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Complete Mitochondrial Genome of *Fulvetta cinereiceps* (Sylviidae: Passeriformes) and Consideration of its Phylogeny within Babblers

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JG and CZ designed the research. BC collected the samples. JG, GW and XS conducted the experiment. JG and MP wrote the manuscript, and GW, CZ, JM, XZ and BY revised the manuscript.

Key words
***Fulvetta cinereiceps*, Babblers, Mitochondrial genome, Phylogeny, Gene order**

ABSTRACT

Fulvetta cinereiceps (grey-hooded fulvetta) has been classified as belonging to the multi-clade babblers within Passeriformes. The complete mitochondrial genome of *F. cinereiceps* is lacking although there has been considerable phylogenetic research on babblers. Therefore, we aimed to determine *F. cinereiceps'* mitogenome and investigate its phylogenetic relationships within the babblers and superfamily Sylvioidea. *F. cinereiceps'* mitogenome is typically circular, 16,969 bp in size with a rich A+T content (52.7%), 13 protein-coding genes (PCGs), 22 tRNAs genes, 2 rRNA genes, a control region (CR) and a non-coding region (NC). We found strong support for *F. cinereiceps* being placed within Sylviidae (superfamily Sylvioidea) and for babblers being separated into two families, Sylviidae and Timaliidae, with four subfamilies within Timaliidae. This is one of many taxonomic arrangements for babblers and there is likely to be continuous debate until a consensus is reached. Consequently, our study's complete mitochondrial genome of *F. cinereiceps* can be added to newly sequenced complete mitochondrial genomes and allow babbler taxonomy to be mapped with confidence.

INTRODUCTION

Fulvetta cinereiceps (grey-hooded fulvetta) is a Chinese fulvetta found in central and south-east China (BirdLife International, 2019). The medium-sized (12 cm) fulvetta is grouped with the babblers and is common and widespread, inhabiting undergrowth and thickets of forests (Collar and Robson, 2019). Despite considerable research on babblers, the complete mitochondrial genome of *F. cinereiceps* is lacking. Additionally, there has been taxonomic uncertainty regarding its place within the multi-clade babblers. *Fulvetta cinereiceps* was originally classified as *Siva cinereiceps* (Global Biodiversity Information Facility, 2020), family Timaliidae, and later within the genus *Alcippe* (Pasquet *et al*., 2006). However, Cibois (2003a) argued that *Alcippe* was polyphyletic, which was supported by Pasquet *et al*. (2006) and Huang *et al*. (2015), forming a clade with *Alcippe ruficapilla, Alcippe striaticollis* and *Alcippe vinipectus*. Pasquet *et al*. (2006) and Moyle *et al*. (2012) assigned *F. cinereiceps* to *Fulvetta* and Moyle *et al*. (2012) stated that *F. cinereiceps* should be more correctly allocated to the family Sylviidae, along with *Sylvia* and *Paradoxornis*. However, Cai *et al*. (2019) propose that *Fulvetta* be placed in Paradoxornithidae along with *Paradoxornis* and *Suthora*, with only *Sylvia* and *Curruca* remaining in Sylviidae.

The phylogenetic uncertainty of *F. cinereiceps* is unsurprising given the debate surrounding the multi-clade babblers (>450 species; ~5 families) (Moyle *et al*., 2012; Gill and Donsker, 2019). The babblers are a widely distributed and morphologically diverse Passerine taxonomic grouping with species found mostly in Africa, Indo-China and South-east Asia, with a few in the Palearctic and New World (del Hoyo *et al*., 2016; Gill and Donsker, 2019). Morphological characteristics vary considerably across the babblers, such as size, bill shape and plumage coloration (Cibois, 2003a). The pre-molecular babblers were often referred to as the "scrap basket" because the birds did not fit well within other taxa and were therefore grouped in a 'miscellaneous' family, Timaliidae (Mayr and

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Amadon, 1951). With the rapid development of molecular biology, the traditional morphological classification of babblers has been challenged across several studies with many genera and species being added or removed and shifts of internal phylogenetic relationships. For example, Sibley and Ahlquist (1992; as cited in Cibois 2003a), Cibois (2003a), Alström *et al*. (2006) and Pasquet *et al*. (2006) have used varying molecular techniques to map the phylogenetic relationships of these taxa. The most recent phylogenetic analysis of the babblers mapped the relationships of 402 species (ca. 89%) and have proposed a taxonomic revision with seven families and 64 genera (Cai *et al*., 2019).

Reduction of phylogenetic uncertainty requires additional genetic information be mapped and published. Previous phylogenetic trees including *F. cinereiceps* were constructed using a single mitochondrial gene or mitochondrial DNA fragments (e.g. Cytochrome b, 12S rRNA, 16S rRNA, ND2 and COI) and would inevitably result in erroneous conclusions. Thus, we aimed to determine the complete mitochondrial genome of *F. cinereiceps* and investigate its phylogenetic relationships within the multi-clade babblers and superfamily Sylvioidea. Our study will provide more detailed information needed to better understand *F. cinereiceps'* phylogeny and future studies on babbler taxonomy.

MATERIALS AND METHODS

Sample collection and DNA extraction

A sample of muscle tissue was collected from an individual of *F. cinereiceps* that had died of natural causes from Laojunshan National Nature Reserve (Pingshan County, Yibin, Sichuan Province, China). The total genomic DNA was obtained from the muscle tissue according to the instructions of the DNA Extraction Kit (Tiangen, Beijing, China).

Amplification and sequencing

The entire mitochondrial genome of *F. cinereiceps* was amplified using Polymerase Chain Reaction (PCR). A total of 11 primer pairs (Table I). Primers A1, A2, A7, A10 and A11, were acquired from Zhou *et al*. (2017), the other primers were designed by aligning with the relatively conserved areas of *Alcippe morrisonia hueti* (KX376475.1). PCR (25 µl) was undertaken as follows: 0.5 µl total genomic DNA, 1 µl of each of the upstream and downstream primers, 12.5 µl T3 Super PCR Mix (TSINGKE, Beijing, China), and 10 µl ddH_2O. The PCR conditions were: 2 mins at 98ºC (pre-denaturation), running 35 cycles with a temperature profile including 10 s at 98ºC, 15 s at 55ºC, and 15~60 s (determined by the length of the assumed fragments) at 72ºC, followed by 5 mins extension period at 72ºC. The PCR products were sequenced in Tsingke Biotecnology Company (Chengdu, Sichuan Province, China).

Sequence analysis

The amplified sequences were assembled into a complete circular mitochondrial genome by SeqMan and SeqBuilder program (DNAStar Inc., Madison, Wisc.). The position of 13 PCGs and two rRNA genes were determined by comparison with related sequences (*A. morrisonia hueti* and *Paradoxornis gularis*: KX397391). The analysis of the complete mitochondrial genome sequence of *F. cinereiceps* was completed using software MEGA 6.0 (Tamura *et al*., 2013). The AT skew was calculated using the formula AT skew = [A - T]/[A + T] (Perna and Kocher 1995). Using the tRNAscan-SE 2.0 (http://lowelab.ucsc.edu/tRNAscan-SE/), the possible cloverleaf structures of tRNA genes were identified (Lowe and Chan, 2016).

