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Characterization of Two New *Apodemus* Mitogenomes (Rodentia: Muridae) and Mitochondrial Phylogeny of Muridae

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Abstract: *Apodemus* is the most common small rodent species in the Palearctic realm and an ideal species for biogeographical research and understanding environmental changes. Elucidating phylogenetic relationships will help us better understand species adaptation and genetic evolution. Due to its stable structure, maternal inheritance, and rapid evolution, the mitogenome has become a hot spot for taxonomic and evolutionary studies. In this research, we determined the mitochondrial genome of *Apodemus agrarius ningpoensis* and *Apodemus draco draco* and studied the phylogeny of Muridae using ML and BI trees based on all known complete mitogenomes. The mitochondrial genome of *Apodemus agrarius ningpoensis* was 16,262 bp, whereas that of *Apodemus draco draco* was 16,222 bp, and both encoded 13 protein-coding genes, 2 ribosomal RNA genes, and 22 transfer RNA genes. Analysis of base composition showed a clear A-T preference. All tRNAs except $tRNA^{Ser}$ and $tRNA^{Lys}$ formed a typical trilobal structure. All protein-coding genes contained T- and TAA as stop codons. Phylogeny analysis revealed two main branches in the Muridae family. *Apodemus agrarius ningpoensis* formed sister species with *Apodemus chevrieri*, whereas *Apodemus draco draco* with *Apodemus latronum*. Our findings provide theoretical basis for future studies focusing on the mitogenome evolution of *Apodemus*.

Keywords: mitochondrial genome; *Apodemus agrarius*; *Apodemus draco*; phylogeny; Muridae

Citation: Wu, D.; Zhou, L.; Xue, J.; Xia, Q.; Meng, L. Characterization of Two New *Apodemus* Mitogenomes (Rodentia: Muridae) and Mitochondrial Phylogeny of Muridae. *Diversity* **2022**, *14*, 1089. https://doi.org/10.3390/d14121089

Academic Editor: Franc Janžekovič

Received: 16 October 2022
Accepted: 6 December 2022
Published: 9 December 2022

1. Introduction

With the continuous improvement of molecular biology technology in recent years, the construction of molecular phylogenetic trees has become an indispensable component of systematics and taxonomy [1]. An increasing number of species differentiation analyses rely on molecular phylogenetic analyses. Therefore, establishing reliable phylogenetic relationships is a prerequisite for accurately distinguishing between species and revealing the mechanisms of species formation.

Many researchers have attempted to determine the evolutionary relationships of species using different methods. Early traditional taxonomies that relied on morphology severely underestimated species richness [2]. Later researchers began to use protein electrophoresis, DNA hybridization and other molecular biology tools to explore the relationship between biological systems [3]. In recent decades, mitochondrial gene sequences have become a hotspot of taxonomic and evolutionary research of different animal groups [4–6]. Mitochondria have their own unique genetic system and thus have become a widely used molecular marker in the study of phylogeny of species because of their matrilineal inheritance and high mutation rate [7–13]. Mitochondria provide energy for the daily cellular activities, and their genes change under the influence of the environment in which the species lives [14,15]. Physiological changes and evolutionary pressures on species are directly reflected in their mitochondrial genome sequence [14]. However, single molecular markers of mitochondrial gene sequences often have limited phylogenetic information [6,16–18]. Increasing use of two, three, or more mitochondrial markers has been

found in phylogenetic studies [19,20]. Researchers can gain more information from the whole mitotic genome sequences owing to their multiple structural genomic characteristics in different groups [21], which are reflected in functionally dependent differences associated with structural differences or phylogenetically distant organisms [22]. The mitochondrial genome contains information on gene rearrangements, tRNAs structure, genetic system that are lacking in single gene fragments [16], hence improving the precision of the study of evolutionary relationships among related species [6,10,23,24]. The ongoing refinement of the composition and structural characteristics of the mitochondrial genome of species help us understand the evolutionary history of species. This will better promote the study of species taxonomy [16,25].

The genus *Apodemus* of the Muridae family species is abundant and widely distributed in the Palearctic [26]. Members of this genus are highly adaptable in almost all habitats in the mountains, and are the most common small rodent in the field [4,27,28]. However, these rodents are the most important hosts of zoonotic diseases and parasites [12,29] and have been associated with multiple hazards to humans, such as disease transmission and crop destruction [30]. Their wide distribution and unique adaptations make members of the genus *Apodemus* ideal candidates for understanding the relationship between environmental change, biogeography, and rodent species evolution [27]. Approximately 7.17–9.95 Mya ago, *Apodemus* underwent two stages of radiation evolution to produce the *agrarius* and *draco* subgroups [28]. Moreover, detailed descriptions of different genetic lineages are lacking, which is not conducive to identifying these species based on morphology alone [12].

Apodemus agrarius ningpoensis and *Apodemus draco draco* are the two common *Apodemus* subspecies in the northern margin of the Oriental and southern margins of the Palearctic in eastern China. However, their molecular characteristics are limited.

Phylogenetic relationships of *Apodemus agrarius ningpoensis* and *Apodemus draco draco* are difficult to determine owing to the paucity of mitochondrial genomic data. Typically, phylogenetic errors in the taxonomy of *Apodemus* have been attributed to recessive cytochrome b pseudogenes [31]. In this study, we sequenced the mitogenomes to explore their phylogenetic position and the composition and evolution of the mitochondrial genomes of *Apodemus agrarius ningpoensis* and *Apodemus draco draco*. Their genome sequences, nucleotide composition, genome size, codon usage, gene overlap, and tRNA secondary structure were analyzed. The mitochondrial genome data generated herein provide a reference for future research focusing on the evolution of *Apodemus* mitochondrial genome. In addition, our study will lay a foundation for the identification and classification of species, better promote the study of rodent taxonomy.

2. Materials and Methods

2.1. Experimental Samples

Specimens of *Apodemus agrarius ningpoensis* and *Apodemus draco draco* were collected from two counties in Dabie Mountains, Yue-xi and Huo-shan, in eastern China. Samples were collected using the clip-night method, with fresh peanut kernel as bait. Combining with habitat characteristics, 100 standard iron plate clips were placed in each sample plot. The clips were placed at dusk and collected next morning. The captured rodents were first weighed and numbered, and then the conventional data such as head–body length, tail length, ear length, and hind foot length were measured, and the sex was identified. After morphological identification, BLAST analysis in NCBI (http://ncbi.nlm.gov/nuccore?db=nucleotide, accessed on 3 February 2022) using DNA barcoding sequences verified their classification. Specimens were stored in 95% ethanol and placed in a −80 °C refrigerator.

2.2. DNA Extraction and Sequencing

The SPIN easy DNA Kit for Tissue and Bacteria (with lysing matrix) (MP Biomedicals, Hefei, China) was used to obtain the required DNA from muscle tissue. High-quality DNA samples were library constructed and sequenced by the Genepioneer Biotechnology (Nanjing, China) on the Illumina HiSeq2500 platform [32].

2.3. Assembly and Annotation

The raw sequences in FASTA format were extracted from the FASTQ format obtained by sequencing using Python. The mitochondrial genome was assembled using the SPAdes 3.15.3 software [33] by referring to the KY851939.1 sequence in NCBI. Using the MITOS web server (http://mitos.bioinf.unileipzig.de/index.py, accessed on 10 May 2022) [34] to annotate the assembled sequence. Annotation results were compared with those of related species, the final annotation results were obtained after manual correction [35]. tRNA structures were predicted using the Mitos web server and tRNAscan-SE 2.0 [36,37]. The mitochondrial genome of the two *Apodemus* obtained in this experiment was mapped using OGDRAW (https://chlorobox.mpimp-golm.mpg.de/OGDraw, accessed on 15 May 2022).

2.4. Sequence Analysis

The base composition of the mitogenome of the two *Apodemus* species was calculated by MEGA X [38]. AT-skew = (A − T)/(A + T) and GC skew = (G − C)/(G + C) [39] was used to calculate the relative numbers of AT skew and GC skew, respectively. The relative synonymous codon usage (RSCU) of the PCGs of the two *Apodemus* species was obtained using PhyloSuite v1.2.2 [40]. After using the PhyloSuite v1.2.2 to obtain the coding sequence (CDS) and remove the stop codon from the sequence based on the codon matching pattern of the MAFFT algorithm and G-INS-i (accurate) strategy, the specific value of nonsynonymous to synonymous substitutions Ka/Ks values of the two *Apodemus* subspecies was calculated using the DnaSP software [41].

