative regions through TBtools - II.

Select pressure detection

PAML software was used to calculate the ratio of nonsynonymous replacement rate (dN) and synonymous replacement rate (dS) (ω=dN/dS) to measure the selection pressure of leptin gene in the process of evolution. The single ratio (M0) and free ratio (M1) branching models that rely on likelihood ratio test (LRT) in Codeml program are used to detect the overall selection pressure of leptin gene and whether there is heterogeneity of selection pressure in different evolutionary branches of leptin gene.

RESULTS AND DISCUSSION

Bioinformatics analysis of leptin in *E. miletus*

Signal peptide assay

It showed that there was a highly reliable Sec/SPI type signal peptide in the leptin protein of *E. miletus* (Fig 1). The probability distribution of different regions of signal peptide is high and the probability of non-signal peptide region is low, which is consistent with the judgment of signal peptide.

Table 1: Basic information of 56 species of mammals and NCBI entry numbers.

Specie latin name	Order	Family	NCBI Accession No.	GC content	GC3 content	ENC
Cercocebus atys	Primates	Cercopithecidae	XM_012087965.1	54.17	73.81	45.13
Macaca mulatta	Primates	Cercopithecidae	U58492.1	53.97	73.81	45.08
Macaca thibetana thibetana	Primates	Cercopithecidae	XM_050782581.1	54.56	73.16	43.33
Rhinopithecus bieti	Primates	Cercopithecidae	XM_017896214.1	54.37	74.40	44.86
Rhinopithecus roxellana	Primates	Cercopithecidae	XM_010378726.1	54.37	74.40	44.86
Trachypithecus francoisi	Primates	Cercopithecidae	XM_033192942.1	54.17	73.81	44.28
Gorilla gorilla gorilla	Primates	Hominidae	XM_004046161.4	54.76	73.21	45.33
Homo sapiens	Primates	Hominidae	NM_000230.3	54.56	72.02	46.92
Pan paniscus	Primates	Hominidae	XM_003806045.5	54.56	72.62	46.53
Pan troglodytes	Primates	Hominidae	U96450.1	55.56	75.51	42.56
Pongo pygmaeus	Primates	Hominidae	U72873.1	55.56	74.83	42.66
Microcebus murinus	Primates	Cheirogaleidae	XM_012789083.1	56.35	73.81	47.50
Hylobates moloch	Primates	Hylobatidae	XM_032753530.2	54.76	72.62	46.61
Symphalangus syndactylus	Primates	Hylobatidae	XM_055281456.1	54.56	72.02	47.66
Octodon degus	Rodentia	Abrocomidae	XM_004642767.2	48.90	64.07	55.54
Heterocephalus glaber	Rodentia	Abrocomidae	XM_004856402.2	50.40	65.48	46.76
Eothenomys miletus	Rodentia	Cricetidae	PV838002	54.76	71.43	42.54
Alexandromys fortis	Rodentia	Cricetidae	XM_050141585.1	55.16	72.02	46.33
Arvicola amphibius	Rodentia	Cricetidae	XM_038321033.1	55.36	71.43	46.74
Cricetulus griseus	Rodentia	Cricetidae	XM_027391330.2	53.79	67.26	52.32
Meriones unguiculatus	Rodentia	Cricetidae	XM_021659887.2	56.13	75.29	44.02
Mesocricetus auratus	Rodentia	Cricetidae	XM_005078071.4	54.56	69.64	48.70
Microtus ochrogaster	Rodentia	Cricetidae	XM_005365724.2	54.76	70.83	47.53
Microtus oregoni	Rodentia	Cricetidae	XM_041675323.1	53.97	68.45	51.11
Myodes glareolus	Rodentia	Cricetidae	XM_048451023.1	55.16	72.62	42.61
Peromyscus californicus insignis	Rodentia	Cricetidae	XM_052721409.1	56.05	72.63	47.48
Peromyscus leucopus	Rodentia	Cricetidae	XM_028866260.2	54.76	70.83	49.49
Phodopus roborovskii	Rodentia	Cricetidae	XM_051175915.1	54.56	70.83	49.67
Castor canadensis	Rodentia	Castoridae	XM_020182860.1	52.98	69.05	43.56
Chinchilla lanigera	Rodentia	Chinchillidae	XM_005402367.2	53.49	71.26	47.46
Apodemus sylvaticus	Rodentia	muridae	XM_052172451.1	54.37	69.64	42.19
Arvicanthis niloticus	Rodentia	muridae	XM_034518822.1	54.34	68.45	44.19
Mastomys coucha	Rodentia	muridae	XM_031380792.1	53.57	68.45	47.64
Mus caroli	Rodentia	muridae	XM_021165722.1	53.37	67.26	42.54
Mus musculus	Rodentia	muridae	NM_008493.3	53.97	68.45	47.47
Mus pahari	Rodentia	muridae	XM_029535370.1	54.96	69.64	44.45
Onychomys torridus	Rodentia	muridae	XM_036180469.1	55.36	72.02	52.24
Peromyscus eremicus	Rodentia	muridae	XM_059257460.1	56.55	75.00	49.79
Peromyscus maniculatus bairdii	Rodentia	muridae	XM_006979375.3	55.16	72.02	47.33
Psammomys obesus	Rodentia	muridae	XM_055608191.1	56.15	74.40	44.18
Rattus norvegicus	Rodentia	muridae	NM_013076.3	55.95	73.21	45.43
Rattus rattus	Rodentia	muridae	XM_032905243.1	56.15	73.81	44.29
Ictidomys tridecemlineatus	Rodentia	Sciuridae	XM_047562352.3	54.17	66.67	48.81
Marmota marmota marmota	Rodentia	Sciuridae	XM_015488308.2	54.17	66.67	48.81
Marmota monax	Rodentia	Sciuridae	XM_046422457.1	54.37	66.67	49.31
Sciurus carolinensis	Rodentia	Sciuridae	XM_015488308.1	55.56	71.43	49.50
Jaculus jaculus	Rodentia	Dipodidae	XM_004658616.1	54.17	71.43	45.10
Cavia porcellus	Rodentia	Caviidae	XM_003475050.3	50.60	65.66	58.39