Rates and patterns of protein-coding gene evolution across the 22 babbler species

Using MEGA 6.0 (Tamura *et al*., 2013), the variable sites (var. sites), transition-to-transversion ratios (ts/tv), model, nucleotide diversity (π), nonsynonymous rates (dN), synonymous substitution rates (dS) and nonsynonymous to synonymous ratio (dN/dS; ω) of 13 PCGs were calculated based on the mitogenomes of 22 babbler species (see Table II, including *F. cinereiceps*). Babblers were α-priori classified according to international ornithological authorities del Hoyo *et al*. (2019) and Gill and Donsker (2019) within families Sylviidae, Zosteropidae, Timaliidae, Pellorneidae and Leiothrichidae.

Control region and gene order

The control regions (positions and the fragment lengths) and gene orders of 54 species (see Table II for species list) from GenBank that were used to construct the phylogenetic trees were summarized. The 54 species included the 36 species from the superfamily Sylvioidea (i.e. 22 babblers + 14 non-babblers) and 18 passerine species from other superfamilies. And sequence identity (%) analyses were conducted using DNAMAN to identify distinct control regions within species from Sylvioidea.

Phylogenetic analysis

The nucleotide sequences of the 13 PCGs from 53 available mitochondrial genomes of Passeriformes species (Table II excluding *F. cinereiceps*) were extracted and translated into amino acid sequences for alignment with the amino acid sequences of the PCGs of *F. cinereiceps* by using MEGA 6.0 with the default settings (Nikaido *et al*., 2001).

Table I. The primers used to amplify the mitochondrial genome of *F. cinereiceps*.

Primer name	F (5' to 3')	R (5' to 3')	Location (bp) from	to
A1[a]	TACATGCAAGTATCCGCG	TTGCTCCCATTCCATAGG	1	686
A2[a]	AACTCTAAGGACTTGGCGG	CCTCGTTTAGCCATTCATACT	479	1985
A3	AACCCGACAGAGGAGCGT	GGATGGCGATGGAGATGT	1753	4862
A4	GAACGCCATAAGGGTCAC	ATCGGTTAATGAATGTCACAGGTA	3682	5057
A5	AAGACCCGCAGGACATTA	GTTTATGCGGTTGGCTTG	5001	6887
A6	TGCCACGACGATACTCAG	GAAGATTCGTTTGCGGAT	6600	10438
A7[a]	AAGACAGTTGATTTCGGC	CTTTCACTTGGATTTGCAC	9810	11736
A8	TAAACAACCTCCACTACCC	ATCATGTTGCGAATTGTAG	10914	12063
A9	CCCCATTATCTTTCCA	TTGAGCGTAGTTTGGA	11798	14512
A10[a]	GACCCAGAAAATTTCACGC	AACTCCTGCTACGCACTGG	14323	15518
A11[a]	GCATACTTTCCCTCTTACCC	AACTACTGCTAATACCCGT	15294	131

a. From Zhou *et al*. (2017).

Stop codon, gaps, and ambiguous sites adjacent to gaps were removed. *Pericrocotus ethologus* (NC_024257.1) and *Nucifraga columbiana* (NC_022839.1) were treated as an out-group. The Bayesian phylogenetic analysis, running for 10,000,000 generations sampling per 1,000 generations, was performed using MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003). According to the Akaike Information Criterion (AIC) (Posada and Buckley, 2004), GTR + I + G was chosen as the best nucleotide substitution by using jModelTest V.2.1.1 (Darriba *et al*., 2012) model for Bayesian analysis. The Bayesian posterior probabilities estimation was performed using the Markov chain Monte Carlo (MCMC) sampling method. The GTR (best-fitting model) was chosen and 1,000 ML bootstrap replicates were estimated to implement Maximum Likelihood (ML) analysis using PhyML 3.0 (Guindon, 2010). A majority-rule consensus tree was obtained from the remaining trees.

The genetic distances between 13 PCG sequences from the 22 babbler species were calculated by Kimura 2-parameter model using MEGA 6.0 to better demonstrate the phylogenetic relationship between *F. cinereiceps* and other babbler species.

RESULTS

F. cinereiceps *sequence composition*

The complete circular mitochondrial genome of *F. cinereiceps* consisted of 16,969 bp (Genbank accession number: MG833030), and its base composition was G 15.0%, T 23.8%, A 28.9%, and C 32.2% with a greater content of A+T (52.7%). We found the same high A + T content in all the 54 Passeriformes species' mitochondrial genomes, which ranged from 51.6% (*Garrulax ocellatus*) to 57.7% (*Pericrocotus ethologus*) (Table II).

The mitogenome of *F. cinereiceps* contained 22 tRNAs, two rRNAs, 13 PCGs, a control region (CR) and a non-coding (NC) region (Fig. 1). The $tRNA^{Thr}$ possessed the highest A+T content (68.6%), while the COX3 lowest (48.2%; Table III). The AT skew of the *F. cinereiceps* mitogenome was 0.095. The highest AT skew was observed in ND6 (0.417), while the lowest in CR (-0.099), and $tRNA^{Leu\ (UUR)}$ showed no AT skew (AT skew = 0). Most of the PCGs and tRNA genes were located on the H-strand, but ND6 and 8 tRNA genes ($tRNA^{Gln}$, $tRNA^{Ala}$, $tRNA^{Asn}$, $tRNA^{Cys}$, $tRNA^{Tyr}$, $tRNA^{Ser\ (UCN)}$, $tRNA^{Pro}$ and $tRNA^{Glu}$) were encoded on the L-strand. The lengths of 12S rRNA and 16S rRNA were 983 bp and 1,597 bp, respectively. The A+T content and AT skew of 16S rRNA were greater than 12S rRNA. The longest intergenic spacer was between $tRNA^{Leu\ (UUR)}$ and ND1 (15 bp). Several genes shared 1~8 bp with each other (Table III).

F. cinereiceps *protein coding genes*

A total of 3,789 codons (excluding termination codons) were identified. The codons CUA (7.15%) and AUC (5.38%) were most common, and codon AGU was the least frequent (0.13%). The frequency of the four bases were similar at the first codon position (U 20.5%, G 23.9%, A 27.4%, C 28.2%). Base U (40.2%) had the highest and G (12.8%) the lowest frequency at the second codon position. While at the third position, G was the least common base (G 7.0%, U 13.8%, A 35.6%, C 43.6%). Leu was the most common amino acid, accounting for 17.21% of all amino acids.

Table II. The A + T (%) content and control regions (CR) of the 54 Passerine species (*F. cinereiceps* + 53 species' sequences obtained from GenBank) used for phylogenetic analysis. The 21 additional babbler species are listed below *F. cinereiceps*, with current babbler families denoted by lettered subscripts (α-priori taxa del Hoyo *et al.*, 2019; Gill and Donsker, 2019). Species in the left section of the table belong to the superfamily Sylvioidea, while the species on the right are Passerines from other superfamilies.