2.5. Phylogenetic Analysis

There are 21 genera in the Muridae family that are distributed in China [42]. To determine the phylogenetic position of *Apodemus agrarius ningpoensis* and *Apodemus draco draco*, we constructed a phylogenetic tree using the whole mitogenome sequences from 63 species of 14 genera in the Muridae family provided by NCBI and this study. *Ratufa bicolor* and *Pteromys volans* of the Sciuridae family were selected for outgroups [43] (Table 1). Mitochondrial genome sequences of 67 species were aligned using the MAFFT plug-in in PhyloSuitev1.2.2 based on the codon pattern and G-INS-i strategy for comparison. The aligned data were trimmed using trimA [41]. Bayesian inference (BI) phylogenetic analysis was based on the GTR model using MrBayes v3.2.2 [44,45]. The Markov chain Monte Carlo (MCMC) for BI analysis was started with a random tree, run 4 Markov chains, 1,000,000 generations, taking a sample every 1000 generations with a burn-in of 25% trees. The RAxMLv.7.3.0 [46] was used to construct the maximum likelihood (ML) tree, with GTRGAMMA as the default model; 1000 bootstrap repeat tests were used to verify branch reliability. The ChiPlot (https://www.chiplot.online/, accessed on 12 June 2022) was used to visualize and beautify the phylogenetic trees.

Table 1. Mitogenomes used in the analyses.

Genus	Species	Accession No.
Apodemus	*Apodemus chevrieri*	NC_017599.1
Apodemus	*Apodemus latronum*	NC_019585.1
Apodemus	*Apodemus flavicollis*	MN122902.1
Apodemus	*Apodemus peninsulae*	NC_016060.1
Apodemus	*Apodemus draco*	NC_019584.1
Apodemus	*Apodemus chejuensis*	NC_016662.1
Apodemus	*Apodemus agrarius*	NC_016428.1
Apodemus	*Apodemus sylvaticus*	NC_049122.1
Rattus	*Rattus andamanensis*	NC_046686.1
Rattus	*Rattus tiomanicus*	NC_029888.1
Rattus	*Rattus hoogerwerfi*	NC_049040.1
Rattus	*Rattus baluensis*	NC_035621.1
Rattus	*Rattus sordidus*	NC_014871.1

Table 1. *Cont.*

Genus	Species	Accession No.
Rattus	*Rattus lutreolus*	NC_014858.1
Rattus	*Rattus villosissimus*	NC_014864.1
Rattus	*Rattus tunneyi*	NC_014861.1
Rattus	*Rattus fuscipes*	NC_014867.1
Rattus	*Rattus leucopus*	NC_014855.1
Rattus	*Rattus niobe*	NC_023347.1
Rattus	*Rattus rattus*	NC_012374.1
Rattus	*Rattus praetor*	NC_012461.1
Rattus	*Rattus norvegicus*	NC_001665.2
Rattus	*Rattus tanezumi*	NC_011638.1
Rattus	*Rattus exulans*	NC_012389.1
Rattus	*Rattus nitidus*	NC_040919.1
Hapalomys	*Hapalomys delacouri*	MZ159976.1
Niviventer	*Niviventer cremoriventer*	NC_035822.1
Niviventer	*Niviventer lotipes*	NC_065402.1
Niviventer	*Niviventer sacer*	MZ935252.1
Niviventer	*Niviventer excelsior*	NC_019617.1
Niviventer	*Niviventer confucianus*	NC_023960.1
Niviventer	*Niviventer fulvescens*	NC_028715.1
Niviventer	*Niviventer andersoni*	NC_060500.1
Leopoldamys	*Leopoldamys sabanus*	NC_035819.1
Leopoldamys	*Leopoldamys edwardsi*	NC_025670.1
Mus	*Mus musculus musculus*	NC_010339.1
Mus	*Mus cervicolor*	NC_025269.1
Mus	*Mus cookii*	NC_025270.1
Mus	*Mus fragilicauda*	NC_025287.1
Mus	*Mus terricolor*	NC_010650.1
Mus	*Mus musculus helgolandicus*	KP877610.1
Mus	*Mus musculus castaneus*	NC_012387.1
Mus	*Mus musculus domesticus*	NC_006914.1
Mus	*Mus musculus molossinus*	NC_006915.1
Mus	*Mus spretus*	NC_025952.1
Mus	*Mus famulus*	NC_030342.1
Mus	*Mus pahari*	NC_036680.1
Mus	*Mus caroli*	NC_025268.1
Berylmys	*Berylmys berdmorei*	NC_036730.1
Brachiones	*Brachiones przewalskii*	KT834972.1
Maxomys	*Maxomys ochraceiventer*	NC_056988.1
Maxomys	*Maxomys surifer*	NC_036732.1
Maxomys	*Maxomys whiteheadi*	NC_049119.1
Bandicota	*Bandicota bengalensis*	NC_057104.1
Bandicota	*Bandicota indica*	KT029807.1
Micromys	*Micromys erythrotis*	NC_060316.1
Micromys	*Micromys minutus*	NC_027932.1
Chiropodomys	*Chiropodomys gliroides*	NC_049121.1
Meriones	*Meriones unguiculatus*	KF425526.1
Meriones	*Meriones tamariscinus*	NC_034314.1
Meriones	*Meriones meridianus*	NC_027684.1
Meriones	*Meriones libycus*	NC_027683.1
Rhombomys	*Rhombomys opimus*	MK359635.1
Pteromys	*Pteromys volans*	NC_019612.1
Ratufa	*Ratufa bicolor*	NC_023780.1

3. Results

3.1. Features of Mitogenomes

The mitochondrial genome length of *Apodemus agrarius ningpoensis* was 16,262 bp, and *Apodemus draco draco* was 16,222 bp. Both mitochondrial genomes were composed

of 37 genes (13 protein-coding genes (PCGs), 22 tRNAs, and 2 rRNAs) (Figures 1 and 2). Of these 37 genes, the light chain encoded 9 genes *ND6*, $tRNA^{Glu}$, $tRNA^{Pro}$, $tRNA^{Gln}$, $tRNA^{Ala}$, $tRNA^{Asn}$, $tRNA^{Cys}$, $tRNA^{Tyr}$, and $tRNA^{Ser}$, whereas the rest were detected in the heavy chain.

The overlapping and gapping regions in the mitogenomes of *Apodemus agrarius ningpoensis* and *Apodemus draco draco*, ranged from 1 bp to 7 bp. *ATP8* and *ATP6* (Table 2) had the longest overlap area. The completed mitochondrial genomes had a similar composition, sequences, and localization to other vertebrates. Interestingly, both mitochondrial genome sequences were biased toward A and T. The AT content of *Apodemus agrarius ningpoensis* was 63.74%, and that of *Apodemus draco draco* was 64.12%. The AT-skew value was greater than 0, whereas the GC skew value was less than 0, indicating that the base composition of *Apodemus agrarius ningpoensis* and *Apodemus draco draco* showed a strong A-bias and C-bias (Tables 3 and 4).

