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Complete Mitochondrial Genome of *Turdus merula* (Aves: Passeriformes: Turdidae) and Related Species: Genome Characteristics and Phylogenetic Relationships

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LJ, and WC conceived and planned the experiments. ZZ, XZ, HQ, LM, and QG performed the experiments, analyzed and interpreted the data. ZY, JL and HM analyzed the sequence data. LJ, BD, and WC wrote the manuscript with input from all authors. All authors read and approved the manuscript.

Key words
***Turdus merula*, Muscicapoidea, Passeriformes, Mitochondrial genome, Phylogenetics**

ABSTRACT

Mitochondrial genome is a very useful marker for determining the phylogenetic relationships. Hence in this study, the complete mitochondrial genome of *Turdus merula* was sequenced, described, and analyzed with Sanger sequencing technology. The complete mitochondrial genome of *T. merula* was 16,734 bp in length and encoded 37 genes, including 13 protein-coding genes, 22 tRNAs, 2 rRNA gene fragments, a control region (D-loop region) and gene arrangement was identical to that of other Passeriformes mitogenomes. The overall base composition included A, 29.34%; C, 32.50%; G, 14.82% and T, 23.34%. The motifs obtained by sequence comparison, "ATGAACCTAA" between ATP8 and ATP6, and "ATGCTAA" between ND4L and ND4, and "CAAGAAAGG" between COXI and tRNA$^{Ser(UCN)}$ were highly conservative in Passeriformes species. The monophyly of Passeriformes is divided into four major clades: Musicicapoidea, Sylvioidea, Passeroidea, and Corvoidea. The phylogeny analyses of Passerida was conducted with the clear support of dividing the group into three superfamilies: the Muscicapoidea, the Sylvioidea, and the Passeroidea, and Passeroidea is a sister taxon for Muscicapoidea and Sylvioidea, which are closely related to each other. We suggest that the genus *Paradoxornis* will be classified as family Sylviidae, while these two species (*Luscinia cyanura* and *Monticola gularis*) are placed in the family Muscicapidae. Moreover, Turdidae formed a sister group with Muscicapidae, which indicates that they are closely related and form the superfamily Muscicapoidea together with the Sturnidae families. The relationships between some species of the order Passeriformes may remain difficult to resolve despite an effort to collect additional characters for phylogenetic analysis. Current research of avian phylogeny should focus on adding molecular markers and taxa samples and use both effectively to reconstruct a better resolution for disputed species.

INTRODUCTION

The fast evolutionary rate, relatively conserved gene content and organization, maternal inheritance, and limited recombination make mitochondrial genomes an useful neutral molecular marker for studies related to species identification, verification taxonomic levels, and identification phylogenetic relationships (Boore and Brown, 1998; Anmarkrud and Lifjeld, 2017; Ingman *et al*., 2000; Wolstenholme, 1992; Bernt *et al*., 2013). The new sequencing and PCR technologies have made the utilization of the mitochondrial genome easier and more

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frequent in recent decades (Powell *et al*., 2013; Alam *et al*., 2014; Mu *et al*., 2015). Since the identification first complete mitochondrial genome of birds (Desjardins and Morais, 1992), nearly 2000 mtDNA sequence of birds have been submitted to NCBI (http://www.ncbi.nlm.nih.gov/). The vertebrate mtDNA is a compact double-stranded closed circular molecule that contains 2 ribosomal RNAs (rRNA), 13 protein-coding genes (PCGs), 22 transfer RNAs (tRNA), and a non-coding control region (CR) (Simon and Frati, 1994). Some of these genes such as *Cytb, COXI*, and *COXII* have frequently been used for population genetic studies (Alam *et al*., 2014; Velez-Zuazo and Agnarsson, 2011).

Nevertheless, genetic information may lack sufficient resolution and may not be always rightly based on these short mitochondrial regions (Alam *et al*., 2014; Velez-Zuazo and Agnarsson, 2011). Compared with single or partial mitochondrial gene fragments, such as COX I and Cytb, mitochondrial genome sequence can provide more information and faster substitution rate and more insight and better resolution from higher-level groups to closely related species (Powell *et al*., 2013; Mu *et al*., 2015).

