Short Communication

Complete mitochondrial genome and codon usage of the Nepalese whiskered bat *Myotis muricola* (Vespertilionidae)

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ABSTRACT. We sequenced and characterized the complete mitogenome of the Nepalese whiskered bat *Myotis muricola* (Vespertilionidae) to provide more data for comparative mitogenomics and codon usage in the genus *Myotis* (Vespertilionidae). The mitogenome of *M. muricola* is a circular molecule of 17,224 bp, consisting of a control region and a conserved set of 37 genes containing 13 protein-coding genes (PCGs), 22 tRNA genes, and two rRNA genes (*12S rRNA* and *16S rRNA*). The mitogenome of *M. muricola* is AT-biased, with a nucleotide composition of 33.6% A, 29.7% T, 23.3% C, and 13.4% G. The total length of the 13 mitochondrial PCGs, excluding stop codons, is 11,376 bp, or 3792 amino acids. The relative synonymous codon usage (RSCU) of codons ending in A/T was generally higher than that for codons ending in G/C. The most frequently used codons are CTA(Leu) and CGA(Arg), with RSCU values greater than 2.0. The most rarely used codons, all terminating in G, are TCG(Ser), CCG(Pro), GCG(Ala), AAG(Lys), TGG(Try), CGG(Arg), and ACG(Thr), with RSCU values below 0.2. TCG(Ser) occurs only five times, and has the lowest

RSCU value (0.091). These results are valuable for a better understanding of the molecular evolution of mitogenomes in the genus *Myotis*.

Key words: Mitochondrial genome; Codon usage; Nepalese whiskered bat; *Myotis muricola*; Vespertilionidae

INTRODUCTION

Animal mitogenomes are double-stranded circular molecules, generally 16 to 18 kb in size, encoding 13 protein-coding genes (PCGs), two ribosomal RNA genes (*12S rRNA* and *16S rRNA*), 22 transfer RNA genes (*tRNAs*), and a non-coding control region (CR) that are essential for the initiation of transcription and DNA replication (Boore, 1999). Because mitogenomes differ to nuclear genomes in many ways (e.g., they are inherited maternally, experience higher mutation rates, and do not undergo recombination), they are useful in studies of phylogenetics, population genetics, and molecular evolution (Moritz et al., 1987). A few rapidly evolving regions within the mitogenome (e.g., the CR) are being used with increasing frequency to study phylogenetic relationships and population genetics. Genome-level analyzes that include nucleotide composition, codon usage, gene order arrangement, and secondary structures of tRNAs and rRNAs, are powerful tools for inferring higher-level phylogenies and investigating molecular evolution (Boore, 1999).

With about 1,240 species, bats are the second largest order of mammals and represent around 20% of all classified mammal species (Myers et al., 2008). Of the 18 bat families, the Vespertilionidae contain over 300 species and represent the largest and best-known bat family (Schnitzler and Kalko, 2001; Simmons, 2005). To date, however, the mitogenomes of only 17 vespertilionid species have been completely sequenced and analyzed. In this study, we sequenced and characterized the complete mitogenome of the Nepalese whiskered bat *Myotis muricola* (Vespertilionidae) to provide more data for comparative mitogenomics and codon usage in the genus *Myotis* (Vespertilionidae).

MATERIAL AND METHODS

Specimen collection and DNA extraction

M. muricola is widespread in Southeast Asia, ranging from East India to South China, Indonesia, and Malaysia (Simmons, 2005; Wiantoro et al., 2012). A *M. muricola* individual was caught in the academic research forests of the University Putra Malaysia (Malaysia) using a mist-net (Avinet, Dryden, NY, USA). A fresh wing membrane tissue sample was preserved in 100% ethanol and stored at -20°C. Total genomic DNA was extracted using a DNeasy® Blood & Tissue Kit (Qiagen, Valencia, CA, USA) according to the manufacturer protocol.