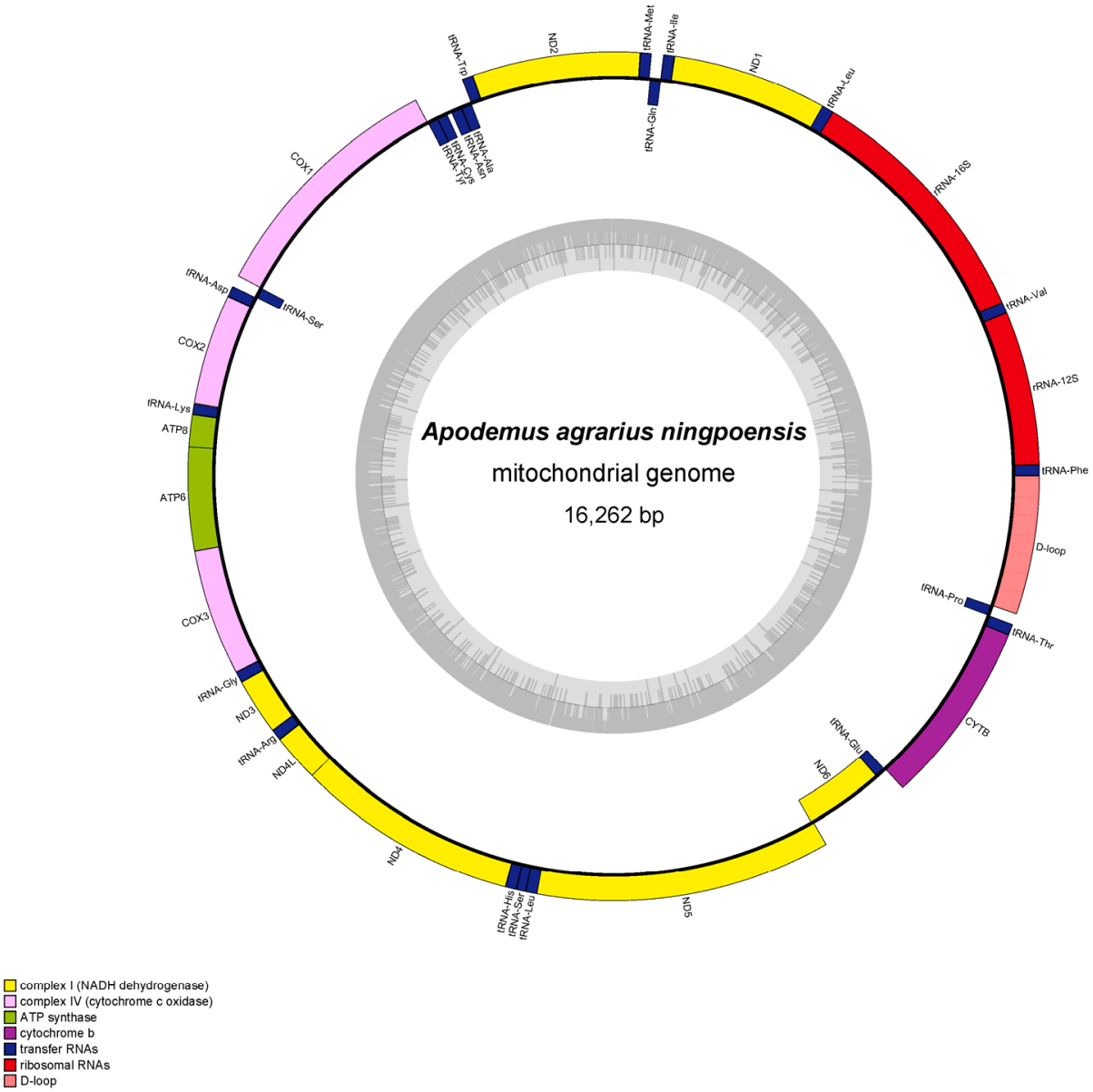

Figure 1. Annotated mitogenome of *Apodemus agrarius ningpoensis*.

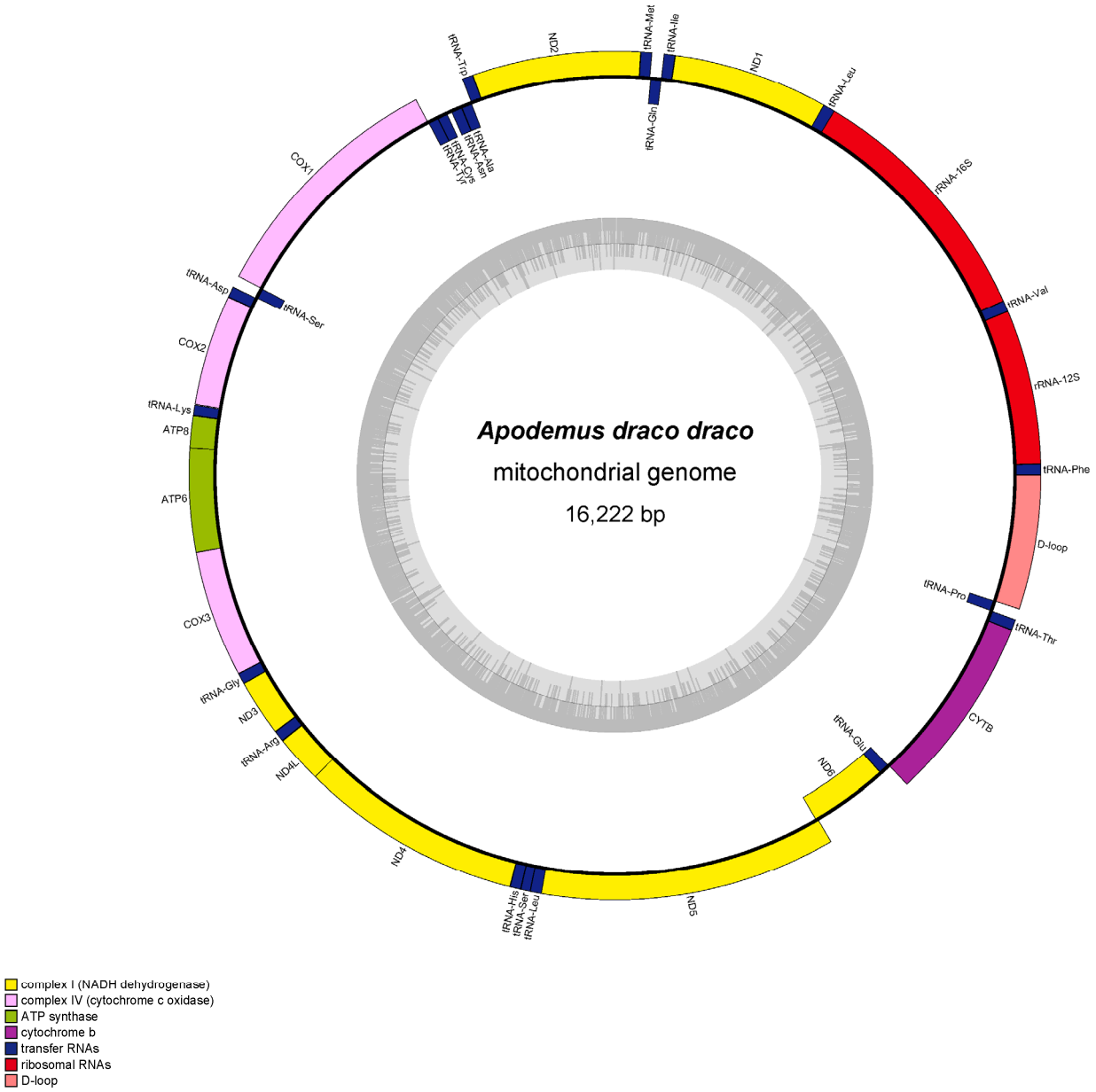

Figure 2. Annotated mitogenome of *Apodemus draco draco*.

3.2. Protein-Coding Genes and Codon Usage

The full length of the protein-coding genes of *Apodemus agrarius ningpoensis* and *Apodemus draco draco* was 11,406 bp and 11,202 bp, respectively. In the *Apodemus agrarius ningpoensis* mitogenome, the start codon of all PCGs was ATN, ATG was the most common start codon, identified in 9 PCGs (*COX1*, *COX2*, *ATP8*, *ATP6*, *COX3*, *ND4L*, *ND4*, *ND6*, and *CYTB*), whereas ATA was the start codon in the remaining PCGs. We observed that the *ND1*, *ND2*, *COX1*, *COX3*, *ND4*, and *CYTB* genes had the stop codon T-, whereas in the remaining seven PCGs had TAA. Interestingly, we detected that the third codon of PCGs tended to select base A. In the case of *Apodemus draco draco*, *ND1*, *ND2*, *ND3*, and *ND5* started with an ATT codon, unlike the rest PCGs that used ATG as the start codon. Similar to *Apodemus agrarius ningpoensis*, *ND1*, *ND2*, *COX1*, *COX3*, *ND4*, and *CYTB* had the stop codon T-, whereas the rest had TAA.

Table 2. Mitochondrial composition of *Apodemus agrarius ningpoensis* (left) and *Apodemus draco draco* (right).