Species	A+T (%)	CR1 (bp)	CR2 or NC (bp)	Identity	Species	A+T (%)	CR (bp)
		between $tRNA^{Thr}$ and $tRNA^{Pro}$	between $tRNA^{Glu}$ and $tRNA^{Phe}$				between $tRNA^{Glu}$ and $tRNA^{Phe}$
Fulvetta cinereicep[a]	52.7	1215	250	9.69%	*Sylviparus modestus*	53.0	1493
Paradoxornis webbianus[a]	53.8	1134	255	10.18%	*Parus major*	52.3	1186
Paradoxornis fulvifronsa	53.9	1235	269	8.01%	*Parus monticolus*	52.2	1187
Paradoxornis nipalensis[a]	54.5	1167	268	10.50%	*Ficedula zanthopygia*	53.2	1213
Sylvia atricapilla[a]	55.5	1107	1260	91.22%	*Cyanoptila cyanomelana*	53.0	1241
Sylvia crassirostris[a]	53.8	1116	524	19.68%	*Sturnus cineraceus*	52.5	1249
Yuhina diademata[b]	54.1	1077	1184	86.84%	*Gracula religiosa*	52.4	1249
Zosterops poliogastrus[b]	54.3	1087	1149	73.39%	*Turdus migratorius*	52.1	1110
Pomatorhinus ruficollis[c]	53	1055	385	16.20%	*Turdus naumanni naumanni*	52.8	1199
Stachyris ruficeps[c]	54.1	1092	231	13.20%	*Turdus eunomus*	52.7	1182
Alcippe morrisonia hueti[d]	53.6	1090	1131	88.76%	*Turdus hortulorum*	52.6	1204
Napothera epilepidota[d]	53.9	1094	1236	83.44%	*Turdus merula*	52.7	1177
Minla ignotincta[e]	54	1123	1174	92.37%	*Turdus rufiventris*	52.3	1113
Leiothrix lutea[e]	54	1077	958	72.55%	*Luscinia calliope*	52.3	1257
Leiothrix argentauris[e]	53.9	1093	1164	88.93%	*Regulus regulus*	55.5	1257
Garrulax formosus[e]	54.1	1136	1155	93.10%	*Nucifraga columbiana*	55.8	1318
Garrulax affinis[e]	53.1	1135	1158	92.58%	*Pericrocotus ethologus*	57.7	1329
Garrulax elliotii[e]	53.3	1137	1163	89.03%	*Turdus philomelos*	53.3	1191
Garrulax canorus[e]	52.2	1109	1152	92.33%			
Garrulax sannio[e]	52.2	1129	1147	91.90%			
Garrulax ocellatus[e]	51.6	1092	1148	90.42%			
Garrulax cineraceus[e]	52.3	1086	1156	89.73%			
Megalurus pryeri	53.7	1128	1265	87.51%			
Megalurus punctatus	53.7	1120	1274	76.28%			
Megalurus punctatus	52.1	1122	1206	90.96%			
Pycnonotus sinensis	54.0	1112	229	10.20%			
Pycnonotus melanicterus	55.1	1117	315	12.22%			
Pycnonotus xanthorrhous	54.4	1113	260	11.83%			
Spizixos semitorques	55.5	1100	360	16.09%			
Hirundo rustica gutturali	54.1	1195	1290	80.87%			
Progne chalybea	53.2	1071	1380	72.93%			
Tachycineta albilinea	53.4	1079	1267	78.58%			
Alauda arvensis	52.3	1152	327	13.53%			
Phylloscopus inornatu	53.5	1071	239	10.70%			
Aegithalos bonvaloti	54.5	1159	1209	87.62%			
Aegithalos caudatus	54.5	1155	1205	86.17%			

[a] Sylviidae; [b] Zosteropidae; [c] Timaliidae; [d] Pellorneidae; [e] Leiothrichidae.

Table III. Characteristics of the *F. cinereiceps* mitogenome, containing 22 tRNAs, two rRNAs, 13 protein coding genes (PCGs), a control region (CR) and a non-coding region (NC). The length, location, A+T content and AT skew of different regions are shown.

Gene	Size (bp)	Inc	Location (bp)		Anticodon	Codon		Skewness	A+T (%)	Strand
			From	To		Start	Stop	AT		
$tRNA^{phe}$	70		1	70	GAA			0.258	50.0	H
12S rRNA	983		71	1053				0.171	50.9	H
$tRNA^{Val}$	70		1054	1123	TAC			0.056	51.4	H
16S rRNA	1597	2	1124	2720				0.223	54.7	H
$tRNA^{Leu\ (UUR)}$	75	15	2723	2797	TAA			0	53.3	H
ND1	978	6	2813	3790		ATG	AGA	0.004	49.9	H
$tRNA^{Ile}$	74	6	3797	3870	GAT			0.095	56.8	H
$tRNA^{Gln}$	71	-1	3877	3947	TTG			0.116	60.6	L
$tRNA^{Met}$	69		3947	4015	CAT			0.134	53.6	H
ND2	1041	-1	4016	5056		ATG	TAA	0.054	52.0	H
$tRNA^{Trp}$	70	1	5056	5125	TCA			0.171	58.6	H
$tRNA^{Ala}$	69	10	5127	5195	TGC			0.111	52.2	L
$tRNA^{Asn}$	73	2	5206	5278	GTT			0.077	53.4	L
$tRNA^{Cys}$	66		5281	5346	GCA			0.295	51.5	L
$tRNA^{Tyr}$	70	1	5347	5416	GTA			0.350	57.1	L
COX1	1551	-8	5418	6968		GTG	AGG	0.046	52.2	H
$tRNA^{Ser\ (UCN)}$	73	2	6960	7032	TGA			0.100	54.8	L
$tRNA^{Asp}$	69	7	7035	7103	GTC			0.158	55.1	H
COX2	684	5	7111	7794		ATG	TAA	0.149	50.9	H
$tRNA^{Lys}$	70	1	7796	7865	TTT			0.235	48.6	H
ATP8	168	-8	7867	8034		ATG	TAA	0.053	56.5	H
ATP6	684	6	8025	8708		ATG	TAG	0.047	53.1	H
COX3	784		8715	9498		ATG	T	-0.005	48.2	H
$tRNA^{Gly}$	69		9499	9567	TCC			0.043	66.7	H
ND3	351	1	9568	9918		ATA	TAA	-0.027	53.3	H
$tRNA^{Arg}$	70	1	9920	9989	TCG			0.023	61.4	H
ND4L	297	-7	9991	10287		ATG	TAA	0.053	50.5	H
ND4	1378		10281	11658		ATG	T	0.089	52.2	H
$tRNA^{His}$	70	-2	11659	11728	GTG			0.131	65.7	H
$tRNA^{Ser\ (AGY)}$	66		11729	11794	GCT			0.177	51.5	H
$tRNA^{Leu\ (CUN)}$	71	-2	11795	11865	TAG			0.116	60.6	H
ND5	1824	4	11866	13689		ATG	TAA	0.095	53.2	H
CytB	1143	3	13694	14836		ATG	TAA	0.054	52.0	H
$tRNA^{Thr}$	70		14840	14909	TGT			0.042	68.6	H
CR	1146		14910	16055				-0.099	53.3	H
$tRNA^{Pro}$	70	6	16056	16125	TGG			0.210	61.4	L
ND6	519		16132	16650		ATG	TAG	0.417	52.2	L
$tRNA^{Glu}$	69		16651	16719	TTC			0.022	62.3	L
NC	250		16720	16969				0.401	58.8	H
Overall of genome	16969		1	16969				0.095	52.7	

Table IV. Rates of mitochondrial protein coding gene (PCG) evolution across the 22 babbler species.