Primer design, polymerase chain reaction (PCR), and sequencing

Primers for PCR amplification were designed based on multiple alignments of complete mitogenomes of *Myotis ikonnikovi* (KF111724) and *M. macrodactylus* (KF440685) accessed from GenBank (Nam et al., 2013; Yoon et al., 2013). PCR amplification was performed in a final 25 µL reaction volume containing 10 mM Tris-HCl (pH 8.4), 50 mM KCl, 4 mM $MgCl_2$, 200 mM each dNTP, 50 pmol each primer, 2 U ExTaq polymerase, and 1 µL DNA sample, using the following

protocol: denaturation for 5 min at 94°C, followed by 35 cycles of denaturation for 1 min at 94°C, annealing for 30 s at 48°-56°C, and extension for 1 min at 72°C; and a final extension for 10 min at 72°C. PCR products were resolved by electrophoresis on 1.0% agarose gel, and extracted using a DNA Gel Extraction Kit (Qiagen, Valencia, CA, USA). Extracted DNA was sent to Biomedic Co. Ltd. (Bucheon, South Korea) for sequencing using a primer-walking strategy.

Genome annotation

The complete mitogenome of *M. muricola* (KT213444) was aligned with those of *M. ikonnikovi* and *M. macrodactylus* using the Clustal-W program found in Geneious Pro 5.5.9 (Biomatters, Auckland, New Zealand), and the *M. muricola* mitogenome was annotated based on gene organization information from the latter two mitogenomes. The 13 PCG sequences were translated into amino-acid sequences using the vertebrate mitogenome genetic code, and the pattern of relative synonymous codon usage (RSCU) in the PCGs was analyzed in DAMBE version 4.2.13 (Xia and Xie, 2001). The tRNA scan-SE search server and the ARWEN web server with default parameters were used to identify tRNA genes and locate potential stem-loop secondary structures within these genes (Lowe and Eddy, 1997; Bernt et al., 2013). Skewness of nucleotide composition was calculated according to the following formulas: AT skew = [A − T]/[A + T] and GC skew = [G − C]/[G + C] (Lobry, 1996). Tandem repeats were located in the CR using Tandem Repeats Finder (Benson, 1999).

RESULTS AND DISCUSSION

Mitogenome organization

The complete mitogenome of *M. muricola* contains 17,224 bp, which is similar in size to other mitogenomes in the genus *Myotis* (Kim et al., 2011; Nam et al., 2013; Yoon et al., 2013; Wang et al., 2014). It consists of a CR and a conserved set of 37 vertebrate mitochondrial genes including 13 PCGs, 22 tRNA genes, and two rRNA genes (*12S rRNA* and *16S rRNA*) (Table 1). The order and orientation of these genes are identical to those in other *Myotis* species (Kim et al., 2011; Nam et al., 2013; Yoon et al., 2013; Wang et al., 2014). *Nd6* and eight *tRNAs* are located on the light strand, while the other 12 PCGs, 14 *tRNAs*, and two *rRNAs* are located on the heavy strand (Figure 1). The control region is located between $tRNA^{Pro}$ and $tRNA^{Phe}$, as seen in other *Myotis* mitogenomes (Kim et al., 2011; Nam et al., 2013; Yoon et al., 2013; Wang et al., 2014).

Nucleotide composition

The mitogenome of *M. muricola* is AT-biased, with a nucleotide composition of 33.6 A, 29.7 T, 23.3 C, and 13.4% G (Table 2). In all regions of the *M. muricola* mitogenome, the AT skew is positive and the GC skew is negative. This is similar to the bias seen in other *Myotis* mitogenomes (Kim et al., 2011; Nam et al., 2013; Yoon et al., 2013; Wang et al., 2014). Intergenic spacers have a higher AT content than other regions. *tRNAs* consist of 64.4% AT and the CR consists of 30% AT. The 13 PCGs are AT-biased, with an average AT content of 63.6 ± 2.9%, ranging from 59.4% in *Cox3* to 72.1% in *Atp8* (Table 3). The AT skew was positive in eight PCGs (*Nd1, Nd2, Cox2, Atp8, Atp6, Nd4, Nd5*, and *Nd6*) while the GC skew was negative in all 13 PCGs. In codons within the PCGs, A is more common than other bases at the 1st and 3rd positions, while T is the most

common base found at the 2nd position. The AT content at the 3rd position (70.7%) is higher than that at the 1st (56.5%) or at the 2nd (62.2%) position. In the 13 PCGs, the highest AT content is found in *Atp8*, while relatively low AT content is found in *Cox3* (Table 3). This asymmetrical base composition may be due to codon usage bias (Foster et al., 1997; Singer and Hickey, 2000).

Table 1. Gene organization and characterization of the *Myotis muricola* mitogenome.