Gene	Direction	Position (bp)	Size (bp)	Start Codon	Stop Codon	Intergenic Nucleotides
$tRNA^{Phe}$	+	1–67\1–68	67\68			0\0
12S rRNA	+	68–1022\69–1023	955\955			0\0
$tRNA^{Val}$	+	1023–1091\1024–1091	69\68			0\0
16S rRNA	+	1092–2665\1092–2660	1547\1569			0\0
$tRNA^{Leu}$	+	2664–2738\2659–2733	75\75			−2\−2
ND1	+	2736–3693\2731–3688	958\958	ATA\ATA	T-\T-	−3\−3
$tRNA^{Ile}$	+	3694–3762\3689–3757	69\69			0\0
$tRNA^{Gln}$	−	3760–3830\3755–3825	71\71			−3\−3
$tRNA^{Met}$	+	3835–3903\3830–3898	69\69			4\4
ND2	+	3094–4939\3899–4934	1036\1036	ATA\ATA	T-\T-	0\0
$tRNA^{Trp}$	+	4940–5007\4935–5002	68\68			0\0
$tRNA^{Ala}$	−	5009–5077\5004–5072	69\69			1\1
$tRNA^{Asn}$	−	5079–5149\5074–5144	71\71			1\1
$tRNA^{Cys}$	−	5181–5248\5176–5241	68\66			31\31
$tRNA^{Tyr}$	−	5249–5315\5242–5308	67\67			0\0
COX1	+	5317–6859\5310–6852	1543\1543	ATG\ATG	T-\T-	1\1
$tRNA^{Ser}$	−	6859–6927\6852–6920	69\69			−1\−1
$tRNA^{Asp}$	+	6931–6998\6924–6991	68\68			3\3
COX2	+	7000–7683\6993–7676	684\684	ATG\ATG	TAA\TAA	1\1
$tRNA^{Lys}$	+	7687–7750\7680–7743	64\64			3\3
ATP8	+	7752–7955\7745–7948	204\204	ATG\ATG	TAA\TAA	1\1
ATP6	+	7913–8593\7906–8586	681\681	ATG\ATG	TAA\TAA	−43\−43
COX3	+	8593–9376\8586–9369	784\784	ATG\ATG	T-\T-	−1\−1
$tRNA^{Gly}$	+	9377–9444\9370–9436	68\67			0\0
ND3	+	9445–9792\9437–9784	348\348	ATA\ATT	TAA\TAA	0\0
$tRNA^{Arg}$	+	9794–9861\9786–9853	68\68			1\1
ND4L	+	9864–10160\9856–10152	297\297	ATG\ATG	TAA\TAA	2\2
ND4	+	10154–11531\10146–11523	1378\1378	ATG\ATG	T-\T-	−7\−7
$tRNA^{His}$	+	11532–11599\11524–11590	68\67			0\0
$tRNA^{Ser}$	+	11600–11658\11591–11649	59\59			0\0
$tRNA^{Leu}$	+	11658–11728\11649–11719	71\71			−1\−1
ND5	+	11720–13549\11711–13540	1830\1830	ATA\ATA	TAA\TAA	−9\−9
ND6	−	13536–14054\13527–14042	519\516	ATG\ATG	TAA\TAA	−14\−14
$tRNA^{Glu}$	−	14055–14123\14043–14111	69\69			0\0
CYTB	+	14127–15270\14115–15258	1144\1144	ATG\ATG	T-\T-	3\3
$tRNA^{Thr}$	+	15271–15339\15259–15326	69\68			0\0
$tRNA^{Pro}$	−	15340–15406\15327–15393	67\67			0\0
D-loop	+	15407–16262\15394–16222	856\829			0\0

Table 3. Base content of the mitochondrial genome of *Apodemus agrarius ningpoensis*.

Apodemus agrarius ningpoensis	Size (bp)	A%	T%	G%	C%	A + T%	G + C%	AT Skew	GC Skew
Mitogenome	16,262	34.01	29.74	12.47	23.79	63.74	36.26	0.067	−0.312
PCGs	11,406	32.05	31.45	12.1	24.4	63.5	36.5	0.01	−0.337
tRNAs	1503	34.13	30.81	18.43	16.63	64.94	35.06	0.051	0.051
rRNAs	2529	37.92	26.49	16.77	18.82	64.41	35.59	0.177	−0.058
D-loop	856	32.71	31.66	11.92	23.71	64.37	35.63	0.016	−0.331

Table 4. Base content of the mitochondrial genome of *Apodemus draco draco*.

Apodemus draco draco	Size (bp)	A%	T%	G%	C%	A + T%	G + C%	AT Skew	GC Skew
Mitogenome	16,222	35.15	28.97	12.08	23.8	64.12	35.88	0.096	−0.327
PCGs	11,403	33.33	30.64	11.68	24.34	63.97	36.03	0.042	−0.352
tRNAs	1498	34.31	31.51	18.29	15.89	65.82	34.18	0.043	0.07
rRNAs	2524	39.26	25.79	16.32	18.62	65.06	34.94	0.207	−0.066
D-loop	829	31.97	29.67	13.15	25.21	61.64	38.36	0.037	−0.314

The codon usage of PCGs was evaluated based on RSCU values (Tables 5 and 6, Figures 3 and 4), CUA of Leu, CGA of Arg, and UCA of Ser were the most frequent codons among all 13 PCGs in *Apodemus agrarius ningpoensis*. In contrast, CGA of Arg, CCA of Pro, and UCA of Ser were the most commonly used codons in *Apodemus draco draco* PCGs.

Table 5. The codon usages of *Apodemus agrarius ningpoensis*.

Codon	No.	RSCU	Codon	No.	RSCU	Codon	No.	RSCU
UAA(*)	7	4	AAA(K)	98	1.98	CGU(R)	10	0.62
GCA(A)	93	1.62	AAG(K)	1	0.02	AGC(S)	36	0.72
GCC(A)	66	1.15	CUA(L)	258	2.58	AGU(S)	20	0.4
GCG(A)	4	0.07	CUC(L)	80	0.8	UCA(S)	138	2.77
GCU(A)	67	1.17	CUG(L)	19	0.19	UCC(S)	54	1.08
UGC(C)	20	1.38	CUU(L)	109	1.09	UCG(S)	2	0.04
UGU(C)	9	0.62	UUA(L)	123	1.23	UCU(S)	49	0.98
GAC(D)	37	1.04	UUG(L)	10	0.1	ACA(T)	161	2.13
GAU(D)	34	0.96	AUA(M)	198	1.7	ACC(T)	60	0.79
GAA(E)	78	1.68	AUG(M)	35	0.3	ACG(T)	4	0.05
GAG(E)	15	0.32	AAC(N)	92	1.1	ACU(T)	78	1.03
UUC(F)	97	0.79	AAU(N)	76	0.9	GUA(V)	85	2.01
UUU(F)	148	1.21	CCA(P)	125	2.51	GUC(V)	25	0.59
GGA(G)	101	1.92	CCC(P)	28	0.56	GUG(V)	9	0.21
GGC(G)	40	0.76	CCG(P)	3	0.06	GUU(V)	50	1.18
GGG(G)	26	0.5	CCU(P)	43	0.86	UGA(W)	93	1.81
GGU(G)	43	0.82	CAA(Q)	77	1.88	UGG(W)	10	0.19
CAC(H)	53	1.1	CAG(Q)	5	0.12	UAC(Y)	60	0.92
CAU(H)	43	0.9	CGA(R)	45	2.81	UAU(Y)	70	1.08
AUC(I)	146	0.79	CGC(R)	7	0.44			
AUU(I)	225	1.21	CGG(R)	2	0.12			

Asterisk "*" indicates that it is a stop codon and has no corresponding amino acid.

Table 6. The codon usages of *Apodemus draco draco*.

Codon	No.	RSCU	Codon	No.	RSCU	Codon	No.	RSCU
UAA(*)	7	4	AUU(I)	216	1.16	CGU(R)	7	0.44
GCA(A)	89	1.6	AAG(K)	3	0.06	CGA(R)	46	2.88
GCC(A)	76	1.36	AAA(K)	99	1.94	AGU(S)	10	0.2
GCG(A)	1	0.02	CUC(L)	66	0.67	UCA(S)	163	3.21
GCU(A)	57	1.02	CUG(L)	8	0.08	UCC(S)	37	0.73
UGC(C)	16	1.1	CUU(L)	104	1.06	UCG(S)	5	0.1
UGU(C)	13	0.9	UUA(L)	134	1.37	UCU(S)	46	0.9
GAC(D)	48	1.33	UUG(L)	13	0.13	AGC(S)	44	0.87
GAU(D)	24	0.67	CUA(L)	264	2.69	ACC(T)	71	0.92
GAA(E)	84	1.79	AUG(M)	28	0.23	ACG(T)	4	0.25
GAG(E)	10	0.21	AUA(M)	211	1.77	ACU(T)	61	0.79
UUC(F)	116	0.95	AAU(N)	72	0.88	ACA(T)	173	2.24
GGA(G)	118	2.26	CCC(P)	16	0.32	GUG(V)	10	0.24
GGC(G)	27	0.52	CCU(P)	36	0.73	GUU(V)	38	0.91
GGG(G)	19	0.36	CCA(P)	145	2.94	GUA(V)	101	2.42
GGU(G)	45	0.86	CAG(Q)	1	0.02	UGA(W)	98	1.9
CAC(H)	60	1.21	CAA(Q)	81	1.98	UGG(W)	5	0.1
CAU(H)	39	0.79	CGC(R)	10	0.62	UAC(Y)	59	0.91
AUC(I)	157	0.84	CGG(R)	1	0.06	UAU(Y)	70	1.09

Asterisk "*" indicates that it is a stop codon and has no corresponding amino acid.