Table 1: Continue...

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Perognathus longimembris pacificus	Rodentia	Heteromyidae	XM_048340174.1	53.89	71.26	43.52
Cynocephalus volans	Dermoptera	Cynocephalidae	XM_063101311.1	53.77	67.86	47.42
Galeopterus variegatus	Dermoptera	Cynocephalidae	XM_008593523.1	53.17	67.86	50.48
Tupaia chinensis	Scandentia	Tupaiidae	XM_006146862.2	55.36	70.24	48.37
Ochotona curzoniae	Lagomorpha	Ochotona	XM_041002478.1	56.94	72.62	45.85
Ochotona princeps	Lagomorpha	Ochotona	XM_004592505.2	56.94	72.62	45.31
Lepus europaeus	Lagomorpha	Leporidae	XM_062198399.1	61.51	81.55	38.70
Oryctolagus cuniculus	Lagomorpha	Leporidae	DQ983191.1	61.11	80.36	40.56

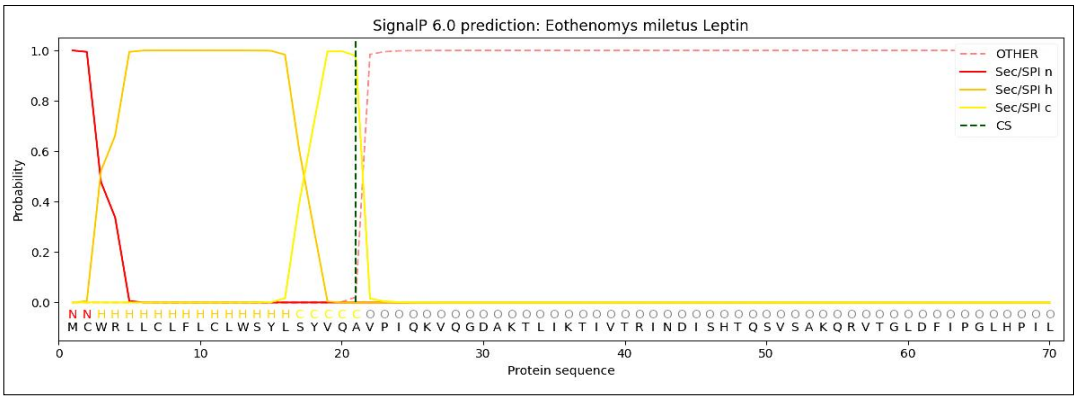


Fig 1: Prediction results of leptin signal peptide in the *E. miletus.*

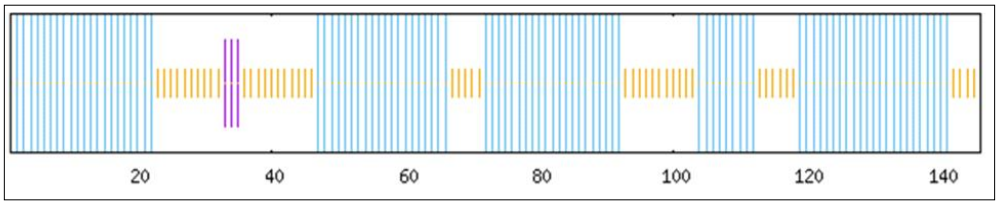


Fig 2: The distribution of α-spiral (blue vertical line), random curl (orange short bar) and extended strand (purple vertical line) in the sequence.

Cleavage site: It is predicted to be between the 21st and 22nd amino acids, with a probability of up to 0.978790. It has high reliability and the cleavage site is clear, which supports the functional localization of its secreted protein. Signal peptide type: it belongs to Sec/SPI signal peptide and the probability of signal peptide is 0.9998.

Protein secondary and tertiary structure prediction

protein secondary structure prediction results

After removal of the signal peptide sequence, the mature leptin protein of *E. miletus* consists of 146 amino acid residues. Secondary structure prediction using SOPMA (Fig 2) revealed a predominance of α-helices (h), comprising 94 residues and accounting for 64.38% of the structure (Fig 3). Random coils (c) were the secondary major structural element, with 50 residues, while the proportion of extended strands (e) was minimal at only 1.37%.

Prediction results of protein tertiary structure

SWISS-MODEL was used to construct the tertiary structure model of leptin protein in *E. miletus* (Fig 4). The core template: leptin LEPR trimer structure (8 × 80.1. C leptin) was selected to establish the model. The sequence consistency was as high as 82.04%, the global model quality score (GMQE) was 0.76 and qmeandico global was 0.76±0.07, indicating that the quality of the model was good and the construction was reasonable. The spiral structure is prominent in the modeling and the model presents a spatial folding shape dominated by α - helix, with less random curl.

