Complete Mitochondrial Genomes of Three Nuthatches From the Genus *Sitta* (Aves: Passeriformes: Sittidae) and Mitogenome-Based Phylogenetic Analysis

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Abstract

Background

Nuthatches (genus *Sitta*) comprise a group of Passeriformes. With the publication of more mitochondrial genome data, there has been considerable focus on the taxonomic status of the nuthatches. To understand the phylogenetic position of *Sitta* and phylogenetic relations within this genus, we sequenced and analyzed the complete mitochondrial genomes of three species, *S. himalayensis*, *S. nagaensis* and *S. yunnanensis*, making this the first account of complete mitochondrial genomes (mitogenomes) for this genus.

Results

The mitochondrial genomes of three *Sitta* species are 16,822-16,830 bp in length and consisted of 37 genes and a control region. This study recovered the same gene arrangement found in the mitogenomes of *Gallus gallus*, which is considered the typical ancestral avian gene order. All tRNAs were predicted to form the typical cloverleaf secondary structures. Bayesian inference and maximum likelihood phylogenetic analyses of sequences of 18 species obtained a well-supported topology. The family Sittidae is the sister-group of Troglodytidae, and the genus *Sitta* can be divided into 3 major clades. We demonstrated the phylogenetic relationships within genus *Sitta* (*S. carolinensis* + (*S. villosa* + *S. yunnanensis* + (*S. himalayensis* + (*S. europaea* + *S. nagaensis*)))).

Background

The avian mitochondrial genome is characterized with a small size, relatively fast rate of evolution, and strict maternal inheritance in genetics, and is easy to extract and amplify, which makes it an ideal marker for the evolutionary analyses at the molecular level [1, 2, 3]. The avian mitogenome is a double-stranded circular molecule composed of a light strand (L-strand) and a heavy strand (H-strand), ranging from 16.3-23.1 kb in length [4], and consists of 1 or 2 control region(s), 13 protein-coding genes (PCGs), 22 transfer RNA genes (tRNAs) and 2 ribosomal RNA genes (rRNAs) [5, 6, 7]. The mitogenome plays an important role in revealing the origin and phylogeny of birds [8].

The genus *Sitta*, which belongs to the subfamily Sittinae (Passeriformes, Sittidae), includes 29 species of genus *Sitta* known around the world, 11 of which are distributed in China [9]. Although many molecular data have been published, the phylogenetic relationship of the family Sittidae remains controversial. Ericson and Johansson (2003) placed the family Sittidae within Sylvioidea [10]. In recent years, it has also been proposed that Sittidae belongs to Certhioidea [11, 12]. Johansson et al. (2008) supported that Sittidae is sister to Polioptilidae and Troglodytidae [11], while Treplin et al. (2010) proposed that Sittidae is sister to Certhiidae and Troglodytidae [13]. Later research results showed that Sittidae and Troglodytidae were closely related [14]. These controversies are mainly generated by limited mitochondrial genome data. Therefore, more molecular data are necessary to reconstruct precise phylogeny [15].

Currently, the data of complete mitogenome from the genus *Sitta* are very scarce. In order to better understand the relationships among the species of *Sitta*, we sequenced three mitogenomes of the genus *Sitta* and compared them with related species in terms of mitochondrial structure and gene rearrangement. In this study, we report the properties of the mitogenomes of three *Sitta* species, and infer the phylogenetic relationships of the species with available mitogenome sequences using all PCGs.

Results And Discussion

Genome organization

The complete mitogenomes of the three newly sequenced *Sitta* species are very similar to each other. All three mitogenomes are closed circular molecules ranging from 16,822 to 16,830 bp in length, consisting of 13 PCGs, 22 tRNAs, 2 rRNAs and a control region (Fig. 1). The gene order of mitogenomes of all three species analyzed are highly conserved (Fig. 1), which is also identical to the gene order found in the mitogenome of *Gallus gallus* [16]. For the whole mitogenomes of the three species, one PCG (*nad6*) and eight tRNAs (*trnQ*, *trnA*, *trnN*, *trnC*, *trnY*, *trnS2*(*UCN*), *trnP*, *trnE*) are encoded by the L-strand, while all the other genes are encoded by the H-strand. The comparison of the three *Sitta* species discovered the longest overlap (10 bp) between *atp8* and *atp6*. The longest intergenic spacer (21 bp) is located between *trnV* and *rrnL* in mitogenome of *S. himalayensis* (Table 1). Similar to other typical vertebrates [17], the mitogenomes of the three *Sitta* species show a significant bias towards A and T, with the nucleotide composition of A and T ranging from 53.1–55.7%. The AT-skew and the GC-skew of the whole mitogenomes of three *Sitta* species are 0.10 to 0.13 and -0.39 to -0.35, respectively (Table 2).