	Var. sites	ts/tv	Model	π	dN	dS	dN/dS
ND1	446 (0.4574)	1.958	HKY+G+I	0.168205	0.038	0.920	0.041
ND2	739 (0.7310)	2.016	TN93+G+I	0.221505	0.223	0.921	0.242
COX1	534 (0.3450)	1.95	GTR+G+I	0.121459	0.010	0.704	0.014
COX2	306 (0.4513)	2.034	GTR+G+I	0.161246	0.061	0.744	0.082
ATP8	90 (0.5455)	1.594	TN93+G	0.187249	0.112	0.730	0.153
ATP6	321 (0.4714)	1.686	HKY+G+I	0.164248	0.046	0.781	0.059
COX3	305 (0.3895)	1.781	GTR+G+I	0.140909	0.035	0.727	0.048
ND3	176 (0.5057)	1.93	HKY+G+I	0.167351	0.070	0.724	0.097
ND4L	140 (0.4762)	2.057	HKY+G+I	0.159715	0.041	0.818	0.050
ND4	646 (0.4691)	1.879	GTR+G+I	0.158412	0.052	0.723	0.072
ND5	864 (0.4768)	1.772	HKY+G+I	0.161831	0.058	0.753	0.077
CYTB	458 (0.4018)	1.599	GTR+G+I	0.143405	0.036	0.726	0.050
ND6	267 (0.5164)	2.219	HKY+G+I	0.182867	0.074	0.809	0.091

See Table II for babbler species names. Rates of the 13 PCGs are represented by variable sites (var. sites), transition-to-transversion ratio (ts/tv), nucleotide diversity (π), nonsynonymous rate (dN), synonymous rate (dS) and non-synonymous-to-synonymous substitution rates (dN/dS). Model abbreviations refer to HKY (Hasegawa *et al*., 1985), TN93 (Tamura and Nei, 1993), I (evolutionarily invariable), GTR (General Time Reversible) and G (γ distribution). CYTB refers to cytochrome B; COX1, COX2 and COX3 refer to the cytochrome C oxidase subunit 1, 2 and 3, respectively; ATP refers to ATP synthase subunit gene; and ND (4L, 1-6) refers to the NADH dehydrogenase subunit (4L, 1-6) gene.

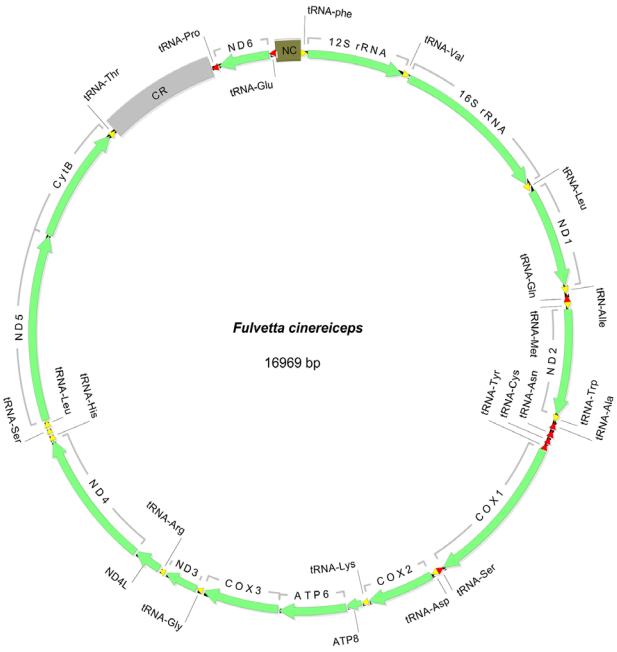

Fig. 1. Circular map of *F. cinereiceps* mitogenome. CytB refers to cytochrome B; COX1, COX2 and COX3 refer to the cytochrome C oxidase subunit 1, 2 and 3, respectively; ATP refers to ATP synthase subunit gene; and ND (4L, 1-6) refers to the NADH dehydrogenase subunit (4L, 1-6) gene.

ATG was the most frequent start codon, although COXI started with GTG and ND3 began with ATA. Most protein genes terminated with TAA, whereas ND1 terminated with AGA, COXI ended with AGG, TAG was the stop codon of ATP6 and ND6, and COX3 and ND4 terminated with T (incomplete stop codon).

F. cinereiceps *tRNA genes*

The A nucleotide content was more than the T content in the 22 tRNA genes (0.022≤AT skew≤0.350, Table III). The greatest A+T content was discovered in $tRNA^{Thr}$ (68.6%), and the lowest was found in $tRNA^{Lys}$ (48.6%). We predicted the secondary structure of the 22 tRNAs (Fig. 2) and they ranged in length from 66 bp to 74 bp. The 22 tRNAs fold into the typical clover leaf structure, except for $tRNA^{Ser\ (AGN)}$ lacking the dihydrouridine arm (DHU arm).

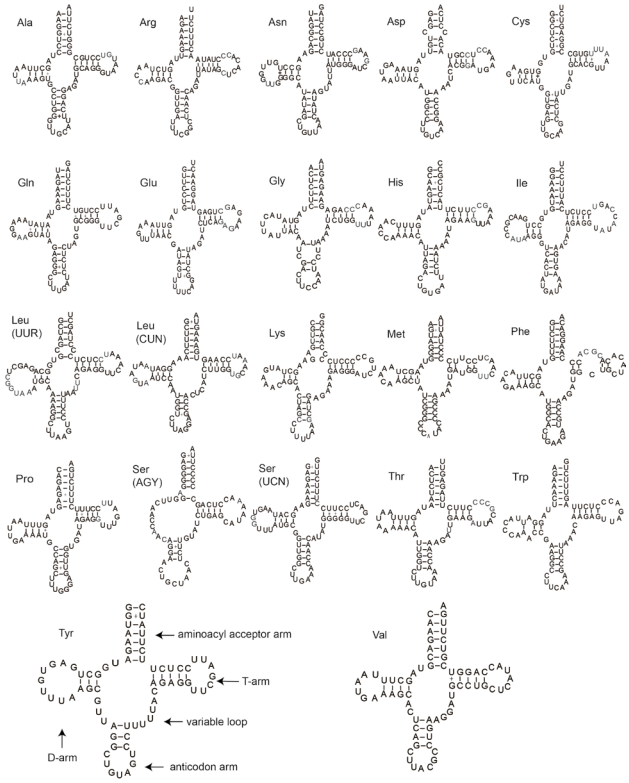

Fig. 2. The clover leaf structures for all tRNA genes of *F. cinereiceps* mitogenome. "-" showed Watson-Crick base pairings and "+" indicated the pairing between G and U in tRNAs.

Rates and patterns of protein coding gene evolution across the 22 babbler species

The COX1 gene had the lowest variable sites proportion, π, dN, dS, dN/dS, while the highest of these same values were observed in ND2 gene (Table IV). The

transversion ratio (ts/tv) was highest in ND6 (2.219) and lowest in ATP8 (1.594) (Table IV). We found that dN/dS ratio was <1 for all 13 PCGs (Fig. 3).

Control region and gene order

We found that the 36 species of Sylvioidea either had one single region (CR1 located between $tRNA^{Thr}$ and $tRNA^{Pro}$) with a NC (located between $tRNA^{Glu}$ and $tRNA^{Phe}$), or two control regions (CR1 located between $tRNA^{Thr}$ and $tRNA^{Pro}$; CR2 located between $tRNA^{Glu}$ and $tRNA^{Phe}$) with little difference in length (Table II). Non-Sylvioidea species' control region (CR) was found between $tRNA^{Glu}$ and $tRNA^{Phe}$.