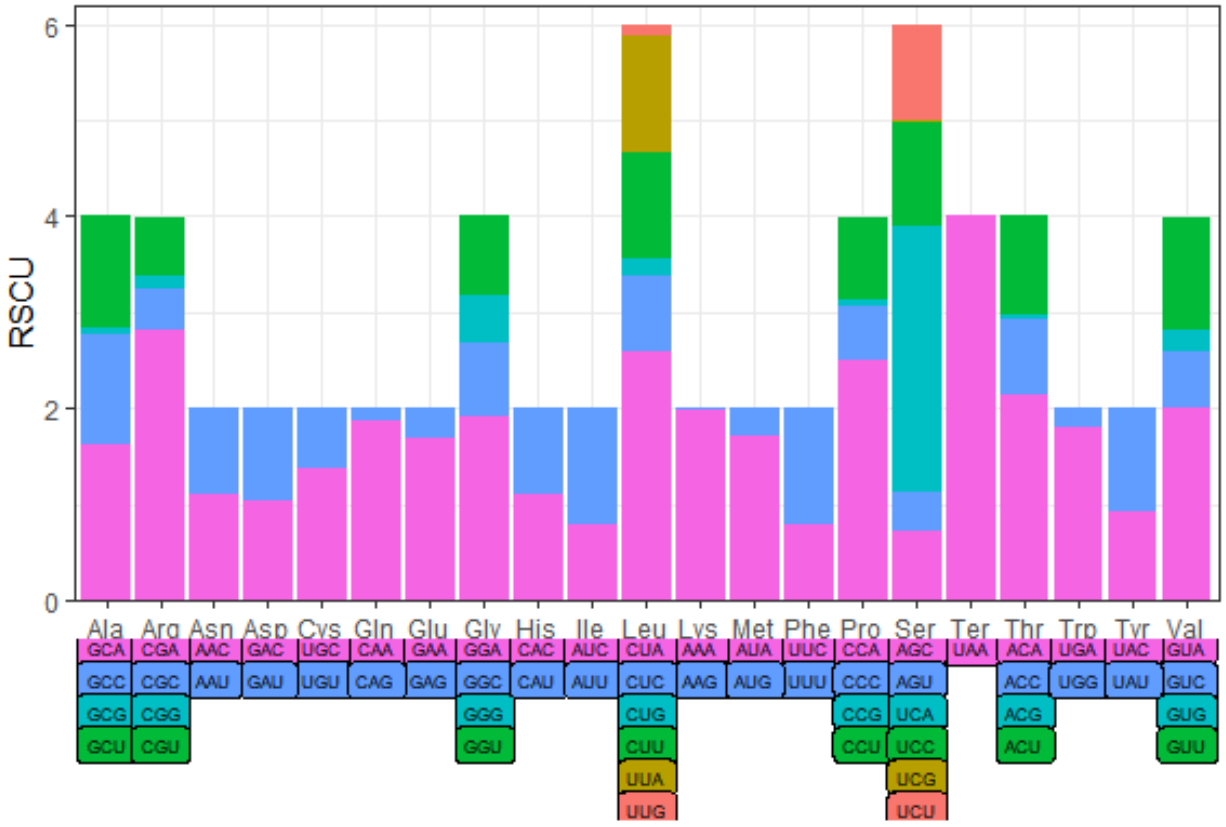

Figure 3. RSCU values of *Apodemus agrarius ningpoensis*.

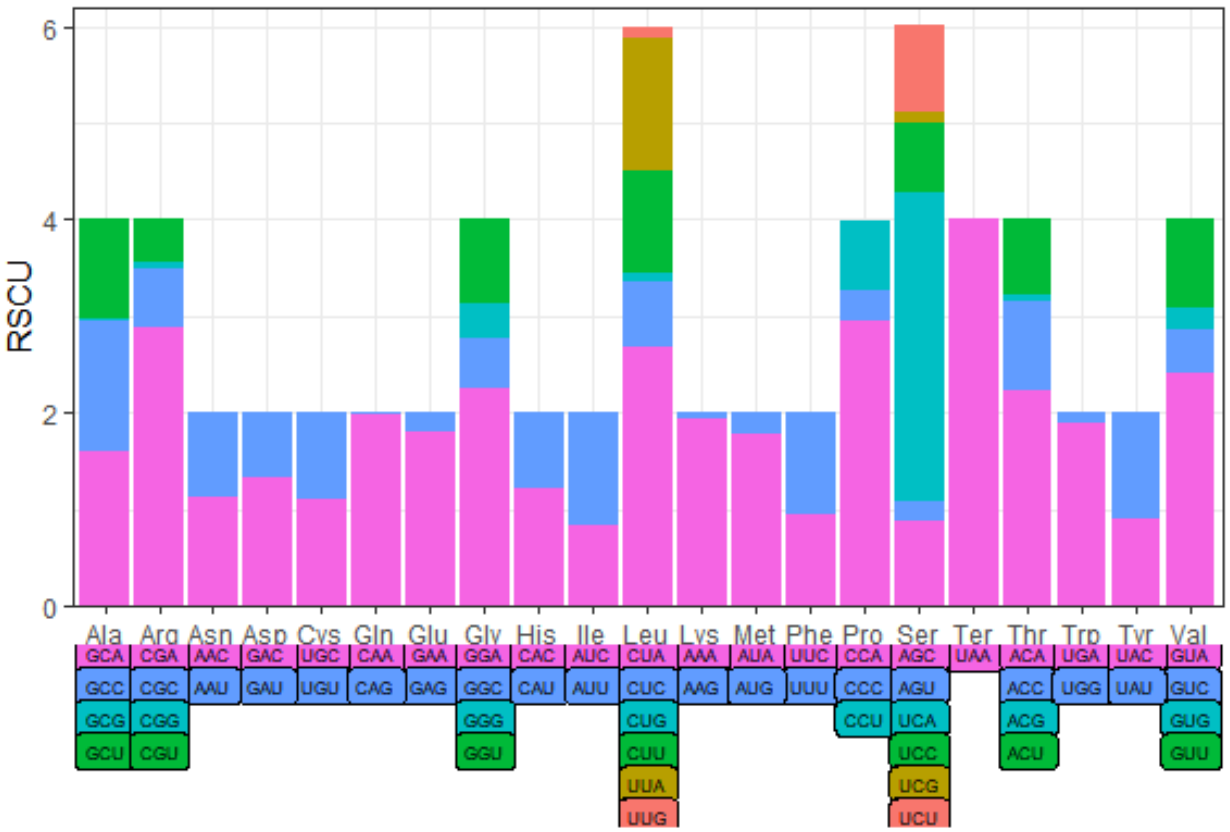

Figure 4. The RSCU values of *Apodemus draco draco*.

We calculated the Ka/Ks values for 13 PCGs from 65 Muridae species (Figure 5). All Ka/Ks values were smaller than 1, indicating genes subjected to purification selection. In particular, the mean and median Ka/Ks values were the highest for *ATP8*, suggesting that *ATP8* had the highest evolution speed, the lowest for *COX1*, indicating the slowest evolution.