Table 1
Annotation of the complete mitogenome of the three *Sitta* species in this study

Gene	Start codon	Stop codon	Anti-codon	Strand	Intergenic Nucleotides (IGN)
trnF			GAA	H	-1
rrnS				H	0(a)/-1(b,c)
trnV			TAC	H	21(a)/2(b,c)
rrnL				H	2(a)/1(b)/7(c)
trnL2(*UUR*)			TAA	H	11(a,c)/12(b)
nad1	ATG	TAA/TA(A)/TAG		H	7(a,c)/10(b)
trnI			GAT	H	6
trnQ			TTG	L	4(a)/3(b)/2(c)
trnM			CAT	H	1(a,b)/0(c)
nad2	ATG	TAA		H	1
trnW			TCA	H	1
trnA			TGC	L	10
trnN			GTT	L	2(a,c)/11(b)
trnC			GCA	L	-1
trnY			GTA	L	1
cox1	GTG	AGG		H	-9
trnS2(*UCN*)			TGA	L	4
trnD			GTC	H	10(a)/11(b,c)
cox2	ATG	TAA		H	1
trnK			TTT	H	1
atp8	ATG	TAA		H	-10
atp6	ATG	TAA		H	9(a,b)/11(c)
cox3	ATG	TA(A)		H	-1
trnG			TCC	H	0
nad3	ATG	TAA		H	-1
trnR			TCG	H	1
nad4L	ATG	TAA		H	-7
nad4	ATG	T(AA)		H	0
trnH			GTG	H	0
trnS1(*AGN*)			GCT	H	-1
trnL1(*CUN*)			TAG	H	1(a)/0(b,c)
nad5	ATG	AGA		H	11
Cytb	ATG	TAA		H	6(a)/3(b,c)
trnT			TGT	H	7(a)/8(b,c)
trnP			TGG	L	14(a,b)/6(c)
nad6	ATG	TAG		L	0
trnE			TTC	L	4(a)/5(b,c)
CR				H	279(a)/282(b)/266(c)

Note: H and L refer to the heavy and light strands, CR = control region. '/' indicate type of intergenic nucleotides in *Sitta* species. *S. himalayensis* (a), *S. nagaensis* (b), *S. yunnanensis* (c).

Table 2
Nucleotide composition of the mitochondrial genome of the three *Sitta* species in this study

Species	Whole genome			PCGs			tRNAs			16S rRNA			12S rRNA			Control region	
	A+T (%)	AT skew	GC skew	A+T (%)	AT skew	GC skew	A+T (%)	AT skew	GC skew	A+T (%)	AT skew	GC skew	A+T (%)	AT skew	GC skew	A+T (%)	AT skew
S. himalayensis	53.9	0.13	-0.38	53.1	0.07	-0.41	58.3	0.03	0.02	55.6	0.24	-0.12	51.2	0.18	-0.11	54.6	-0.15
S. nagaensis	53.1	0.13	-0.39	52.0	0.09	-0.42	58.0	0.04	0.01	55.6	0.24	-0.11	51.5	0.18	-0.11	53.3	-0.12
S. yunnanensis	55.7	0.10	-0.35	55.2	0.04	-0.38	58.3	0.04	0.01	56.4	0.23	-0.10	52.2	0.18	-0.11	55.5	-0.15