The mitogenome of *G. formosus* had two control regions with the highest sequence identity (93.1%; Table II). Other species with sequence identities >90% were *S. atricapilla* (91.22%), *Minla ignotincta* (92.37%), *G. affini* (92.58%), *G. canorus* (92.33%), *G. sannio* (91.90%), *G. ocellatus* (90.42%) and *Me. punctatus* (90.96%). No species had identities >95%. *F. cinereiceps* control regions had low sequence identity (9.69%).

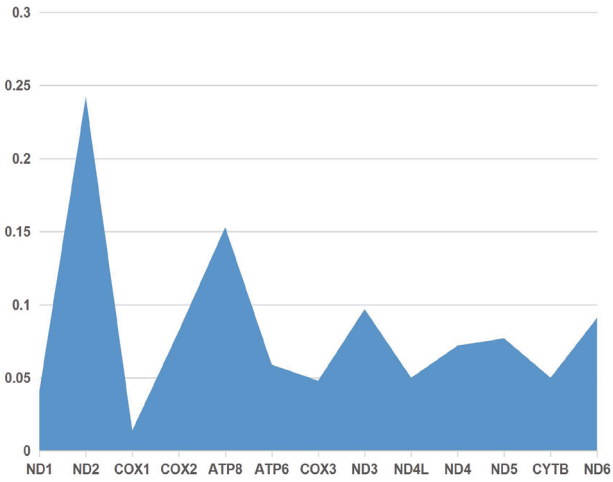

Fig. 3. dN/dS ratios (ω) for mitochondrial PCGs of the 22 babbler species (see Table II for list). Gene abbreviations are given in Table IV.

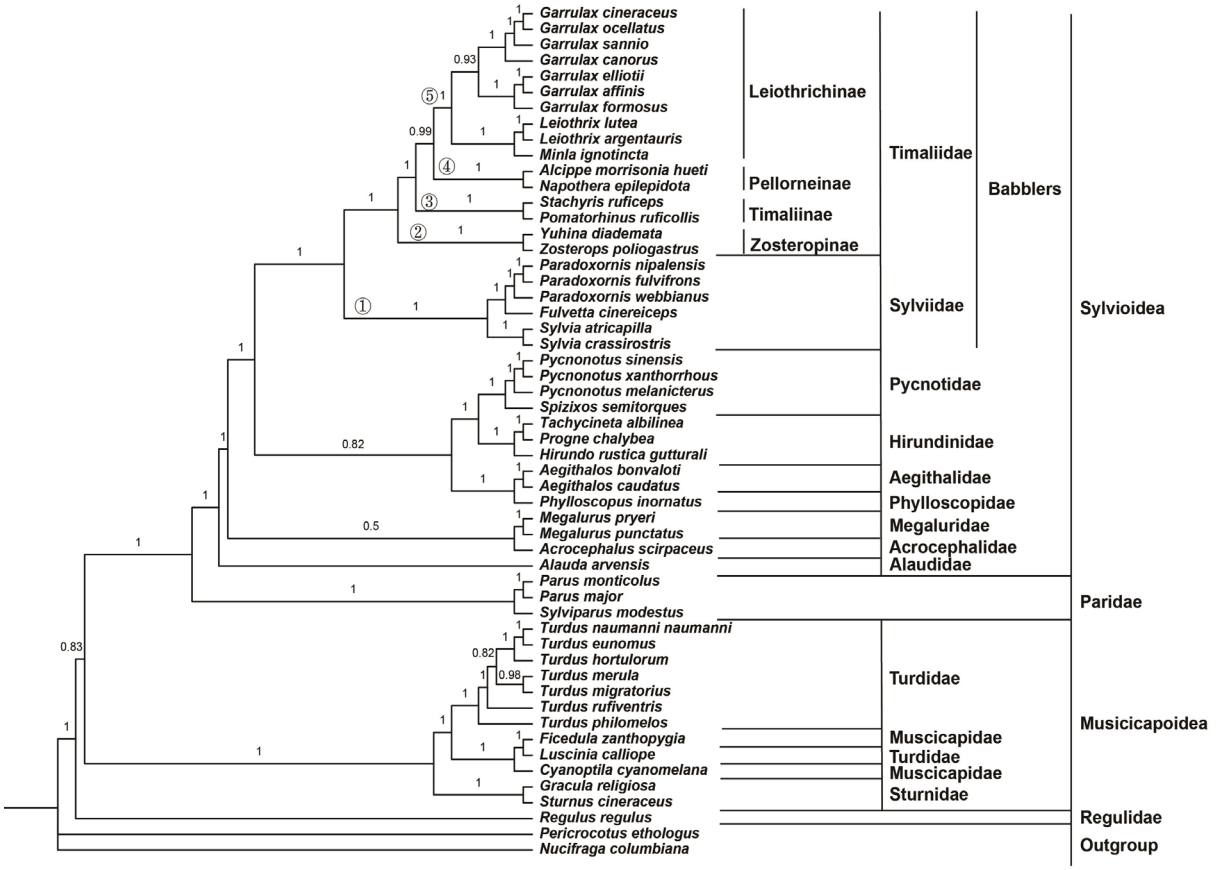

Fig. 4. Phylogenetic relationships based on nucleotide sequences of 13 protein-coding genes of from 54 mitochondrial genomes of Passeriformes were analyzed by Bayesian inference. The numbers abutting branches were Bayesian posterior probabilities (BPP).