Determination and analysis of physical and chemical properties

The analysis results of physical and chemical properties of rat leptin protein showed that the number of amino acids was 127, including 20 essential amino acids, of which leucine accounted for the highest proportion and serine accounted for the second highest proportion (Table 2). Its molecular formula is $C_{824}H_{1353}N_{221}O_{252}S_8$, with a total atomic number of 2658 and a molecular weight of 18644.62. The theoretical isoelectric point of protein is 5.86, which is acidic. The theoretical half-life of *E. miletus* leptin protein *in vitro*

(mammalian reticulocytes) was 30 h, in yeast > 20 h and in *Escherichia coli* > 10 h. The aliphatic index of the protein was 119.04; Average hydrophilicity (GRAVY): 0.113, instability index: 50.89, which is an unstable hydrophilic protein.

Lipophilicity/hydrophobicity of leptin protein in *E. miletus*

The leptin proteins of *O. princeps* inhabiting alpine tundra, *T. belangeri* from tropical regions, *H. sapiens* widely distributed across temperate zones and *E. miletus* were analyzed and compared (Fig 5). The four species share similar hydrophobic/hydrophilic fluctuation patterns in their leptin protein sequences. The leptin proteins of the North American pika and the large-eared vole exhibit stronger hydrophobicity, while those of humans and the *T. chinensis* display weaker hydrophobicity.

Comparison and analysis of leptin gene sequences in 56 mammalian species

phylogenetic analysis of leptin gene

The phylogenetic tree based on leptin gene sequences of 56 mammalian species (Fig 6) shows: Some Microtus species and *E. miletus* cluster into a small branch, with close genetic relationship and distant relationship with Dermoptera. Rodents of the same family cluster closely; squirrels, beavers and other rodents have longer branches and earlier differentiation than *E. miletus*. Ochotona and *Lepus europaeus* have short branches, adjacent to but independent of Rodentia. *T. belangeri* is close to Primates. Dermoptera species cluster into one branch, adjacent to Scandentia and Primates branches.

Table 2: Amino acid composition of leptin protein in *E. miletus*.