Protein-coding genes and codon usage

In the three *Sitta* species, the scope of A + T content in PCGs is between 52.0% and 55.2% (Table 2). The start codons and stop codons of the 13 PCGs are mostly the same among the three species. *cox1* uses GTG as the start codon, while the rest twelve PCGs initiate strictly with the standard start codon ATG (Table 1). There are six kinds of stop codons (TAA, AGG, AGA, TAG, TA*, T**) included in the mitogenome of the three *Sitta* species. For the incomplete stop codons, the missing nucleotides may be the result of post-transcriptional polyadenylation [18], which is common in animal mitogenomes and could produce functional stop codons by polycistronic transcription cleavages and polyadenylation mechanisms [18, 19]. The PCG *nad1* in the mitochondrial genome of *S. nagaensis* contains the incomplete stop codon TA*, while TAN (N represents A, G) occurs in the other two species. Except for *nad1*, all of the other PCGs use the same stop codon across the three *Sitta* species.

The relative synonymous codon usage (RSCU) of 13 PCGs in the three newly sequenced mitogenomes was calculated. As shown in Fig. 2, CGA (Arg) and CUA (Leu1) are most commonly used in all three *Sitta* species. The highest value of RSCU is 3.86 of CGA in *S. yunnanensis* and the lowest value of RSCU is 0.03 of UAG in *S. himalayensis* and *S. nagaensis*. In addition, analysis of the RSCU values for the 13 PCGs indicates an AT bias. As for PCGs, the AT bias can be attributed to the frequent use of NNA and NNU (N represents A, T, C, G) codons [20]. The A + T content in PCGs of *S. yunnanensis* is slightly higher than that of the other two species, and the use of NNA and NNU codons is also more common in *S. yunnanensis*.

The tRNA genes and rRNA genes

The 22 tRNAs of the three *Sitta* species are typical and include all 20 types of amino acids, ranging from 66 to 75 bp in size. And the total length of the 22 mitogenome tRNAs of *S. nagaensis* is 1542 bp, which is the same as that of *S. yunnanensis* and only one-base different from that of *S. himalayensis*. The A + T content of the total mitogenome tRNAs of *S. nagaensis* is 58.0%, which is lower than that of *S. himalayensis* (58.3%) and *S. yunnanensis* (58.3%) (Table 2). The tRNAs of three *Sitta* species were all predicted to fold into typical cloverleaf secondary structures. Furthermore, mismatched base pairs were identified in the stems of 22 different tRNAs, most of which were G-U pairs.

In the mitogenome of the three *Sitta* species, the *16S rRNA* is located between *trnV* and *trnL2*(*UUR*), ranging from 1575 to 1592 bp in length, while the *12S rRNA* is located between *trnF* and *trnV*, ranging from 977 to 980 bp. The longest *16S rRNA* was found in *S. nagaensis* and the shortest in *S. himalayensis*, while the longest *12S rRNA* was discovered in *S. yunnanensis* and the shortest in *S. himalayensis*. The A + T contents of *16S rRNA* and *12S rRNA* range from 55.6–56.4% and from 51.2–52.2% respectively (Table 2).

The control region

The control region of the three species is located between *trnE* and *trnF* genes (Fig. 1). The size of control region of *S. yunnanensis* is 975 bp, which is longer than that of *S. himalayensis* (945 bp) and *S. nagaensis* (945 bp). The A + T content of the control region ranges from 53.3–55.5% (Table 2). The AT-skew is -0.15 to -0.12 and the GC-skew is -0.22, and the A + C content is higher than the T + G content. In this study, we analyzed the control region of three *Sitta* species and the predicted structures are shown in Figure 3. The entire control region contains three structural domains, namely Domain □, Domain □ and Domain □. Domain □ is relatively conservative, while Domain □ and Domain □ are heterogeneous across species in terms of nucleotide composition and size [21].

Domain □ includes extended termination-associated sequences such as ETAS1 and ETAS2 and CSB1-like sequences. Domain □ is the central conserved domain in the control region, including six conserved sequence blocks (F-box, E-box, D-box, C-box, b-box and B-box). Domain □ includes CSB1 sequence and light/heavy strand promoter (LSP/HSP), which are located at 911-929 bp in the control region (Figure 3).