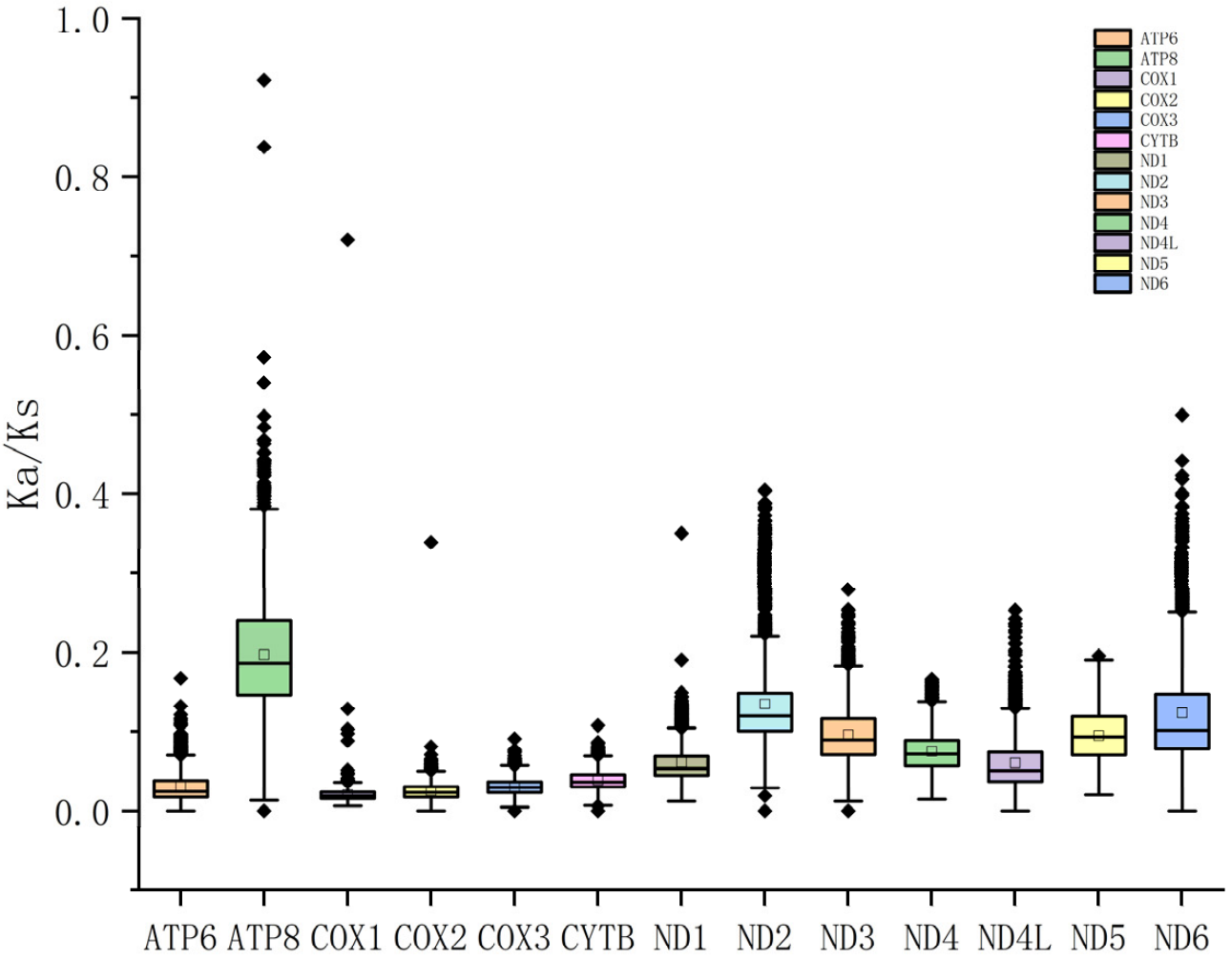

Figure 5. The Ka/Ks values for the 13 PCGs of 65 Muridae mitogenomes.

3.3. tRNAs and rRNAs

As expected, both *Apodemus agrarius ningpoensis* and *Apodemus draco draco* had 22 tRNA genes in their mitochondrial genomes, of which 9 were encoded on the negative chain, and the remaining 13 were encoded on the positive chain (Figures 6 and 7). The 12S rRNA and 16S rRNA genes were located between the $tRNA^{Phe}$ and $tRNA^{Leu}$ genes and segregated by the $tRNA^{Val}$ gene. In addition, $tRNA^{Ser}$ lacked the entire dihydrouracil (DHU) arm, $tRNA^{Lys}$ had a DHU arm lacking the D-loop to form a stable secondary structure, while the rest could form a cloverleaf structure. We further identified 36 mismatches in the *Apodemus agrarius ningpoensis* mitogenome, including 28 G-U, forming weak hydrogen bonds in tRNAs, as well as atypical A-A2, U-U1, A-G1, U-U2, C-U1, and A-C1. Similarly, there were 33 mismatches in the *Apodemus draco draco* mitogenome, including G-U26, U-U1, A-A2, A-G1, C-A2, and C-U1.

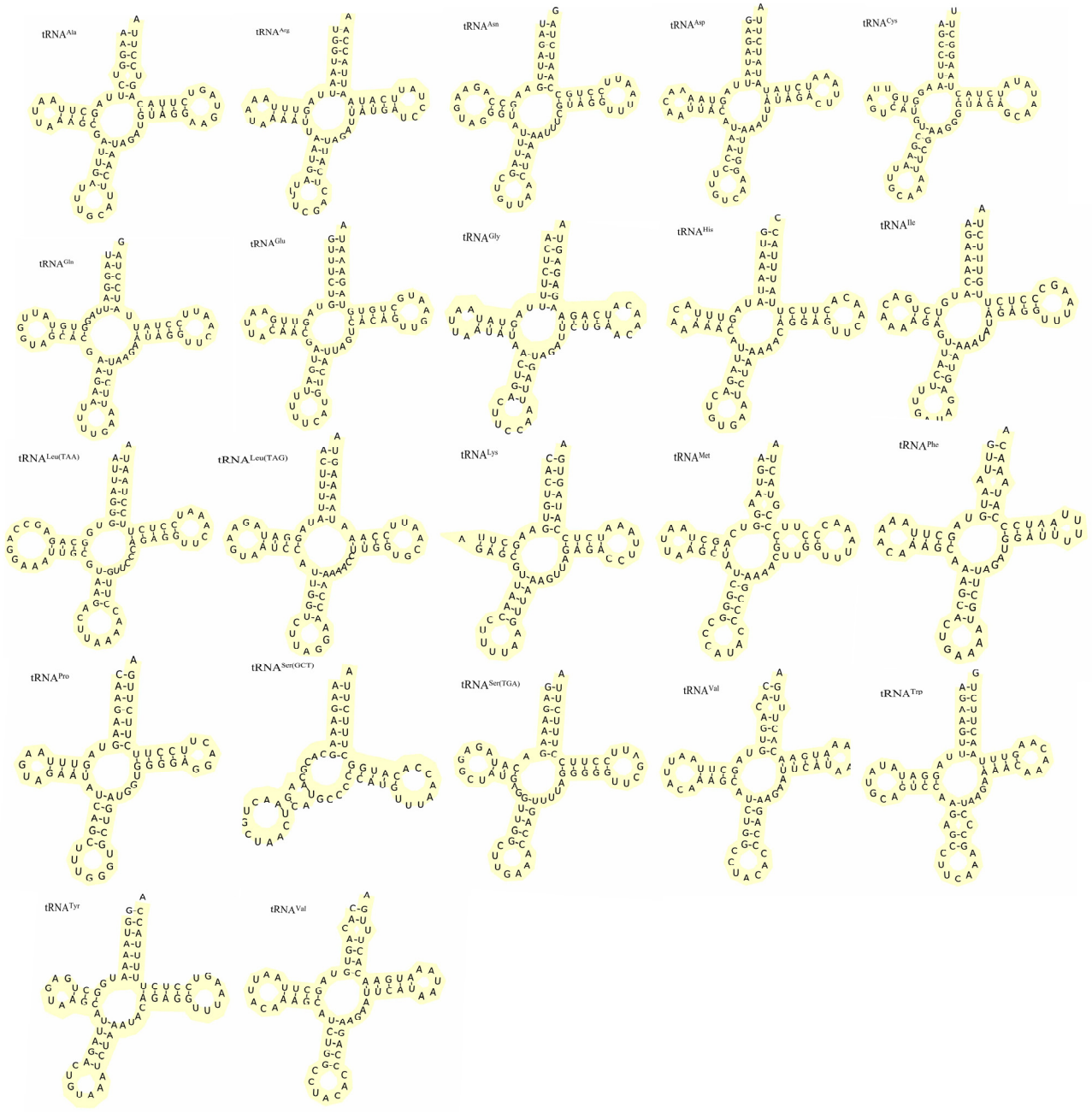

Figure 6. Structures of tRNA of *Apodemus agrarius ningpoensis*.

3.4. Phylogenetic Analysis

Phylogenetic trees were constructed based on ML and BI methods and yielded a consistent topology (Figures 8 and 9). Most branches of the BI and ML trees received high node support. We identified two major clades in the Muridae family. One clade consisted of the genera *Rhombomys*, *Meriones*, *Brachiones*, and *Hapalomys*, whereas the others were composed of the genera *Micromys*, *Chiropodomys*, *Apodemus*, *Mus*, *Maxomys*, *Leopoldamys*, *Niviventer*, *Berylmys*, *Bandicota*, and *Rattus*. Interestingly, the genera *Hapalomys*, *Micromys*, *Chiropodomys*, *Apodemus*, *Mus*, *Maxomys*, *Leopoldamys*, *Niviventer*, *Berylmys*, *Bandicota*, and *Rattus* exhibited monophyly. However, the genus *Meriones* did not form a monophyletic group, and was first grouped with the genus *Brachiones*, followed by the genus *Rhombomys* g, and finally with the genus *Meriones*. The other branch of the relationship was (*Apodemus* + *Mus*) + (*Maxomys* + ((*Leopoldamys* + *Niviventer*) + (*Berylmys* + (*Bandicota* + *Rattus*))). Several members of the genus *Apodemus* formed an evident monophyletic group, which

was divided into two small branches, among which *Apodemus agrarius ningpoensis* and *Apodemus chevrieri* were first clustered into a clade. Subsequently, it was grouped with *Apodemus peninsulae*, constituting a monophyletic group. The second group was composed of *Apodemus flavicollis* and *Apodemus sylvaticus*. The monophyletic lineage of the genus *Apodemus* was well supported by the sisters of the genus *Mus*; moreover, the first genus to be grouped with the genus *Apodemus* was the genus *Mus*.