Amino acid	Quantity/Number	Proportion/%
Alanine (A)	8	4.8
Lysine (K)	7	4.2
Arginine (R	7	4.2
Methionine (M)	3	1.8
Asparagine (N)	4	2.4
Phenylalanine (F)	2	1.2
Aspartic acid (D)	11	6.6
Proline (P)	6	3.6
Cysteine (C)	5	3.0
Serine (S)	21	12.6
Glutamine (Q)	14	8.4
Threonine (T)	9	5.4
Glutamic acid (E)	5	3.0
Tryptophan (W)	2	1.2
Glycine (G)	4	2.4
Tyrosine (Y)	4	2.4
Histidine (H)	3	1.8
Valine(V)	12	7.2
Isoleucine (I)	10	6.0
Leucine (L)	30	18.0

Distribution of 56 species in different temperature zones

The 56 species are clustered by their main distribution across temperature zones (Fig 7). Most primates, *T. belangeri* and Dermaptera cluster together, mainly in tropical areas. Two Ochotonidae pikas cluster, mainly in cold and sub-cold regions, differing from the two Leporidae species, which are mainly in temperate or temperate-subtropical zones. Most rodents are mainly in temperate zones; a few span tropical, subtropical and temperate zones, with some widely distributed from tropical to temperate.

Codon preference and GC content analysis

Analysis of ENC, GC3 and GC contents of leptin genes in 56 species (Table 1) showed: leptin gene GC content ranged 48.90-61.51 (average 54.76); GC3 was 64.07-81.55 (average 71.42), indicating a tendency to use G/C-ending codons (since the third codon rarely changes amino acid type); ENC values were 38.70-58.39 (average ~46.57). Lagomorpha had the highest GC and GC3 contents and the smallest ENC value, while *E. miletus* showed medium levels in all three.

Analysis of conservative areas and functional annotation areas

Multi sequence conservative motif analysis

The conservative motif prediction results of 56 leptin gene sequences were visualized with tbtools- all (Fig 8). Motif 1-5 is stable in most species and its position is relatively concentrated. In rodents, motif 1-3 has more prominent repetition frequency and length; Some motifs are widely distributed in rodents and some primates and the distribution span is from 5′ to 3′. Some motifs only appear in specific groups and species, such as motif 7-11 unique to some Primates Macaca and are more densely distributed at the 3′ end.



Fig 3: Prediction results of secondary structure of leptin protein in *E. miletus*.

Functional annotation analysis of multiple sequence conservative motifs

Functional annotation was performed on motif 1-11, some conservative motifs had no functional annotation (Fig 9). Leptin was concentrated near the 20-25 amino acids. The region of membrane bound protein predicted to be outside the membrane, in the extracellular region, is expected to be located outside the membrane, occupying the posterior segment of the sequence (about 25-50 positions). Motif 1-3 showed the functional region of the conserved motif of the protein sequence encoded by the *E. miletus* leptin gene sequence.

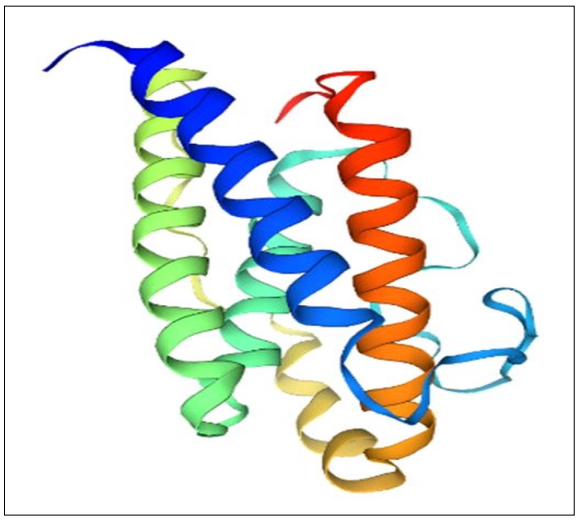


Fig 4: Three-dimensional structure prediction result of leptin protein in *E. miletus.*

Analysis of selection pressure and positive selection effect

The single ratio model selection pressure analysis (Table 3) shows that the overall ω=0.32993<1 and the leptin gene dN (1.5749) is significantly lower than dS (4.7733), indicating the presence of significant purification (negative) selection. *E. miletus* and Microtus gather together. In some branches, synonymous substitutions of rodents decrease, while in others, synonymous substitutions increase. In addition, the average content of GC3 between sequences is 0.7142.