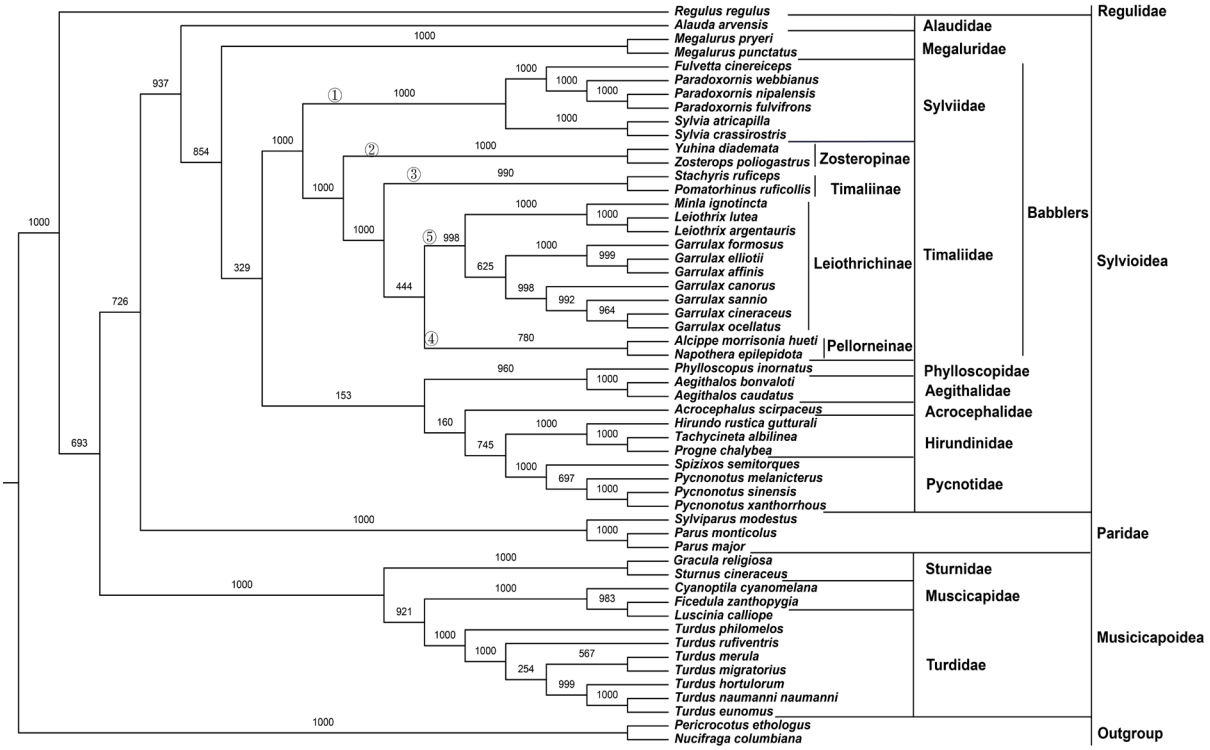

Fig. 5. Phylogenetic tree was inferred based on the nucleotide sequences of 13 PCGs from 54 mitochondrial genomes of Passeriformes using maximum likelihood method. Numbers at each node were bootstrap support values (BS).

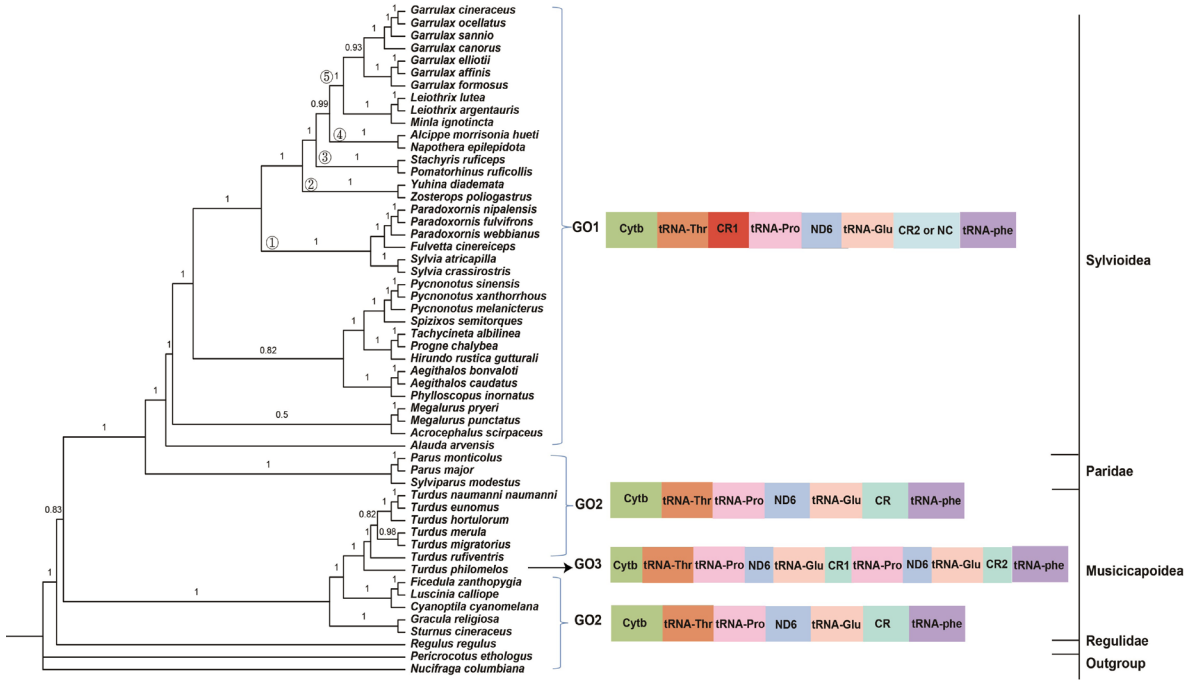

Fig. 6. The distribution of gene orders of 54 mitochondrial genomes from the Passeriformes based on phylogenetic tree. GO1, CYTB/tRNA^{Thr}/CR1/tRNA^{Pro}/ND6/tRNA^{Glu}/CR2 (or NC)/ tRNA^{Phe}; GO2, CYTB/tRNA^{Thr}/tRNA^{Pro}/ND6/tRNA^{Glu}/CR/tRNA^{Phe}; GO3, CYTB/tRNA^{Thr}/ tRNA^{Pro}/ND6/tRNA^{Glu}/CR1/tRNA^{Pro}/ND6/tRNA^{Glu}/CR2/tRNA^{Phe}.

Table V. Genetic distances between 13 protein coding genes (PCGs) of 22 babbler species (see Table II for list) computed by Kimura 2-parameter model. Pairwise genetic distances are shown below the diagonal and the upper numbers are standard error estimates.