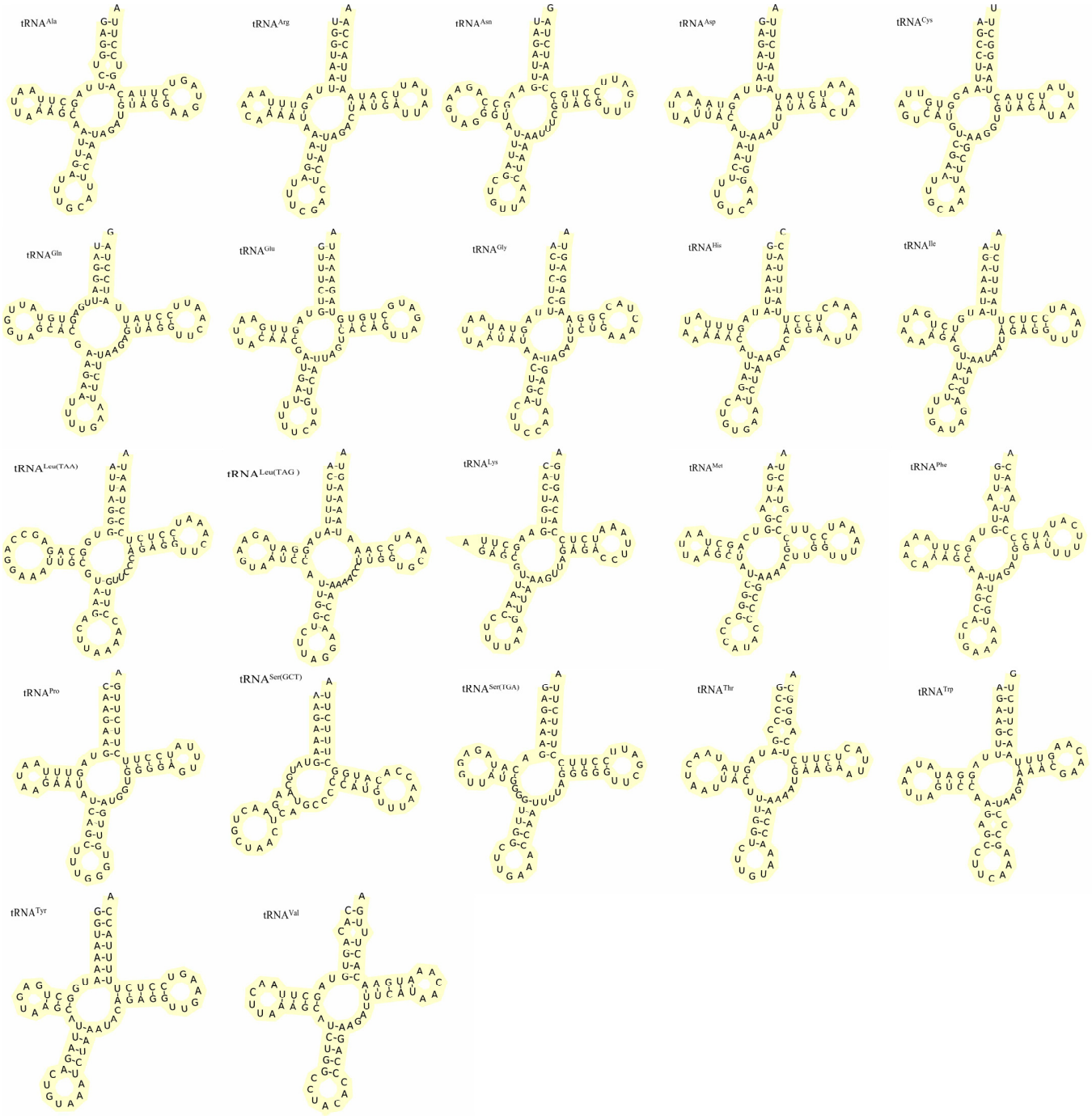

Figure 7. Structures of tRNA of *Apodemus draco draco*.

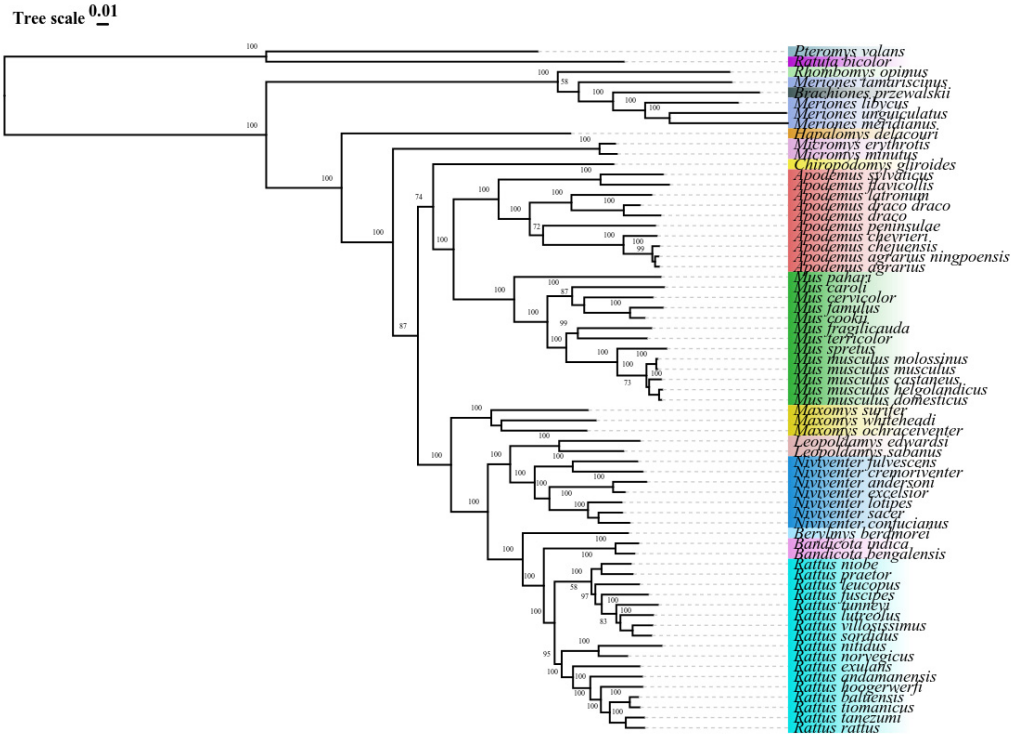

Figure 8. Phylogenetic tree inferred from mitochondrial genome sequences using ML. Bootstrap support values are indicated on branches. Different genera are distinguished by different colors.

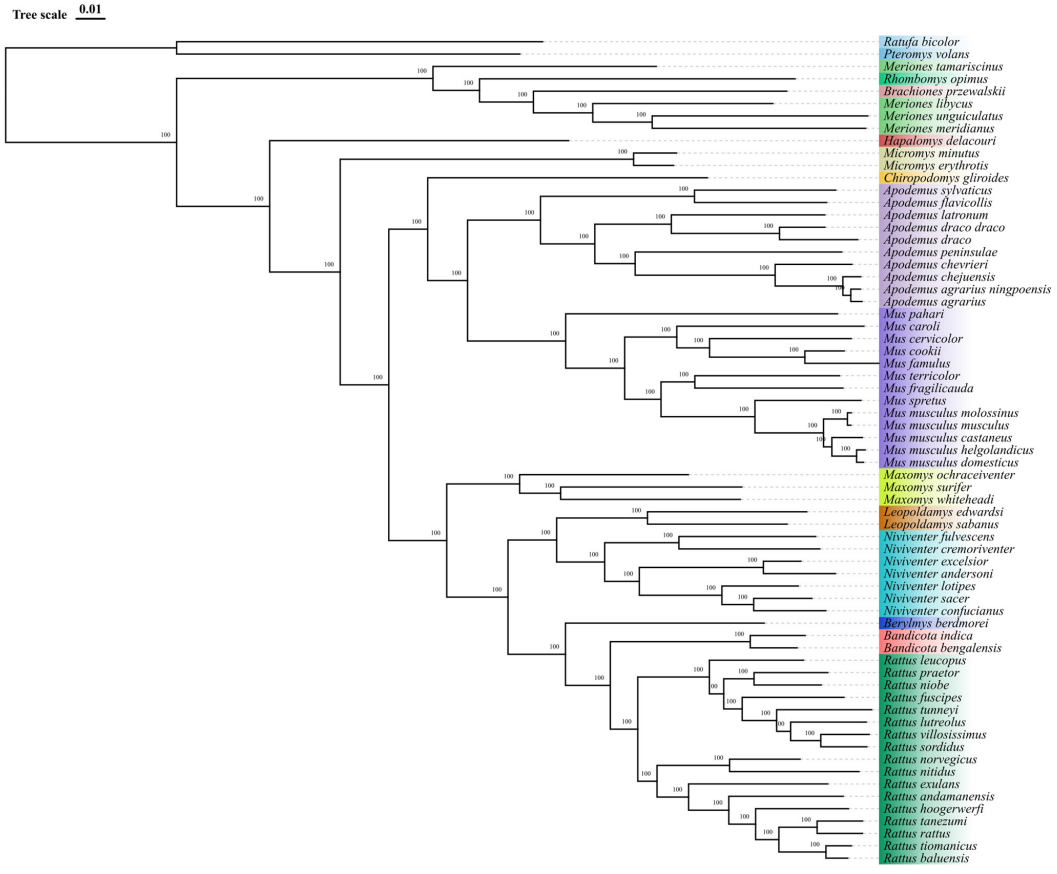

Figure 9. Phylogenetic tree inferred from mitochondrial genome sequences using BI. Bootstrap support values are indicated on branches. Different genera are distinguished by different colors.