Free ratio model-based branch selection pressure analysis showed a better overall likelihood value (LNL = -5630.47) than the single ratio model (LNL = -5721.88). Likelihood ratio test (LRT) confirmed significant heterogeneity in selection pressure among branches. The overall kappa (ts/tv = 4.13) indicated a high transition/ transversion ratio, consistent with mammalian gene evolution characteristics. With total nonsynonymous substitution rate (dN = 1.62) lower than synonymous substitution rate (dS = 4.55; dN < dS), the overall selection remained dominated by purifying selection. Under the free ratio model, Microtus and *E. miletus* (ω = 0.5661) clustered into one branch. Most species underwent strong purifying selection, while some branches showed significant positive selection.

Sequence characteristics of leptin gene and protein structure of *E. miletus* adapted to their living needs

This study undertook an analysis of the leptin gene in *E. miletus*, predicting that its leptin protein contains a Sec/ SPI-type signal peptide with a notably high support probability. Leptin functions by binding to its receptor

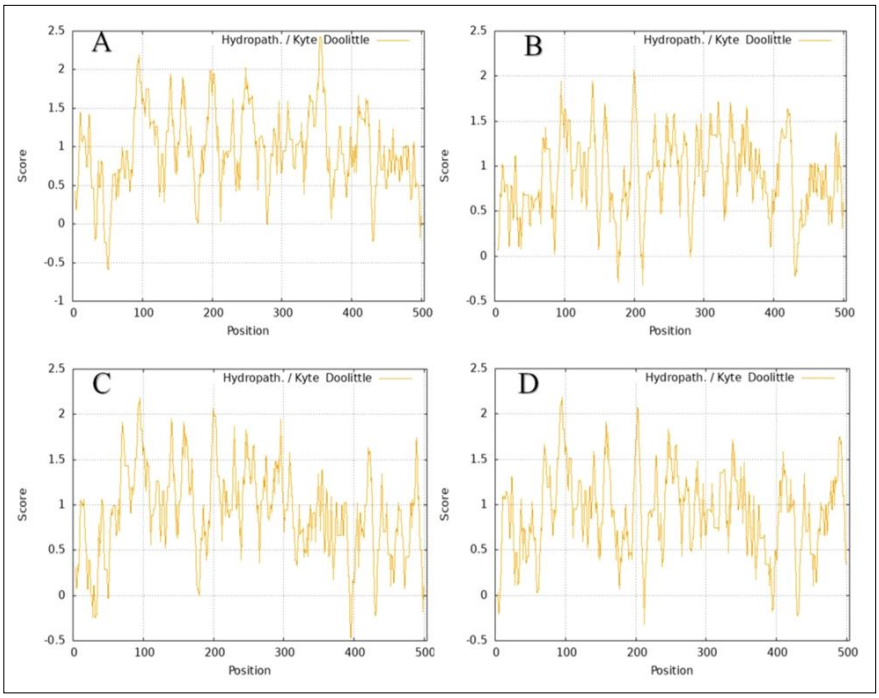


Fig 5: Prediction results of leptin affinity/hydrophobicity of *O. princeps* (A), *E. miletus* (B), *T. chinensis* (C) and *H. sapiens* (D).

(LepR), with α-helices playing a pivotal role in receptor recognition. The leptin of *E. miletus* exhibits a high α-helix ratio, which is conducive to a stable signal transduction domain. Research on Ochotona curzoniae has demonstrated that elevated α-helix ratios enhance receptor affinity and thermogenesis, facilitating adaptation to extreme cold conditions (Yang *et al*., 2011). Compared to humans and mice, *E. miletus* leptin displays a higher α-helix ratio, which is essential for energy regulation in adapting to high-altitude, cold environments. Its low random coil ratio indicates a compact structure, thereby reducing energy consumption. These findings, coupled with high sequence consistency with the leptin-LepR trimer template, confirm structural compatibility and enhance receptor binding efficiency in cold environments.

E. miletus leptin (127 amino acids) has the highest leucine proportion, potentially enhancing energy reserve and utilization in alpine environments (Zhang *et al*., 2009). Its amino acid composition is more "energy-efficient" than human leptin (≈11% leucine), aligning with survival needs in high-altitude, food-deficient habitats. As an unstable hydrophilic protein requiring continuous *in vivo* synthesis, its theoretical half-life indicates eukaryotic stability, ensuring sustained energy regulation signals.