Phylogenetic Analyses

Based on the concatenated nucleotide sequences of 13 PCGs, the phylogenetic analyses of 18 Passeriformes mitogenome sequences were performed, with one of the *Regulus* species as the outgroup (Table 3). BI and ML analyses generated similar tree topologies, so the topology of the BI tree is shown (Fig. 4). The results of this study indicate that Sittidae is closely related to Troglodytidae (1.00 posterior probability and 95% bootstrap value). Muscicapidae and Turdidae were herein corroborated to be sister groups (1.00 posterior probability and 100% bootstrap value). These results are consistent with the work of Barker on the sister groups of Sittidae and Troglodytidae [14].

Table 3
List of 18 species used for the phylogenetic analyses in this study

Species	GenBank NO.	Mitogenome Size (bp)	Total A+T %	References
Sittidae				
Sitta nagaensis	MK343427	16,828	53.1	This study
Sitta europaea	MN356255	16,827	53.2	Unpublished
Sitta himalayensis	MK343426	16,822	53.9	This study
Sitta villosa	MT444149	16,816	55.4	[22]
Sitta yunnanensis	MN052793	16,830	55.7	This study
Sitta carolinensis	NC_024870	16,826	54.8	[23]
Troglodytidae				
Campylorhynchus zonatus	NC_022840	16,780	50.6	[24]
Campylorhynchus brunneicapillus	NC_029482	16,786	50.3	Unpublished
Henicorhina leucosticta	NC_024673	16,727	52.9	[25]
Muscicapidae				
Oenanthe isabellina	NC_040290	16,812	52.7	[26]
Ficedula zanthopygia	JN018411	16,794	53.2	Unpublished
Niltava davidi	NC_039538	16,770	54.2	Unpublished
Cyanoptila cyanomelana	HQ896033	16,802	53.0	Unpublished
Copsychus saularis	NC_030603	16,827	52.7	[27]
Turdidae				
Turdus kessleri	NC_041095	16,754	52.8	[28]
Turdus eunomus	NC_028273	16,737	52.7	Unpublished
Geokichla sibirica	MK377247	16,766	52.3	[29]
Regulidae				
Regulus regulus	NC_029837	16,847	55.5	Unpublished

In the genus *Sitta*, *S. nagaensis* and *S. europaea* were found to be the sister to the *S. himalayensis* (1.00 posterior probability and 100% bootstrap value). *S. villosa* is the sister to *S. yunnanensis* (1.00 posterior probability and 100% bootstrap value). All datasets supported a monophyletic clade of *S. carolinensis*, which was placed at the basal position of the genus *Sitta* (1.00 posterior probability and 100% bootstrap value). These results are generally identical to the previous study conducted by Pasquet et al. [30]. Currently, published mitochondrial genome data of *Sitta* species are very limited, so mitochondrial genomes of more *Sitta* species should be sequenced to better elucidate these phylogenetic relationships.

Materials And Methods

Samples and DNA Extraction

Sample of *Sitta himalayensis* was collected in December 2018 from Gaoligong Mountain Nature Park of Yunnan Province, China, and samples of *Sitta nagaensis* and *Sitta yunnanensis* were collected in September 2018 from Zixi Mountain Provincial Nature Reserve of Yunnan Province, China. Voucher samples of three *Sitta* species were deposited in Department of Biodiversity Conservation, Southwest Forestry University, Kunming. Samples used in this study were preserved in ethanol absolute and stored at -20 °C. The total genomic DNA was extracted from blood using the TIANamp Genomic DNA Kit (DP304, TIANGEN, Beijing, China) according to the manufacturer's protocol.

Genome Sequencing, Assembly and Annotation

As described in previous studies, the mitogenomes were amplified and sequenced [31, 32, 33]. All products of this study were sequenced by Shanghai Personal Biotechnology Co., Ltd (Shanghai, China). The complete mitogenomes of *S. himalayensis*, *S. nagaensis* and *S. yunnanensis* have been deposited in GenBank (Accession Numbers: MK343426, MK343427 and MN052793). Sequences were checked and assembled with SeqMan program of DNASTAR software [31]. Two rRNAs and all PCGs were identified by BLAST searches in NCBI (Available online: http://www.ncbi.nlm.nih.gov), and then confirmed by alignment with homologous genes from other published *Sitta* mitogenomes. The mitogenomic map was depicted with OGDRAW (https://chlorobox.mpimp-golm.mpg.de/OGDraw.html) [34]. Then 22 tRNAs were identified by tRNAscan-SE 2.0 and ARWEN (online version) [35, 36]. MEGA 7.0 was used to calculate the nucleotide composition and RSCU for PCG analysis [37]. The formulas AT-skew = [A - T] / [A + T] and GC-skew = [G - C] / [G + C] were used for composition