4. Discussion

The length of the mitochondrial genome analyzed in this study was consistent with that of mammals, which is generally approximately 16,000 bp long [47]. The mitochondrial genome structure of *Apodemus agrarius ningpoensis* and *Apodemus draco draco* is a double-stranded closed loop, containing a noncoding control region sequence and encoding 37 genes. The distribution of mtDNA genes was not homogeneous. Most mitochondrial genes were encoded on the heavy chain, except for the *ND6* gene and eight tRNA genes, which are encoded on the light chain. The apparent A-T preference in base composition was consistent with the basic characteristics of the Muridae mitochondrial genome, which is related to a base composition bias associated with the shedding of purine or pyrimidine bases from heavy chains during replication [48]. This high AT content and mutation rate of the mitochondrial genome have also been suggested to accelerate the evolutionary process in rodents [49,50]. Most animal mitochondrial genomes usually have obvious strand-specific biases, as expressed by AT and GC skews. The mitogenomes of the two species examined in this research showed positive AT, and negative GC skews, suggesting that the base composition of the whole mitogenomes of *Apodemus agrarius ningpoensis* and *Apodemus draco draco* is biased toward A and C. Synonymous codon frequency is generally biased owing to natural selection or mutational reasons, with different pressures on gene evolution. RSCU calculations showed that the protein-coding gene region of *Apodemus agrarius ningpoensis* and *Apodemus draco draco* had 3800 and 3799 codons, respectively. The codon CUA, CUU, UUA, and UCA determining Leu and Ser with RSCU values greater than 1, occupied a higher percentage in these mitogenomes, thus explaining the negative GC skew exhibited by the PCGs. The RSCU values indicate that there are significantly more bases in the third position of the codon for A and T than for G and C. This may be attributed to the AT preference for selective avoidance of termination codon formation and amino acid loss [51].

The calculation of Ka/Ks values revealed that *ATP8* had the highest mean and median Ka/Ks values, implying that *ATP8* had the fastest evolution among all PCGs, and is thus likely to be under relaxed selective pressure, accumulating more mutations [52]. In contrast, *COX1* had the lowest mean and median Ka/Ks values, indicating it was under greater evolutionary pressure. All 13 PCGs had Ka/Ks values smaller than 1, indicating that sites underwent purification selection. It determines the development of the mitogenome [53].

Among the predicted 22 tRNA structures, we observed that $tRNA^{Ser}$ lacked the DHU arm and the DHU loop of $tRNA^{Lys}$ failed to form a typical cloverleaf structure. Most mammalian mitochondrial genomes lack DHU arms, which might be related to the compensatory mechanism between tRNA arms [22,54,55].

The presence of gene overlap and spacing in both species and gene overlap regions implied that Muridae species need to continuously improve DNA utilization from a supply-side perspective to meet evolutionary demands, which is extremely beneficial to the evolutionary process.

Both sequences had multiple G-U mismatches, which might be mainly attributed to the low free energy of the bond, which makes it stable and neutral [56]. This usually occurs in invertebrates and is rectified by post-transcriptional processing of RNA [57].

Currently, studies on the evolution and taxonomy of the Chinese *Apodemus* populations have concentrated on mitochondrial single gene sequences such as D-loop region and cytb. However, the mitochondrial genome can provide a better understanding of phylogenetic relationships [6,24]. The phylogenetic trees of the gene sequences of 63 rodent species in 14 genera of the Muridae family constructed using the BI and ML methods in this study were largely consistent, with bootstrap values of evolutionary branches >70% being considered indicative of strong relationships. This indicated a close phylogenetic relationship between species. The phylogenetic analysis of Muridae in this study was consistent with a system based on *COB*, *IRBP*, and *GHR* genes [43,58], albeit with the use of different datasets. We identified two major clades in the Muridae family. One clade consisted of the genera *Rhombomys*, *Meriones*, *Brachiones*, and *Hapalomys*, whereas the others were composed of the

genera *Micromys*, *Chiropodomys*, *Apodemus*, *Mus*, *Maxomys*, *Leopoldamys*, *Niviventer*, *Berylmys*, *Bandicota*, and *Rattus*. Except for *Rhombomys opimus*, which originated from the same ancestor with the genera *Brachiones* and *Meriones*, the majority of Muridae species formed their own branches on a genus basis, which was consistent with previous studies [43]. *Brachiones przewalskii* has been reported to be nested in a diverse taxon corresponding to *Rhombomys opimus* and separated from *Meriones* [22]. In addition, *Meriones tamariscinus* formation predated the separation of *Brachiones* from *Rhombomys* [59], with *Meriones tamariscinus* being closely related to *Rhombomys* but more distantly related to any other *Meriones* species. The results of the study by Ito et al. supported our findings [60]. These morphological features might be associated with surrounding habitats. Ecological specialization allows for molecular and phenotypic evolution independent of each other [22,61]. Interestingly, the topology of the phylogenetic tree changed when other mitochondrial genomes were added to the study. The topology of the phylogenetic tree also changed when different species were selected for tree building.

Parallel analyses in the nuclear genome and mitogenome may probably influence phylogenetic studies in the future. The cytogenetic characteristics of the analyzed species are interesting [62]. *Apodemus agrarius* and *Apodemus draco* belong to the *Apodemus* group and *Draco* group, respectively, based on morphological characteristics. The karyotype of *Apodemus agrarius* had 3–5 biarmed metacentric autosome pairs (2n = 48, FNa = 54), whereas that of *Apodemus draco* had one small metacentric pair (2n = 48, FNa = 48). The karyotype of *Apodemus chevrieri*, a sister species of *Apodemus agrarius*, possesses four small metacentric pairs (2n = 48, FNa = 54), which was consistent with the standard karyotype of *Apodemus agrarius*. The sister species of *Apodemus draco*, *Apodemus latronum*, has a similar karyotype to *Apodemus draco*. However, they have different chromosome histotypes, and central heterochromatin is present in many distal center pairs. Two DNA phylogenetic species groups were distinct. Chromosomal rearrangements at the cytological level have contributed to the formation of post-mating reproductive isolation.

5. Conclusions

We reported and analyzed the mitochondrial genomes of *Apodemus agrarius ningpoensis* and *Apodemus draco draco* for the first time. The basic features of the mitogenomes of *Apodemus agrarius ningpoensis* and *Apodemus draco draco*, such as base content, gene arrangement, codon usage, and structures of tRNA, were highly conserved, also showing a relative abundance of AT content and a relative lack of GC content, which were consistent with the basic features of the Muridae mitochondrial genome. The new mitochondrial genome data contributed to shedding light on the phylogenetic relationship of the family Muridae and also revealed the phylogenetic position of *Apodemus agrarius ningpoensis* and *Apodemus draco draco*. The phylogenetic relationships among the various groups are consistent with related studies. It will provide genetic data for further evolutionary study of *Apodemus*.

In the future, developing primers using the mitochondrial genome sequence can help identify the most abundant loci in different rodent polymorphic types and combine the data with the construction of DNA barcode candidate markers to distinguish rodent populations. The data can be used for comparative analysis of mitochondrial genomes of different species, to understand disease information related to the source of the samples, and to provide a reference basis for the conservation and utilization of germplasm resources of the genus *Apodemus*. However, because of their wide distribution, representative *Apodemus* species exhibit particularly high levels of morphological and genetic variations. Expanding the sample size of the geographical population of *Apodemus* will help to better understand the genetic differentiation, dispersal, and phylogenetic relationships of *Apodemus*, as well as the influence of environmental mechanisms on its evolution.

Author Contributions: Conceptualization, L.Z.; data curation, D.W.; methodology, D.W.; software, D.W.; formal analysis, D.W.; visualization, D.W.; validation, D.W.; investigation, D.W., J.X., Q.X. and L.M.; funding acquisition, L.Z.; project administration, L.Z.; resource, L.Z.; supervision, L.Z.; writing—original draft preparation, D.W.; writing—review and editing, D.W. and L.Z. All authors have read and agreed to the published version of the manuscript.

Funding: This research was supported by grants for the National Science and Technology Fundamental Resources Investigation Program of China (2019FY101804).

Institutional Review Board Statement: The animal study protocol was approved by the Institutional Animal Care and Use Committee of Anhui Zoological Society (2022102001) on 20 October 2022.

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

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