The monophyly of the order Passeriformes is strongly supported by the morphological characteristics and molecular data (Raikow, 1986; Edwards, 1991; Sibley and Ahlquist, 1992; Zhang *et al*., 2018). This order is divided into two major clades, the suboscines and oscines. The oscines can be further split into two suborders: Corvida and Passerida. Corvida is further divided into three superfamilies: Menuroidea, Meliphagoidea and Corvoidea. Passerida can be also divided into three superfamilies: Muscicapoidea, Sylvioidea, and Passeroidea (Sibley and Ahlquist, 1992; Zhang *et al*., 2018). However, the taxonomic status of passerines is rather confusing, since most of the evolutionary groups underwent very rapid radiation during the Paleogene (Feduccia, 1995). In addition, in some studies, the monophyly of Sylvioidea has not been supported (Sheldon and Gill, 1996; Barker *et al*., 2002; Alström *et al*., 2006), thus owing to the monophyletic taxonomic relationship of Passeridae and Sylviidae, Paridae should be removed from Sylvioidea. However, Alström *et al*. (2006) thought that the Alaudidae originally belonged to Passeroidea and should therefore be included in the Sylvioidea. These controversies are at a deep level of the taxonomic status between Muscicapoidea, Sylvioidea, Passeroidea, and Paridae (Barker *et al*., 2004; Johansson *et al*., 2008; Nabholz *et al*., 2010; Zhang *et al*., 2018).

Turdus merula, the national bird by Sweden, is a passerine bird of the family Turdidae (Passeriformes), a large family which is composed of 341 species in 24 genera (Dickinson, 2003; Sangster *et al*., 2010). They are widespread in temperate Eurasia, North Africa, the Canary Islands, and South Asia, and have been introduced to Australia and New Zealand (Long, 1981). The *T. merula* may be a resident, partially migratory, or fully migratory depending on latitude (Zheng, 2018). In China, the *T. merula* is a resident bird extending from the Yangtze River to the Tianshan Mountains (in Nanchong, Sichuan, and Zipeng mountain in Hefei province). However, in Hainan province, they are winter migratory birds (Luo *et al*., 2008; Ni, 2014; Zhou *et al*., 2001). In recent years, although *T. merula* population is increasing, due to their natural habitat loss and urbanization part of the *T. merula* has colonized from original forest to urban (Wang and Yin, 2016).Thus the *T. merula* was listed as less concern species by IUCN in 2021 (http://www.iucnredlist.org/search). To strengthen the protection of *T. merula* and marginal species of it, many scholers have studied the classification of *T. merula* and Turdidae, and some achievements have been made. Because of its easy amplification, lack of insertion and deletion, and sufficient variation, the *COX1* gene in mtDNA plays an important role in bird identification and phylogeny (Seutin and Bermingham, 1997). Thus, Xu *et al*. (2010) studied 14 birds of the subfamily Turdidae and explored their phylogenetic relationships with *COX1* genes in the year 2010, and the results showed that the family of Turdidae can be divided into 2 large branches, including *Turdus*, *Zoothera*; *Phoeicurus*, *Tarsiger*, *Enicurus*, and *Myiophonus* (Xu *et al*., 2010). Some phylogenetic relationships strongly suggest that Muscicapidae is not monophyletic or paraphyletic with mitochondrial genes (Zhang *et al*., 2018) and multilocus study (Sangster *et al*., 2010; Payevsky, 2014). Furthermore, the relationships between the family Turdidae species are suspected as a monophyletic group (Payevsky, 2014; Zhang *et al*., 2018). In addition, there is a parallel relationship between generic level in Turdidae or Muscicapidae (Sangster *et al*., 2010; Zhang *et al*., 2018).

In this study, we extracted, measured, and analyzed the characterization of the complete mitochondrial genome sequence of *T. merula.* Moreover we analyzed and compared its complete mitochondrial genome DNA sequence with 78 mitochondrial genome sequences of other Passeriformes to provide insight into their genome evolution as well as the phylogeny. We reconstructed the phylogenetic tree within this order based on 13 PCGs of the selected birds to understand the sequence characteristics and evolutionary status and to infer their higher phylogeny.