Fulvetta cinereiceps		0.004	0.004	0.004	0.004	0.005	0.004	0.004	0.005	0.004	0.004	0.005	0.004	0.005	0.005	0.005	0.005	0.005	0.004	0.004	0.004
Paradoxornis nipalensis	0.162		0.004	0.004	0.004	0.004	0.004	0.005	0.005	0.004	0.005	0.005	0.004	0.005	0.005	0.005	0.005	0.005	0.004	0.004	0.004
Paradoxornis fulvifrons	0.171	0.125		0.004	0.005	0.004	0.005	0.005	0.005	0.005	0.004	0.006	0.005	0.005	0.006	0.005	0.005	0.004	0.004	0.004	0.004
Paradoxornis webbianus	0.165	0.159	0.166		0.005	0.004	0.005	0.004	0.004	0.005	0.004	0.005	0.004	0.006	0.006	0.004	0.005	0.004	0.005	0.004	0.004
Sylvia atricapilla	0.195	0.195	0.207	0.200		0.005	0.005	0.006	0.005	0.005	0.006	0.005	0.005	0.004	0.005	0.005	0.005	0.005	0.005	0.005	0.004
Sylvia crassirostris	0.179	0.184	0.184	0.177	0.172		0.005	0.004	0.005	0.005	0.005	0.005	0.005	0.005	0.006	0.005	0.005	0.005	0.004	0.004	0.005
Garrulax sannio	0.190	0.200	0.202	0.202	0.206	0.188		0.004	0.003	0.004	0.004	0.004	0.004	0.005	0.004	0.004	0.004	0.004	0.004	0.004	0.004
Garrulax elliotii	0.186	0.193	0.195	0.193	0.204	0.180	0.144		0.003	0.004	0.004	0.003	0.004	0.003	0.004	0.004	0.005	0.005	0.004	0.004	0.004
Garrulax affinis	0.186	0.194	0.195	0.191	0.202	0.184	0.148	0.103		0.004	0.003	0.003	0.003	0.004	0.004	0.004	0.005	0.004	0.003	0.004	0.004
Garrulax cineraceus	0.191	0.199	0.204	0.200	0.203	0.185	0.137	0.143	0.145		0.004	0.003	0.005	0.004	0.004	0.004	0.004	0.005	0.004	0.004	0.005
Garrulax formosus	0.184	0.193	0.193	0.192	0.201	0.180	0.150	0.119	0.116	0.146		0.004	0.004	0.005	0.005	0.005	0.005	0.004	0.003	0.004	0.005
Garrulax ocellatus	0.195	0.208	0.209	0.208	0.213	0.190	0.139	0.155	0.152	0.127	0.159		0.003	0.005	0.003	0.004	0.004	0.004	0.003	0.004	0.004
Garrulax canorus	0.192	0.205	0.205	0.201	0.208	0.190	0.146	0.153	0.153	0.142	0.153	0.151		0.004	0.004	0.004	0.004	0.003	0.004	0.004	0.004
Leiothrix lutea	0.188	0.197	0.204	0.204	0.208	0.192	0.162	0.152	0.153	0.156	0.152	0.166	0.161		0.003	0.004	0.005	0.005	0.004	0.003	0.005
Leiothrix argentauris	0.194	0.202	0.207	0.206	0.207	0.193	0.157	0.154	0.155	0.160	0.156	0.168	0.164	0.086		0.004	0.004	0.005	0.004	0.004	0.004
Minla ignotincta	0.188	0.196	0.204	0.202	0.205	0.189	0.158	0.152	0.154	0.158	0.157	0.166	0.164	0.138	0.142		0.005	0.004	0.004	0.004	0.004
Alcippe morrisonia hueti	0.186	0.192	0.196	0.186	0.198	0.185	0.164	0.167	0.158	0.162	0.161	0.171	0.167	0.171	0.169	0.166		0.005	0.004	0.004	0.004
Napothera epilepidota	0.203	0.198	0.203	0.204	0.204	0.193	0.178	0.179	0.173	0.175	0.170	0.183	0.181	0.178	0.187	0.181	0.169		0.004	0.004	0.004
Stachyris ruficeps	0.199	0.209	0.211	0.212	0.200	0.198	0.185	0.181	0.178	0.183	0.180	0.195	0.186	0.184	0.188	0.186	0.177	0.184		0.004	0.004
Pomatorhinus ruficollis	0.189	0.193	0.199	0.199	0.206	0.191	0.171	0.167	0.167	0.165	0.170	0.168	0.156	0.178	0.179	0.179	0.170	0.178	0.172	0.004	0.004
Yuhina diademata	0.186	0.192	0.197	0.193	0.204	0.185	0.170	0.164	0.162	0.150	0.166	0.177	0.176	0.161	0.172	0.170	0.172	0.186	0.186		0.004
Zosterops poliogastrus	0.191	0.196	0.206	0.198	0.205	0.192	0.181	0.177	0.171	0.179	0.177	0.191	0.184	0.186	0.188	0.184	0.177	0.191	0.196	0.160	

The 36 Sylvioidea species shared a common gene order of "CYTB / $tRNA^{Thr}$ / CR1 / $tRNA^{Pro}$ / ND6 / $tRNA^{Glu}$ / CR2 (or NC) / $tRNA^{Phe}$" (GO1 Fig. 6). We found that "CYTB / $tRNA^{Thr}$ / $tRNA^{Pro}$ / ND6 / $tRNA^{Glu}$ / CR / $tRNA^{Phe}$" was a shared by Regulidae, Paridae and other species of Muscicapoidea (18 non-Sylvioidea species; GO2 Fig. 6). The exception to this was *Turdus philomelos* from Muscicapoidea who had the gene order of "CYTB / $tRNA^{Thr}$ / $tRNA^{Pro}$ / ND6 / $tRNA^{Glu}$ / CR1 / $tRNA^{Pro}$ / ND6 / $tRNA^{Glu}$ / CR2 / $tRNA^{Phe}$" (GO3).

Phylogenetic relationships

The phylogenetic trees were constructed using 13 PCGs of the 54 complete mitochondrial genomes from Passeriformes (Fig. 4 and Fig. 5). We found there was strong support for babblers being separated into two families, including five primary Clades (distinct from minor/macro clades by the use of "Clade" ① to ⑤). These two groups have been classified here as Sylviidae (Clade ①) and Timaliidae (Clades ②~⑤; BI = 1.00, ML = 1000), with subfamilies forming Clades ②~⑤. The clade consisting of *F. cinereiceps* and *Paradoxornis* (BI = 1.00, ML = 1000) was a sister to the *Sylvia* (BI = 1.00, ML = 1000), which together formed Clade ①. Within Timaliidae, Clade ② was composed of *Yuhina* and *Zosterops* (subfamily Zosteropinae), which was deeply nested in the basal position of Timaliidae as the sister group of Clade ③ + (Clade ④ + Clade ⑤). Clade ③ included the *Stachyris* and *Pomatorhinus* (BI = 1.0, ML = 990; subfamily Timaliinae). Clade ⑤ comprising *Garrulax* + (*Leiothrix* + *Minla*) (subfamily Leiothrichinae) was sister to the Clade ④ (*A. morrisonia hueti* + *Napothera epilepidota*) (BI = 0.99, ML = 444; subfamily Pellorneinae).

The genetic distance between *F. cinereiceps* and other species of Sylviidae was shorter (0.162 ~ 0.195; average: 0.174) than between *F. cinereiceps* and species Timaliidae (Table V). The genetic distance between *F. cinereiceps* and species of Timaliidae ranged from 0.184 to 0.203 (average: 0.191).

The babblers (Sylviidae + Timaliidae), Pycnonotidae, Hirundinidae, Acrocephalidae, Aegithalidae, Phylloscopidae, Megaluridae and Alaudidae were clustered into a clade constituting Sylvioidea (BI=1.00, ML=937), which formed the sister clade of Paridae (BI=1, ML=726). Within Sylvioidea families, the position of Acrocephalidae was variable with low support, either as a sister to Megaluridae (Fig. 4) or as the sister to Hirundinidae + Pycnotidae (Fig. 5). The Muscicapoidea consisted of Turdidae, Muscicapidae and Sturnidae, and was situated in the basal position of Sylvioidea and Paridae (BI=0.83, ML=693).

DISCUSSION

F. cinereiceps *sequence composition, PCGs and tRNA genes*

We found that *F. cinereiceps* has a circular mitochondrial genome (16,969 bp) with a greater A+T (52.7%) content and this A+T bias was reflected in the 53 other Passeriformes species' mitochondrial genomes (51.6-57.7%). This A+T rich pattern is normal and widespread in most vertebrates (Sun *et al*., 2005). The frequency of nucleotide bases in *F. cinereiceps* mitochondrial genome is C > A > T > G with Guanine being the least common nucleotide. *F. cinereiceps* has a typical vertebrate mitogenome with 22 tRNAs, two rRNAs, 13 PCGs, a control region (CR) and a non-coding (NC) region. Several genes shared a few bases with each other (1~8 bp) and consequently mitochondrial genome sequences were quite compact (Curole and Kocher, 1999). A total of 3,789 codons were identified within the PCGs, where Guanine was the least common base at the third codon position as has been demonstrated in other studies (Webb and Moore, 2005). Similarly, start and stop codons were in congruence with other species, where *F. cinereiceps* stop codons TAA, AGA, AGG, TAG and T (incomplete stop codon) are very common in birds (Wen and Liao, 2016; Liu *et al*., 2017). Our finding that $tRNA^{Ser\ (AGN)}$ lacked the DHU arm has been found in other animals, such as birds (Liu *et al*., 2017) and insects (Du *et al*., 2015).