skew analysis [38]. The Tandem Repeats Finder program (Available online: http://tandem.bu.edu/trf/trf.advanced.submit.html) was used to analyze the tandem repeats of the putative control region [39]. Moreover, genome organization, base composition, intergenic spacers, overlapping regions, codon usage, PCGs, tRNAs, rRNAs and control region of the mitochondrial genomes of three *Sitta* species were compared.

Phylogenetic Analyses

The sequences of 15 published mitochondrial genomes were obtained from NCBI. These sequences, along with three new mitogenome sequences obtained in this study, were used to reconstruct the phylogenetic relationships within the genus *Sitta*, with *Regulus regulus* (Accession No. NC_029837) serving as an outgroup [14]. The sequence information is listed in Table 3.

The mitogenome sequences of the 13 PCGs were aligned using Clustal X in MEGA v.7.0 with the default parameters [40]. The length of the final alignment was 11,380 nucleotides. The substituted saturation of nucleotide sequences was analyzed with DAMBE 5.2.63 [41]. If Iss was significantly lower than Iss.c ($p < 0.05$), the whole PCG nucleotide sequences entered the next step. The Bayesian information criterion (BIC) in jModelTest v.0.1.1 [42] was used to determine the optimal nucleotide substitution model, which was GTR+G+I. Bayesian inferences (BI) and maximum likelihood (ML) analyses were performed using MrBayes v.3.2.1 [43, 44] and RAxMLGUI v.1.5b3 [45], respectively. BI analyses initiated from a random tree, with four Markov chains running simultaneously for 200,000 generations, sampling every 100 generations and discarding the first 25% as burn-in. The average standard deviation of split frequencies was set below 0.01 to ensure that stationarity was reached. [46]. The confidence values of the BI tree were shown as Bayesian posterior probabilities. In ML analyses, a total of 1000 replicates were performed with the GTR+GAMMA substitution model. Finally, FigTree v.1.2.2 was used to visualize the phylogenetic trees [47].

Declarations

Ethics approval and consent to participate

All samples collected in this study were non-invasive sampling. All animal experiments were approved by the Academic Committee of Southwest Forestry University, which includes some regulations on animal ethics, animal welfare, and wildlife conservation. And all methods were performed in accordance with the the Guidelines for the ethical review of laboratory animal welfare People's Republic of China National Standard (GB/T 35892-2018) and the study complies with the ARRIVE guidelines (https://arriveguidelines.org).

Consent for publication

Not applicable.

Availability of data and materials

The newly described mitogenome sequences have been deposited in the NCBI database under the Accession numbers MK343426, MK343427 and MN052793. The datasets generated and/or analysed during the current study are available in the NCBI (https://www.ncbi.nlm.nih.gov/).

Competing interests

The authors declare no conflict of interest.

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Authors' Contributions

Xu Luo provided a sample of *Sitta himalayensis*. Qing-Miao Yuan and Yu-Bao Duan conceived and designed the experiments. Qing-Miao Yuan and Jing Cao performed the experiments. Qing-Miao Yuan analyzed the data and wrote the paper. Yu-Bao Duan revised the manuscript and provided advice and guidance.