The hydrophobic region interacts with the phospholipid bilayer in transmembrane structures and maintains the conformational stability of membrane proteins at low temperatures (Tomczak *et al*., 2002). *E. miletus* exhibits high hydrophobicity, which facilitates protein structural maintenance and receptor signal transduction efficiency in cold environments (Chen *et al*., 2022). Plateau cold-zone species (*O. princeps* and *E. miletus*) showed strong hydrophobicity, ensuring stability and response speed of energy regulation signals. In contrast, temperate *H. sapiens* and tropical *T. belangeri* balance hydrophilicity and hydrophobicity, likely meeting the energy metabolism and environmental stability requirements.

Multidimensional characteristics of *E. miletus* adapted to living environment

Further evolutionary analysis showed that *E. miletus* and Microtus clustered, indicating leptin gene conservation in the same family and genus. Rodentia families have longer branches, with niche differentiation possibly driving leptin

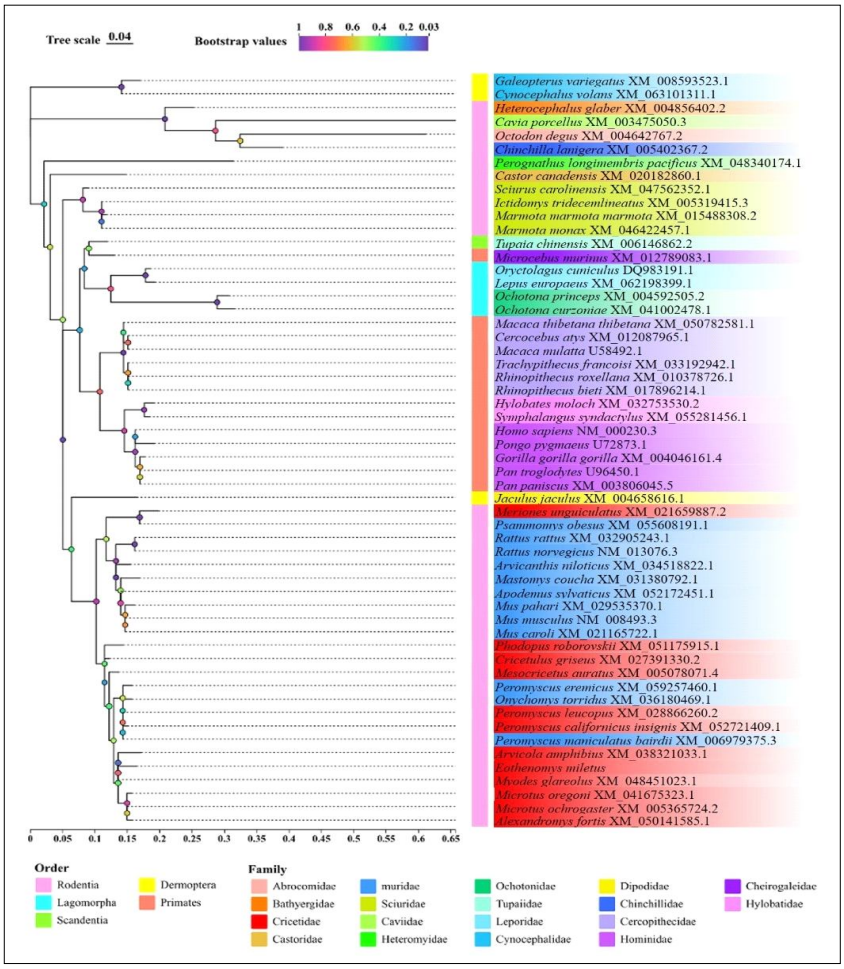


Fig 6: Phylogenetic tree of 56 mammalian leptin genes.

gene differentiation. Primates and hominids cluster closely, reflecting high leptin gene conservation.

In temperature distribution, Primates, *T. belangeri* and Dermoptera are mostly tropical. Ochotonidae and Leporidae differ in differentiation: some species range from tropical to temperate zones, with their leptin genes scattered in temperate clusters, showing environment-adaptive polymorphism to meet energy needs across temperature zones, reflecting molecular-level ″ecological range expansion″ (Londraville *et al*., 2014; Gong *et al*., 2013). *E. miletus*, mainly temperate, uses leptin gene regulation to adapt to the Hengduan Mountains' extreme, climate-variable, large temperature-difference environment.