	Start position	Stop position	Length (bp)	Anticodon	Start codon	Stop codon	Strand
tRNA^{Phe}	1	68	68	GAA			+
12S rRNA	69	1032	964				+
tRNA^{Val}	1033	1099	67	TAC			+
16S rRNA	1100	2675	1576				+
tRNA$^{Leu(CUN)}$	2676	2750	75	TAA			+
Nd1	2756	3712	957		ATG	TAA	+
tRNA^{Ile}	3712	3780	69	GAT			+
tRNA^{Gln}	3778	3851	74	TTG			-
tRNA^{Met}	3852	3921	70	CAT			+
Nd2	3922	4965	1044		ATC	TAG	+
tRNA^{Trp}	4964	5031	68	TCA			+
tRNA^{Ala}	5037	5105	69	TGC			-
tRNA^{Asn}	5107	5178	72	GTT			-
O_R	5179	5213	35				+
tRNA^{Cys}	5211	5276	66	GCA			-
tRNA^{Tyr}	5277	5343	67	GTA			-
Cox1	5345	6889	1545		ATG	TAA	+
tRNA$^{Ser(UCN)}$	6901	6969	69	TGA			-
tRNA^{Asp}	6977	7043	67	GTC			+
Cox2	7044	7727	684		ATG	TAA	+
tRNA^{Lys}	7731	7798	68	TTT			+
Atp8	7800	8003	204		ATG	TAA	+
Atp6	7961	8641	681		ATG	TAA	+
Cox3	8641	9424	784		ATG	T-	+
tRNA^{Gly}	9425	9493	69	TCC			+
Nd3	9494	9840	347		ATA	TA-	+
tRNA^{Arg}	9841	9909	69	TCG			+
Nd4L	9911	10207	297		ATG	TAA	+
Nd4	10201	11578	1378		ATG	T-	+
tRNA^{His}	11579	11646	68	GTG			+
tRNA$^{Ser(AGY)}$	11647	11705	59	GCT			+
tRNA$^{Leu(UUR)}$	11707	11776	70	TAG			+
Nd5	11777	13597	1821		ATA	TAA	+
Nd6	13581	14108	528		ATG	TAA	-
tRNA^{Glu}	14109	14177	69	TTC			-
Cytb	14184	15323	1140		ATG	AGA	+
tRNA^{Thr}	15324	15392	69	TGT			+
tRNA^{Pro}	15392	15458	67	TGG			-
Control region	15460	17224	1765				+

Protein-coding genes and codon usage

The PCGs consist of 11,376 bp excluding stop codons. This is equivalent to 3792 amino acids (Table 2). Some mammalian mitogenomes contain compact overlapping regions between PCGs (Fernandez-Silva et al., 2003). We found a 43-bp overlap between *Atp8* and *Atp6*, a single-base pair overlap between *Atp6* and *Cox3*, a 7-bp overlap between *Nd4L* and *Nd4*, and a 17-bp overlap between *Nd5* and *Nd6* (Table 1), which are consistent with other *Myotis* mitogenomes (Kim et al., 2011; Nam et al., 2013; Yoon et al., 2013; Wang et al., 2014).

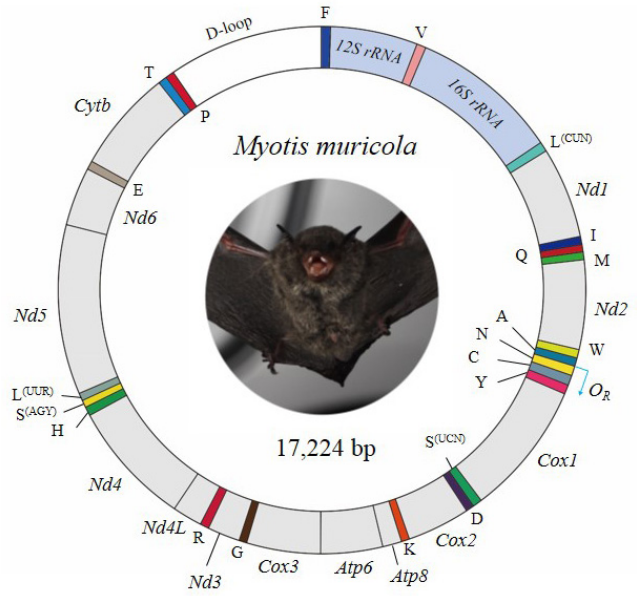

Figure 1. Map of the circular mitogenome of *Myotis muricola*. All protein-coding genes are encoded on the H strand (heavy strand), with the exception of *Nd6*, which is encoded on the L strand (light strand). Genes for tRNAs are denoted by one-letter symbols according to the IUPAC-IUB single-letter amino acid codes. The replication origin is denoted as O_R. The H strand in the outer circle encodes 28 genes, and the L strand in the inner circle encodes nine genes.