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Figures

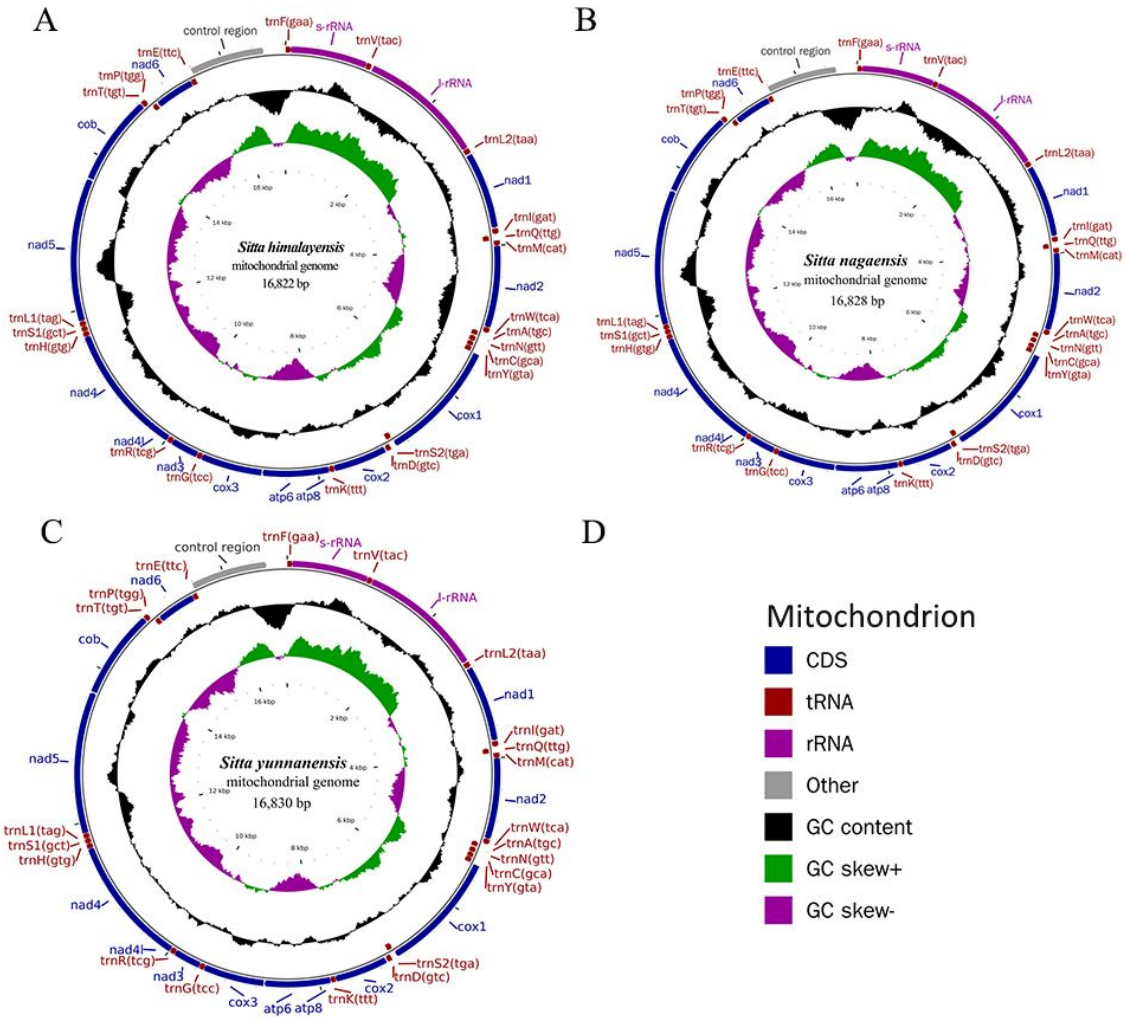

Figure 1

Characters of mitochondrial genomes of the three newly sequenced *Sitta* species. Gene names are annotated using standard abbreviations; single letters indicate corresponding amino acids based on the IUPAC-IUB abbreviation. A: *S. himalayensis* mitochondrial genome. B: *S. nagaensis* mitochondrial genome. C: *S. yunnanensis* mitochondrial genome. The legends are depicted in D.