Molecular-level analyses of codon preference and conserved motifs further support these conclusions. The leptin gene shows a preference for G/C-ending codons;

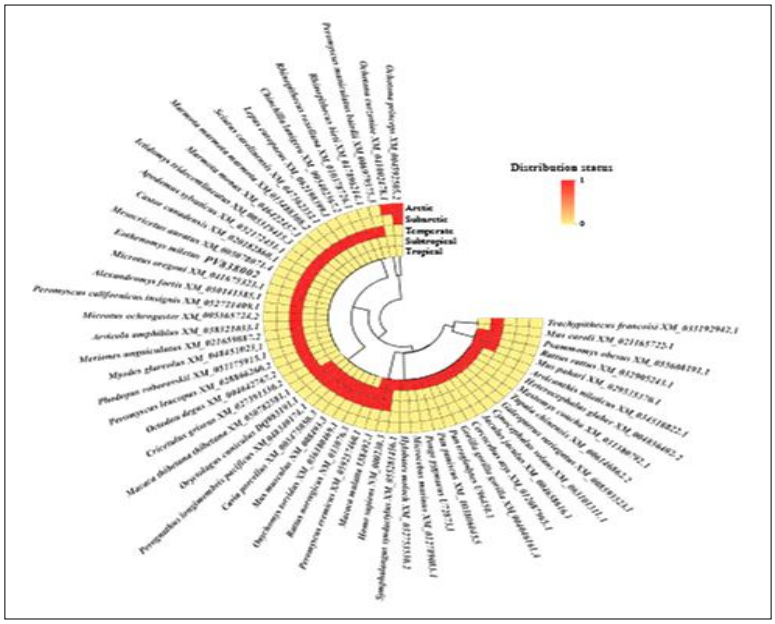


Fig 7: Clustering heat map of the distribution of 56 species in each temperature zone.

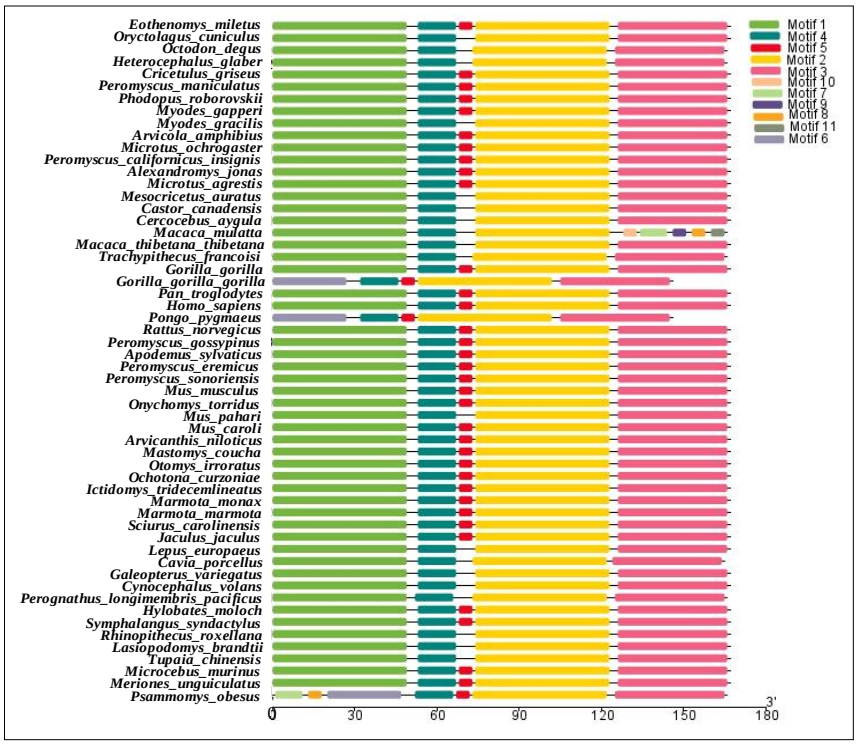


Fig 8: Results of conserved motif determination of 56 leptin gene sequences.