Table 2. Nucleotide composition of the *Myotis muricola* mitogenome.

Gene	No. of nucleotides	Proportion of nucleotides					AT skew	GC skew
		A	T	G	C	A+T content		
Whole mitogenome	17,244	33.6	29.7	13.4	23.3	63.3	0.06	-0.27
Protein-coding genes*	11,376	32.1	31.0	12.4	24.5	63.1	0.02	-0.33
First codon position	3,792	33.3	23.8	20.0	22.9	57.1	0.17	-0.07
Second codon position	3,792	20.0	41.6	11.4	27.0	61.6	-0.35	-0.41
Third codon position	3,792	41.7	28.9	6.5	22.9	70.6	0.18	-0.56
Ribosomal RNA genes (12S *rRNAs* and 16S *rRNAs*)	2,540	38.5	25.2	16.7	19.6	63.7	0.21	-0.08
tRNA genes	1,511	35.0	29.4	15.6	20.0	64.4	0.09	-0.12
Control region	1,756	34.4	28.3	13.4	23.9	62.7	0.10	-0.28
Intergenic spacers	57	36.8	31.6	10.5	21.1	68.4	0.08	-0.34

*indicates the number of nucleotides in the 13 PCGs (34 bp), except for stop codons.

The 13 mitochondrial PCGs in *M. muricola* use the standard start codon (ATN), three stop codons (TAA, TAG, and AGA), and two incomplete stop codons (TA- and T-) for translation initiation and termination (Table 1). The start codon ATG is used in all PCGs except *Nd3* and *Nd5* (ATA), and *Nd2* (ATC). Eight PCGs (*Nd1*, *Cox1*, *Cox2*, *Atp8*, *Atp6*, *Nd4L*, *Nd5*, and *Nd6*) use the stop codon TAA, while TAG and AGA only occur in *Nd2* and *Cytb,* respectively. Incomplete stop codons (T- or T-), which may be completed by poly-adenylation of the 3'-end of the mRNA after transcription (Boore, 1999), are used in the other five PCGs. Incomplete stop codons or overlaps between PCGs may result from selection pressure to reduce the size of the mitogenome (Rand, 1993).

Table 3. Nucleotide composition of the protein-coding genes (PCGs) of the *Myotis muricola* mitogenome.

Gene	Length (bp)	Proportion of nucleotides (%)					AT skew	GC skew
		A	T	C	G	AT content		
Nd1	957	32.2	31.0	24.3	12.4	63.2	0.02	-0.32
Nd2	1044	37.6	26.7	26.2	9.4	64.4	0.17	-0.47
Cox1	1545	27.3	34.1	21.9	16.7	61.4	-0.11	-0.13
Cox2	684	32.2	30.3	23.8	13.7	62.4	0.03	-0.27
Atp8	204	40.7	31.4	21.6	6.4	72.1	0.13	-0.54
Atp6	681	32.3	30.2	25.8	11.6	62.6	0.03	-0.38
Cox3	784	27.4	32.0	25.5	15.1	59.4	-0.08	-0.26
Nd3	347	30.8	32.0	24.2	13.0	62.8	-0.02	-0.30
Nd4L	297	28.6	34.3	23.6	13.5	63.0	-0.09	-0.26
Nd4	1378	32.8	31.1	25.0	11.0	63.9	0.03	-0.39
Nd5	1821	33.2	32.5	23.4	10.9	65.7	0.01	-0.37
Nd6	528	42.6	21.8	28.6	7.0	64.4	0.32	-0.61
Cytb	1140	29.1	32.0	25.2	13.7	61.1	-0.05	-0.30

Codon frequencies and RSCU values are shown in Table 4. All 60 codons are used to encode 20 amino acids. The most common amino acid is Leu (CUN and CUR; 15.72%), followed by Ile (8.97%), Thr (8.20%), Ser (AGN and AGY; 7.23%), and Met (7.04%) (Figure 2A). CTA and CGA have high RSCU values (>2.0). Several codons ending in G (TCG, CCG, GCG, AAG, TGG, CGG, and ACG) have low RSCU values (<0.2). TCG, which is only used five times, has the lowest RSCU value (0.091) (Table 4).