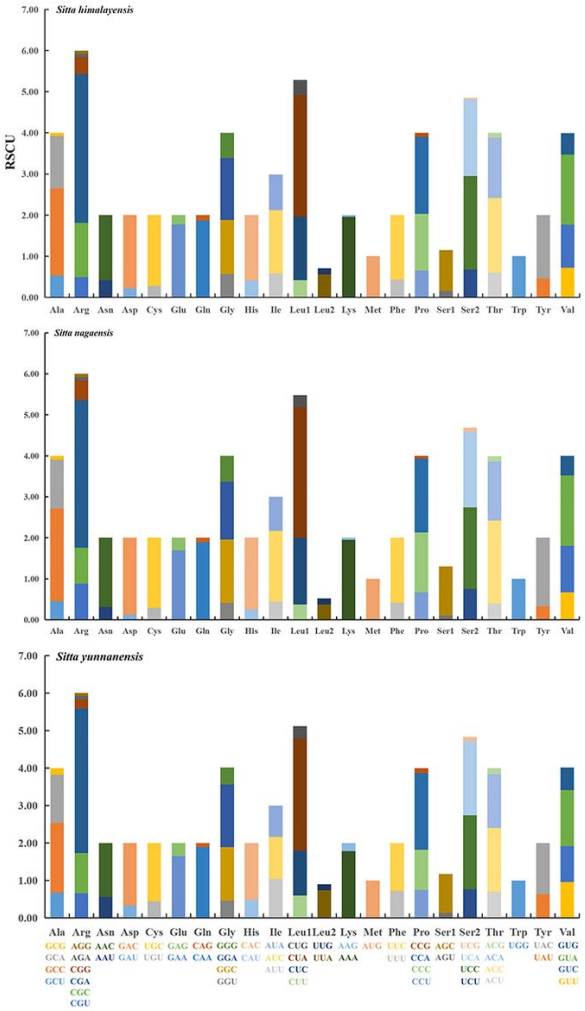

Figure 2

Relative synonymous codon usage (RSCU) for protein-coding genes of the three *Sitta* mitochondrial genomes. Codon families are provided on the x-axis.

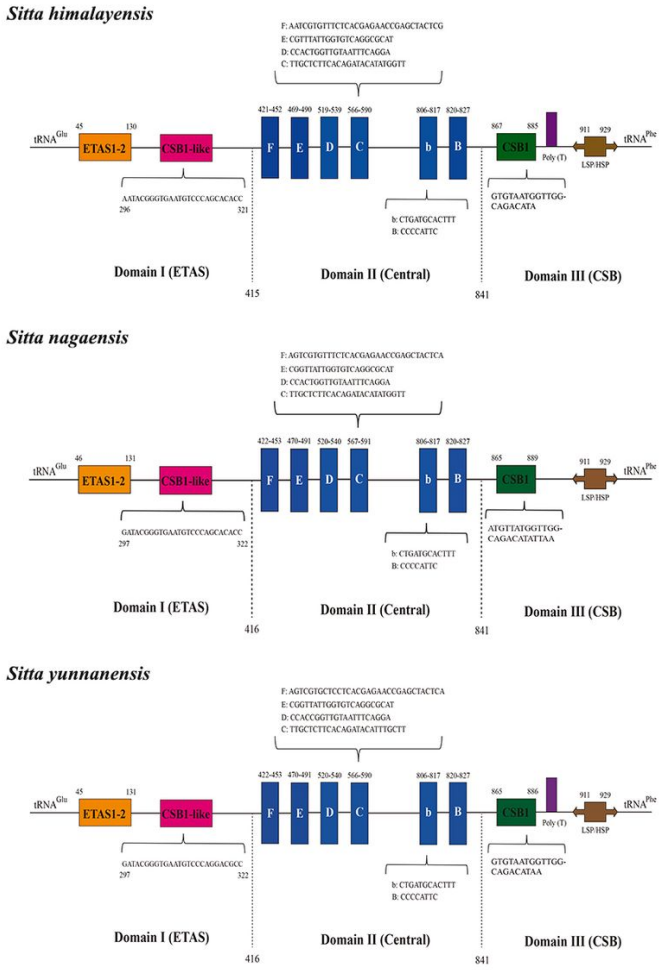

Figure 3

Predicted structural elements in the control region of three *Sitta* species. Extended termination-associated sequences are indicated by orange boxes, and conserved sequence blocks are indicated by blue boxes.