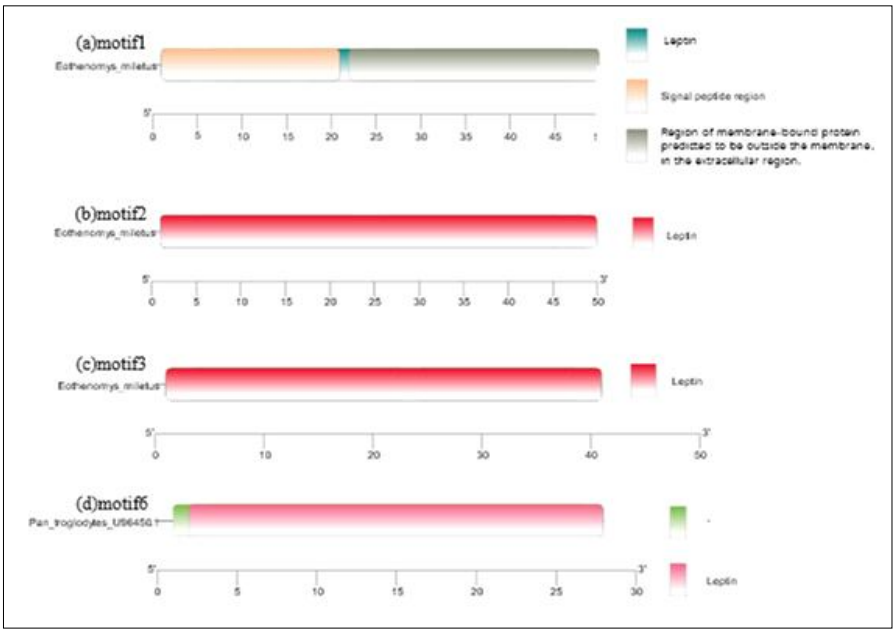


Fig 9: Functional annotation results of each conserved motif.

Table 3: Reference values of leptin gene results under two branch models.

Model	tree length for dN/dS	lnL	tree length	kappa (ts/tv)	GC3 content
M0	1.6241/4.5475	-5630.468199	7.148581	4.12861	C:0.39517 G:0.32513
M1	1.5749/4.7733	-5721.884113	7.214167	4.11744	C:0.39517 G:0.32513

high GC3 content enhances DNA stability, aiding adaptation to UV-intense tropical environments and cold zones with large temperature fluctuations (Sambrook and Russell, 2001). A lower ENC value indicates stronger codon preference and faster translation (Sharp and Li, 1987); the moderate ENC value of *E. miletus* leptin suggests a trade-off between rapid translation and sequence variation, balancing core energy regulation functions with temperature zone specialization demands. Motifs 1-5, as core functional domains, ensure cross-species energy regulatory basics. The distribution of motifs 1-3 reveals *E. miletus* leptin retains signal peptide-guided secretion, metabolic regulation and extracellular membrane protein functions, linked to adaptation to the Qinghai-Tibet Plateau's high-altitude, cold and hypoxic conditions (Chen *et al*., 2022; Gong *et al*., 2022).

Selection pressure analyses showed overall purifying selection on the leptin gene, with highly conserved functions. Some Rodentia branches exhibited higher dS, indicating rapid evolution and sequence divergence, potentially linked to adaptive radiation. Most rodents clustered closely with short branch lengths and low genetic distances. Microtus clusters with *E. miletus*, supporting their close phylogenetic relationships. Leptin function is highly conserved among congeneric rodent species, with consistent patterns observed in other taxa.

CONCLUSION

This study elucidates the molecular mechanisms underlying *Eothenomys miletus* adaptation to high-altitude environments in the Hengduan Mountains. Key findings demonstrate that: Leptin gene evolution reflects long-term selective pressures under plateau conditions, with specific mutations stabilizing energy-regulation signaling pathways while maintaining metabolic flexibility to cope with resource fluctuations. Physiological specialization at the molecular level provides a paradigm for mammalian adaptation to extreme environments, bridging a critical gap between genetic changes and ecosystem-level resilience. These results offer two-fold implications: Theoretically, establishing leptin as a biomarker for high-altitude adaptation, challenging traditional views on metabolic adaptation timelines. Practically, proposing molecular targets for monitoring vulnerable plateau ecosystems, informing conservation strategies under climate change.

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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

The authors declare that there are no conflicts of interest regarding the publication of this article. No funding or sponsorship influenced the design of the study, data collection, analysis, decision to publish, or preparation of the manuscript.

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