Within each synonymous family of codons of type NBN (CTN, TCN, CCN, GCN, CGN, GGN, GTN, and ACN), codons ending in A have the highest RSCU value, while codons ending in G have the lowest RSCU value (Figure 2B). In codons of type NDY (TTY, ATY, AGY, TAY, CAY, AAY, TGY, and GAY), U-ending codons have higher RSCU values, while C-ending codons have lower values. In codons of type NDR (TTR, ATR, CAR, AAR, TGR, and GAR), the A-ending codons have higher values, while codons ending in G have lower values. RSCU values for codons ending with A/T are generally much higher than for those ending in G/C (Figure 2B).

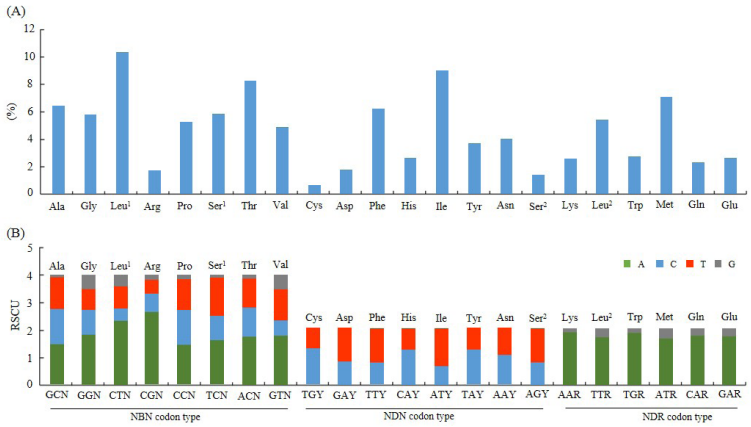

Figure 2. Amino acid composition and codon usage pattern in PCGs in the *Myotis muricola* mitogenome. (**A**) Composition ratios for the 3792 amino acids found in the PCGs. Leu1 and Leu2 are encoded by CTN and CCR, respectively. Ser1 and Ser2 are encoded by TCN and AGY, respectively. (**B**) Relative synonymous codon usage (RSCU) in the PCGs. RSCU values for codons within each synonymous family are indicated using different colors on each bar. Different colors on the bars denote different bases at the 3rd position of codons. N = A/T/G/C; D = A/T/G; R = A/G; and Y = T/C.

Table 4. Codon frequencies and relative synonymous codon usage (RSCU) in the protein-coding genes (PCGs) of the *Myotis muricola* mitogenome.

Codon (aa)	N (RSCU)	Codon (aa)	N (RSCU)	Codon (aa)	N (RSCU)	Codon (aa)	N (RSCU)	Codon (aa)	N (RSCU)
Ala GCA	90 (1.481)	Arg CGA	43 (2.646)	Thr ACA	137 (1.762)	Phe TTC	93 (0.791)	Lys AAA	89 (1.854)
GCC	77 (1.267)	CGC	11 (0.677)	ACC	82 (1.055)	TTT	142 (1.209)	AAG	7 (0.146)
GCT	69 (1.136)	CGT	8 (0.492)	ACT	81 (1.042)	His CAC	62 (1.265)	Leu[2] TTA	171 (1.676)
GCG	7 (0.115)	CGG	3 (0.185)	ACG	11 (0.141)	CAT	36 (0.735)	TTG	33 (0.324)
Gly GGA	100 (1.826)	Pro CCA	73 (1.482)	Val GTA	83 (1.804)	Ile ATC	115 (0.676)	Trp TGA	95 (1.845)
GGC	49 (0.895)	CCC	62 (1.259)	GTC	25 (0.543)	ATT	225 (1.324)	TGG	8 (0.155)
GGT	42 (0.767)	CCT	54 (1.096)	GTT	51 (1.109)	Tyr TAT	86 (1.237)	Met ATA	219 (1.640)
GGG	28 (0.511)	CCG	8 (0.162)	GTG	25 (0.543)	TAC	53 (0.763)	ATG	48 (0.360)
Leu[1] CTA	230 (2.347)	Ser[1] TCA	91 (1.647)	Cys TGC	14 (1.273)	Asn AAT	79 (1.046)	Gln CAA	78 (1.793)
CTC	44 (0.449)	TCC	48 (0.869)	TGT	8 (0.727)	AAC	72 (0.954)	CAG	9 (0.207)
CTT	78 (0.796)	TCT	77 (1.394)	Asp GAC	28 (0.836)	Ser[2] AGT	21 (0.792)	Glu GAA	84 (1.714)
CTG	40 (0.408)	TCG	5 (0.090)	GAT	39 (1.164)	AGC	32 (1.208)	GAG	14 (0.286)