Rates and patterns of PCG evolution in the 22 babbler species

We found that five of the six (excluding ts/tv) rates of variation in mitochondrial PCGs were highest in ND2 and lowest in COX1, while the ts/tv was highest in ND6 and lowest in ATP8. Marshall *et al*. (2013) and Kerr (2011) also found that COX1 had the lowest non-synonymous-to-synonymous substitution ratios (dN/dS or ω), confirming these authors conclusions that functional constraints are strong for this gene rather than the earlier suggestion of recurrent bouts of positive selection. In addition, we found that ω<1 for all 13 PCGs indicating purifying selection (Jiggins *et al*., 2002) of these PCGs across the 22 babbler species.

Control region and gene order

We found that the 36 species of Sylvioidea either had one single region (CR1) with a NC, or two control regions (CR1 and CR2). Non-Sylvioidea species' control region (CR) was found between $tRNA^{Glu}$ and $tRNA^{Phe}$. *F. cinereiceps* control regions had low sequence identity (9.69%), while *G. formosus* had two control regions with the highest sequence identity (93.1%). Meanwhile, *Sylvia crassirostris* with the NC and *Sylvia atricapilla* with the

CR2 shared the identical phylogenetic position (Table II and Fig. 6). Therefore, the control regions may had multiple independent origins in Sylvioidea.

The 36 Sylvioidea species shared a common gene order (Fig. 6). The 18 passerine non-Sylvioidea species also shared gene order, except for *T. philomelos* (Muscicapoidea). Accordingly, the gene order can provide a meaningful reference for phylogenetic analysis to a certain extent.

Phylogenetic relationships

We found strong support for babblers being separated into two families, Sylviidae and Timaliidae, with *F. cinereiceps* belonging to Sylviidae. Our results indicated that Timaliidae should be separated into the subfamilies Zosteropinae, Timaliinae Leiothrichinae and Pellorneinae, contrary to current ornithological authorities who have classified these subfamilies as families (del Hoyo *et al*., 2019; Gill and Donsker, 2019). Previous studies have found support for a range of varying classifications (e.g. Alström *et al*., 2006; Cibois, 2003b; Pasquet *et al*., 2006; Cai *et al*., 2019), consequently the taxonomy of the babblers has been extensively debated. There has been no consensus and a recent phylogenetic analysis of the babblers has proposed a new taxonomic revision with seven families and 64 genera (Cai *et al*., 2019), rather than confirming an existing arrangement and at odds with our study. Our results are supported by Gelang *et al*. (2009) who found strong support for the babblers being from families Sylviidae and Timaliidae. Gelang *et al*. (2009) also divided Timaliidae into the subfamilies Zosteropinae, Timaliinae, Pellorneinae and Leiothrichinae. Given that our results are contrary to many other studies, yet they support Gelang *et al*., (2009), and our study is based on fewer babbler species than, for example Cai *et al*. (2019), we cannot make certain conclusions regarding the phylogeny of babblers. It is assumed that as there has been no consensus across more than a few studies, the broader debate of babbler taxonomy will continue.

We found that *F. cinereiceps* was genetically closer and may be more morphologically (including plumage) similar to other Sylviidae species (Pasquet *et al*., 2006; Zhang *et al*., 2014). *F. cinereiceps* is too genetically distant from Timaliidae to be placed in that family. Consequently, we argue that *F. cinereiceps* should be placed within Sylviidae. Although *F. cinereiceps* was previously classified as belonging to genus *Alcippe*, we confirmed the finding of Moyle *et al*. (2012) that *F. cinereiceps* is more closely related to *Paradoxornis* and *Sylvia* than *Alcippe*.

Mayr and Bock (2002) and Gill *et al*. (2005) stated that a significant criterion for a well-defined taxon was monophyly (Dong *et al*., 2010). However, we found several instances of polyphyly within genera. The polyphyly of Sylviidae genera prompted the reclassification of *F. cinereiceps* into *Fulvetta* and other species into new/old genera (e.g. Cibois, 2003a; Dong *et al*., 2010; Huang *et al*., 2015). Similar to the debate surrounding the higher hierarchical classification of babblers, it is likely that genera classifications within Sylviidae will continue. Nevertheless, our results strongly support classification of *F. cinereiceps* within Sylviidae and our mapping of the complete mitogenome of *F. cinereiceps* will assist with reducing taxonomic uncertainty in future studies. The debate surrounding babbler phylogeny will continue until more species' genomes are mapped and more comprehensive phylogenetic analyses are undertaken.

Similarly, higher hierarchical classifications are not certain, although not as contentious as within the babblers. We found that Sylvioidea consisted of the babblers (Sylviidae + Timaliidae), Pycnonotidae, Hirundinidae, Acrocephalidae, Aegithalidae, Phylloscopidae, Megaluridae and Alaudidae (Figs. 4, 5, 6; GO1). The position of Acrocephalidae is either as a sister to Megaluridae or Hirundinidae + Pycnotidae. Although previously classified in Paridae, the results of phylogenetic analysis and gene order confirmed that Aegithalidae should be classified in Sylvioidea as has been accepted in other studies (Alström *et al*., 2006, 2014). We also confirmed that Muscicapoidea consisted of Turdidae, Muscicapidae and Sturnidae, and was situated in the basal position of Sylvioidea and Paridae, while Regulidae should be excluded from Sylvioidea.

We suggest that gene order can be a useful for confirming results of phylogenetic analyses. However, we question whether gene orders of mitochondrial genomes can distinguish between higher taxa, as has been suggested to separate Passeriformes suborders, such as Passeri (Oscines: songbirds) and Tyranni (Suboscines) (Mindell *et al*., 1998). For example, we found three distinct gene orders (GO1-GO3) within our study only using birds from Passeri. If a study only used gene order to separate (e.g.) *Zosterops poliogastrus* (GO1) and *Parus major* (GO2) they would incorrectly conclude they were from different suborders. Additionally, using gene order may be limited in some instances because we found that one species (*T. philomelos*) had a distinct gene order (GO3) from its congenerics. Therefore, gene order should be used in conjunction with phylogenetic analyses when classifying taxa.

CONCLUSIONS

Our findings provide the first complete mitochondrial genome of *F. cinereiceps* and we found strong support for *F. cinereiceps* being placed within Sylviidae (superfamily Sylvioidea). Of the 36 sampled species of Sylvioidea, we identified that all had the same gene order. However, gene order is limited as a stand-alone taxonomic analysis tool because we also found that species of the same genus and

higher taxonomic classifications had differing gene orders (non-Sylvioidea species). Within Sylvioidea, we found strong support for babblers being separated into two families, Sylviidae and Timaliidae, with four subfamilies within Timaliidae. This is one of many taxonomic arrangements for babblers and there is likely to be continuous debate regarding the taxonomy of babblers until a consensus is reached. Therefore, the complete mitochondrial genome of *F. cinereiceps* that we have provided should reduce uncertainty and with additional complete mitochondrial genomes babbler taxonomy can be mapped with confidence.

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Statement of conflict of interest

All authors declared no conflict of interest.

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