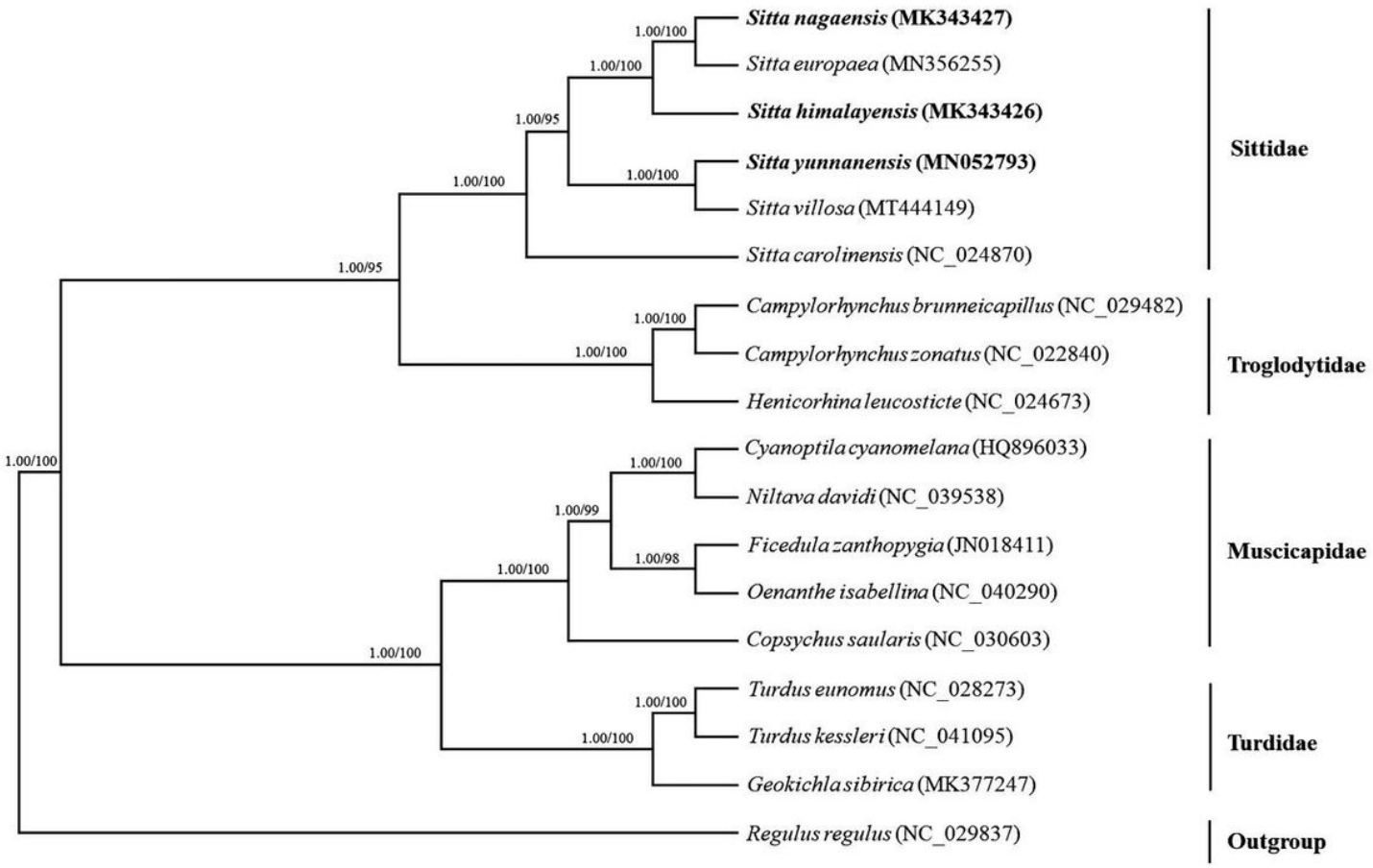

Figure 4

Phylogeny from *Sitta* mitochondrial genome sequences. Bayesian inference (BI) and maximum likelihood (ML) analyses inferred from protein-coding genes supported the same topological structure. Values at nodes are Bayesian posterior probabilities and ML bootstrap values. The tree is rooted with one outgroup (*Regulus regulus*).