- 'N' indicates the number of codons found in the 13 mitochondrial PCGs.

rRNA genes and tRNA genes

As with rRNA genes in other *Myotis* mitogenomes (Kim et al., 2011; Nam et al., 2013; Yoon et al., 2013; Wang et al., 2014), *12S rRNA* and *16S rRNAs* in the *M. muricola* mitogenome are located between *tRNA*^{Phe} and *tRNA*$^{Leu(CUN)}$ and separated by *tRNA*^{Val} (Figure 1). The combined size of the two rRNA genes is 2540 bp. There are a total of 22 tRNA genes for transferring 20 amino acids, ranging in size from 59 bp [*tRNA*$^{Se\ (AGY)}$] to 75 bp [*tRNA*$^{Leu\ (CUR)}$] (Table 1). The tRNA genes include two leucine-tRNA genes [*tRNA*$^{Leu\ (UUR)}$ and *tRNA*$^{Leu\ (CUN)}$] and two serine-tRNA genes [*tRNA*$^{Ser\ (AGY)}$ and *tRNA*$^{Ser(UCN)}$]. Most of the tRNAs can be folded into the canonical cloverleaf secondary structure. In *tRNA*$^{Ser\ (AGY)}$, the DHU (dihydrouridine) arm has been deleted. This is a common condition in the metazoan mitogenome.

Noncoding regions

The metazoan mitogenome contains some non-coding regions, which are important during replication and maintenance (Fernandez-Silva et al., 2003), such as the origin of replication (O_R), some intergenic spacers, and the CR. The mitochondrial O_R of *M. muricola* is 35 bp in length and is located between *tRNA*^{Asn} and *tRNA*^{Cys} in the WANCY region, which consists of a cluster of five tRNA genes (*tRNA*^{Trp}, *tRNA*^{Ala}, *tRNA*^{Asn}, *tRNA*^{Cys}, and *tRNA*^{Tyr}) (Figure 1), as in other *Myotis* species (Kim et al., 2011; Nam et al., 2013; Yoon et al., 2013; Wang et al., 2014). Intergenic spacers are found in 10 regions of the mitogenome, ranging from a single-base pair spacer (between *tRNA*^{Ala} and *tRNA*^{Asn}, *tRNA*^{Tyr} and *Cox1*, *tRNA*^{Lys} and *Atp8*, and *tRNA*^{Arg} and *Nd4L*) to an 11-bp spacer (between *Cox1* and *tRNA*$^{Ser(UCN)}$) (Table 1). The CR of the *M. muricola* mitogenome is 1765-bp long and is located between *tRNA*^{Pro} and *tRNA*^{Phe} (Figure 1). The sequence GTATGC is repeated 58 times near the 3' end region of the CR and accounts for 19.8% of the size of the CR. Similar short tandem repeats in the CR have also been found in *M. macrodactylus* (60 CATACG repeats), *M. davidii* (68 CATACG repeats), and *M. brandtii* (60 CATACG repeats) (Nam et al., 2013; Jiang et al., 2014; Wang et al., 2014). Most of the variation in size between *Myotis* mitogenomes is due to these short tandem repeats.

In conclusion, our study presents mitogenome characteristics and codon usage patterns of the 13 mitochondrial PCGs of *M. muricola*. The gene order and organization of the *M. muricola* mitogenome follow the pattern found in other *Myotis* mitogenomes. In the PCGs, Leu (CUN and CUR) is the most common amino acid, followed by Ile, Thr, Ser (AGN and AGY), and Met. CTA and CGA have high RSCU values, with TCG having the lowest (0.091). RSCU values for codons ending with A/T are generally much higher than those for codons ending in G/C. Codon bias in the *M. muricola* mitogenome might be due to natural selection or mutation bias. These findings contribute to our understanding of the codon usage pattern and molecular evolution of *M. muricola*, and provide better insight into the relationships among species in the genus *Myotis*.

Conflicts of interest

The authors declare no conflict of interest.

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