MATERIALS AND METHODS

Sample and DNA extraction

A single specimen of *T. merula* was obtained from

Zitong County, Mianyang City, Sichuan Province in China (105°4'14.04", 31°49'49.95", 555m above sea level). Tissue samples (2 mL) were obtained and preserved with heparin anticoagulant tube prior to the analysis. The complete mitogenome was extracted and purified from the preserved muscle tissue by the Proteinase K/SDS digest extraction method followed by phenol–chloroform isolation and ethanol precipitation (Sambrock and Russel, 2001). After the quality of the obtained genomic DNA was checked by using 0.9 % agarose gel, it was stored at -20°C until needed for PCR.

Primer design, PCR amplification and sequencing

The twelve overlapping fragments that we amplified with normal PCR and LA-PCR covered the entire mtgenome of *T. merula*, and each of them overlapped more than 120-230 bp. Complete mtDNA was amplified as concatenated sequences using selectively amplified mtDNA template and seven primer pairs derived from the literature (Zhao *et al*., 2012; Jiang *et al*., 2014). The remaining PCR primers were designed based on the alignments of the relatively conserved nucleotide sequences in 6 homologous Turdidae species in GenBank and designed by using Primer (Premier 5.0 software). Twelve pairs of primers (Table I) used for PCR amplification were used in the reaction volume of 25 µl which contained 2.5µl 10×loading buffer, 2.0µl of $MgCl_2$ (2.5mol/L), 1.5µl dNTP mix (2.5mM/L each), 1.0 µL of each primer (10 µmol/L), 1.0 µl DNA template (20 ng/µL), 0.6µl (5U/µL) of LA Taq polymerase and sterilized distilled water. The thermal cycle comprised an initial denaturation at 94 °C for 2.5 min, 33 cycles each of denaturation at 94 °C for 45s, annealing at 50-61 °C for 40 s, extension at 72 °C for 80-180 s, and a final extension at 72 °C for 9 min. The products of PCR were separated by 1.0 % TAE agarose gel electrophoresis and recovered using a Gel Extract Purification System (Omega bio-tek, U.S.A) and sequenced by using an ABI 3730 sequencer, either directly or following sub-cloning into the pUC19 DNA vector (TaKaRa, Japan).

Sequence analysis

The sequence assembly and annotation were performed using DNA Baser Assembler and manual screening (http://www.DNABaser.com). The primary DNA sequence data were homologous alignment using BLAST searches at NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Protein-coding genes (PCGs) and rRNA genes were identified by multiple sequence alignments with previouslysubmitted sequences of three *Turdus* species as well as by secondary structure search (Barker, 2014; Yan *et al*., 2016; Gibb *et al*., 2015). Both transfer RNAs genes and their secondary structures of the stem-loop were identified by tRNAscan-SE Search Server v.1.21 (http://lowelab.ucsc.edu/cgi-bin/tRNAscan-SE2.cgi) using the default arguments (Lowe and Eddy, 1997). And the cloverleaf secondary structures of *T. merula* were painted by using Photoshop. The tandem repeats of noncoding regions were analyzed with the Tandem Repeats Finder program (http://tandem.bu.edu/trf/trf.advanced.submit.html) (Benson, 1999). The base composition of the complete mitochondrial genome, 13 PCGs, 22 tRNAs, 2 rRNAs, D-loop, codon usage of 13 PCGs were analyzed using MEGA 6.06 (Tamura *et al*., 2013). Composition skew was calculated according to the following formulas: AT-skew = [A–T]/[A + T] and GCskew = [G–C]/[G + C], respectively (Lobry, 1996; Perna and Kocher, 1995). The complete mtDNA sequence of *T. merula* reported in this article was deposited in GenBank under the accession number MN248536.

Table I. PCR primers covering the mitochondrial genome of the *T. merula*.

Primer name	Upper primer sequence(5'→3')	Lower primer sequence(5'→3')
T1	GGGCCATCAACTTCATCACTACT	GGAGAAGGTGAATCAGGTTAGGA
T2	GTAACAAGGTAAGTGTACCGGAAGG	GCTAGGGAGAGGATTTGAACCTC
T3	TTCGAAGCAACCCTAATCCCAAC	AGGCCAAATTGAGCGGATTTTCC
T4	GCTGAGARGGNGTRGGAATCATRTC	CCCTCAGAATGATATTTGTCCTCA
T5	AACATCTCCGCATGATGAAA	CTTTTCAAGCCGTAGKYCTYGG
T6	ACAAAAACTACCAGCATACCCC	CGATTACAGAACAGGCTCCTCTA
T7	GAGCAATCCAGGTCGGTTTCTA	GGCTGATTGGGTGAGGAAGTAT
T8	CCCATACCCCGAAAATGATG	CCGAAGAATCAGAATAGGTGTTG
T9	GGMCARTGCTCAGAAATCTGYGG	GGGTCAAATCCGCATTCRTABGG
T10	TTYGAAGCMGCMGCCTGATACTG	GGWGCTTCTACGTGGGCTTTDGG
T11	AAAGCATGGCACTGAAGATG	CTTTCAGGTGTAAGCTGAATGC
T12	CAGTAGAGCACCCATTCATCATC	GCACCGCCAAGTCCTTAGAG

Table II. Mitogenomes of the Passeriformes used in the study.

	Species	Size (bp)	Accession no.
Suborder: Passerida Superfamily: Muscicapoidea Family: Turdidae Genus: *Turdus*			
	1.T. merula	16,734	MN248536*
	2. T. merula	16,730	KT601060
	3. T. merula	16,733	NC_028188
	4. T. abyssinicus	16,707	NC_052843
	5. T. cardis	16,761	NC_046948
	6. T. ruficollis	16,737	MT712159
	7. T. dissimilis	16,761	MW307918
	8. T. mupinensis	16,735	MW338659
	9. T. kesseri	16,754	NC_041095
	10. T. celaenops	16,749	LC541445
	11. T. obscurus	16,739	MZ337397
	12. T. eunomus	16,737	KM015261
	13. T. naumanni	16,750	KJ834096
	14. T. hortulorum	16,759	NC_024552
	15. T. migratorius	16,669	NC_024872
	16. T. rufiventris	16,669	NC_028179
	17. T. philomelos	18,540	NC_029147
Myadestes	*18. M. myadestinus*	16,641	NC_031352
Zoothera	*19. Z. aurea*	16,778	KT340629
Copsychus	*20. C. saularis*	16,827	NC_030603
Cyanoptila	*21. C. cyanomelana*	16,802	HQ896033
Ficedula	*22. F. albicollis*	16,787	KF293721
	23. F. zanthopygia	16,794	JN018411
Calliope	*24. C. calliope*	16,841	HQ690246
Phoenicurus	*25. P. auroreus*	16,772	NC_026066
Luscinia	*26. L. cyanura*	16,803	NC_026067
Monticola	*27. M. gularis*	16,801	NC_033536
Acridotheres	*28. A. cristatellus*	16,820	JF810423
Gracula	*29. G. religiosa*	16,818	JF937590
Sturnus	*30. S. cineraceus*	16,821	HQ896037
	31. S. nigricollis	16,841	JQ003192
	32. S. sericeus	16,823	NC_014455
	33. S. vulgaris	16,822	HQ915864
Acridotheres	*34. A. tristis*	16,793	NC_029360
Abrornis	*35. A. inornata*	16,875	NC_024726
Sylvia	*36. S. crassirostris*	17,207	AM889141
	37. S. atricapilla	17,937	AM889140
Acrocephalus	*38. A. scirpaceus*	17,903	AM889139
Psittiparus	*39. P. gularis*	17,109	NC_039536
Paradoxornis	*40. P. fulvifrons*	17,059	NC_028436
	41. P. nipalensis	16,996	NC_028437
	42. P. webbianus	16,960	NC_024539
	43. P. gularis	17,109	KX397391
Alauda	*44. A. arvensis*	17,018	NC_020425
Melanocorypha	*45. M. mongolica*	17,358	NC_036760
Parus	*46. P. major*	16,776	NC_026293
	47. P. monticolus	16,771	NC_028187
Pardaliparus	*48. P. venustulus*	16,778	NC_026701
Periparus	*49. P. ater*	16,783	NC_026223
Poecile	*50. P. atricapilla*	16,765	NC_024867
	51. P. montanus baicalensis	16,783	KX388479
	52. P. palustris	16,824	NC_026911
Pseudopodoces	*53. P. humilis*	16,758	KP001174
Remiz	*54. R. consobrinus*	16,737	KC463856
Sylviparus	*55. S. modestus*	17,086	KP642167
Hyliota	*56. H. flavigaster*	16,218	NC_024868
Pyrgilauda	*57. P. ruficollis*	16,909	KC836121
Pyrgilauda	*58. P. davidiana*	16,912	NC_025915
Pyrgilauda	*59. P. blanfordi*	16913	NC_025912
Passer	*60. P. ammodendri*	16,782	NC_029344
	61. P. montanus saturatus	16,904	KM577704
	62. P. domesticus	16,802	KM078784
	63. P. montanus	16,887	NC_024821
Prunella	*64. P. montanella*	16,832	NC_027284
Padda	*65. P. oryzivora*	16,817	NC_028441
Montifringilla	*66. M. henrici*	16,924	NC_042414
	67. M. taczanowskii	16,917	NC_025914
	68. M. adamsi	16,912	NC_025913
	69. M. nivalis	16,923	NC_025911
Petronia	*70. P. petronia*	17,426	MF071218
Prunella	*71. P. fulvescens*	16,837	NC_035747
	72. P. strophiata	16,830	NC_031819
Regulus	*73. R. regulus*	16,847	NC_029837
	74. R. calendula	16,859	NC_024866
Callaeas	*75. C. cinereus*	16,711	NC_031350
Creadion	*76. P. carunculatus*	16,827	NC_029143
Corvus	*77. C. frugilegus*	16,931	NC_002069
Pica	*78. P. pica*	16,939	NC_015200
Cyanopica	*79. C. cyanus*	16,893	JN108020
Lanius	*80. L. tephronotus*	16,820	NC_021105
	81. L. schach	16,820	NC_030604
Oriolus	*82. O. chinensis*	16,803	NC_020424
Turnagra	*83. T. capensis*	16,932	KU158197
Rhagologus	*84. R. leucostigma*	17,044	NC_040956
Pericrocotus	*85. P. ethologus*	16,935	NC_024257
Lalage	*86. L. tricolor*	16,952	KY994597
Vireo	*87. V. olivaceus*	17,295	NC_024869
Terpsiphone	*88. T. atrocaudata*	16,954	NC_032725
Menura	*89. M. novaehollandiae*	17,839	NC_007883
Climacteris	*90. C. picumnus*	16,869	KY994598

Note: The asterisk indicates the species in this study.

Phylogenetic analysis

To explore the phylogenetic relationships between *T. merula* and other Aves (Passeriformes: Turdidae, Sturnidae, Muscicapidae and Paridae, and so on), all available complete mitochondrial genomes of them were downloaded at NCBI Genbank. Moreover, two species *Menura novaehollandiae* (NC_007883), and *Climacteris picumnus* (KY994598) were set as outgroup (Table II). All 13 protein coding genes were aligned using MEGA 6.06 with default settings (Tamura *et al*., 2013). Subsequently, all 13 PCG alignments were combined to create a concatenated data set. The optimal nucleotide substitution model was selected using jModeltest v.0.1.1 (Posada, 2008). Maximum Likelihood (with 1000 bootstrap replicates) and Akaike Information Criterion (AIC; Posada and Buckley, 2004) scores (Bayesian Information Criterion) were used for phylogenetic tree construction using PhyML v.3.0 (Guindon and Gascuel, 2003) and the Bayesian inference (BI) analysis was performed with MrBayes v.3.1.2 (Huelsenback and Ronquist, 2001). The best partitioning schemes for each partition were determined using PartitionFinder v2.1.1 (Lanfear *et al*., 2017). The mitogenome data matrix was divided into 39 partitions based on codon positions for each PCGs (13 genes × 3 codons = 39 partitions). The best fitting model of GTR+I+G (nst=6; rates=gamma) was selected for subsequent Bayesian phylogenetic analyses. Four Markov chains (one cold and three hot chains) were simultaneously run at five million generations, sampling every 1000 generations.

RESULTS AND DISCUSSION

Genome annotation and base composition

The complete mitogenomes of *T. merula* was a 16,734 bp in length. The circular DNA molecule (Table III, Fig. 1), which contains 13 protein-coding genes, 2 rRNA genes, 22 tRNA genes, and one non-coding region. Among the 37 fragment genes, 9 genes (including 1 protein-coding genes and 8 tRNA genes) were encoded by L chains, and the rest by H chains. Its overall base composition (H-strand) was: A, 29.34%; C, 32.50%; G, 14.82% and T, 23.34%, so the percentage of A and T (52.67%) was slightly higher than G and C (47.32%), within the range for avian mitogenomes (51.6-55.7%), similar to those of other thrushs (Haring *et al*., 2001; Rong *et al*., 2010; Yan *et al*., 2016; Li *et al*., 2016; Peng *et al*., 2016). The mitochondrial genome sequence of *T. merula* contained 42bp (0.25% of the whole sequence) overlapping nucleotides. Moreover, varied in length from 1 to 10 bp and the largest overlap (10bp) is located between 12S rRNA and tRNA^{Val}, ATP6 and ATP8, respectively. A total of 74bp (0.44% of the whole sequence) intergenic nucleotides (IGN) were dispersed in 18 locations and ranged in size from 1 to 10 bp; the largest gene interval can be found in three places (tRNA^{Asp} and COX2, ND5 and Cytb, tRNA^{Pro} and ND6). The details of gene location were given in Table III.

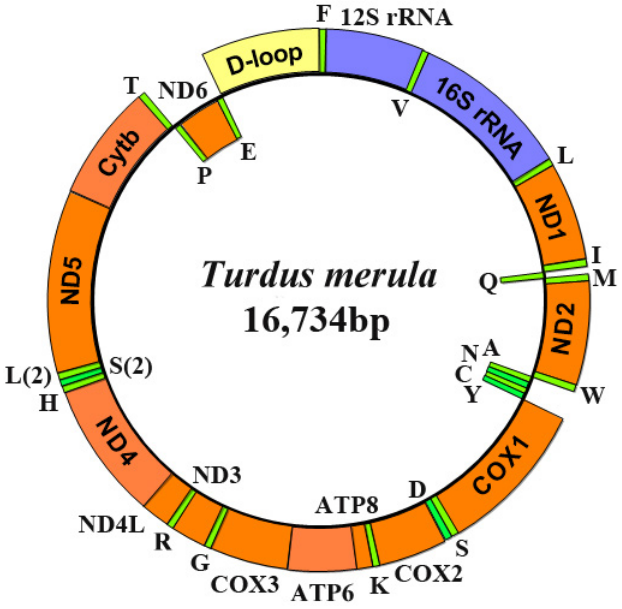

Fig. 1. Gene organization of the *T. merula* mitochondrial genome. All 37 genes are encoded on both strands. Genes for proteins and rRNAs are shown with the standard abbreviation. Genes for tRNAs are designated by a single letter for the corresponding amino acid with two leucine tRNAs and two serine tRNAs differentiated by numerals.

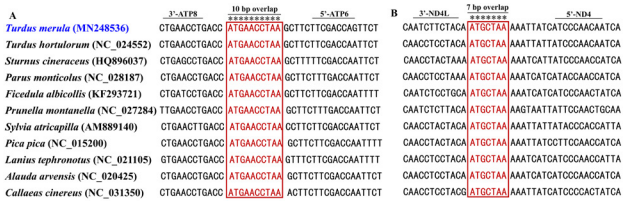

Fig. 2. Sequence alignments of ATP8/ATP6 and ND4L/ND4 of passerine birds. The boxed nucleotides indicate the 10 bp (ATGAACCTAA) and 7 bp (ATGCTAA) highly conserved overlaps.

We identified two long gene overlaps in *T. merula*, namely, 10-bp long overlaps (ATGAACCTAA) and 7-bp long overlaps (ATGCTAA), which are also found in many other Passeriformes species (Fig. 2, Table III). The two overlaps were located between ATP8 and ATP6 and between ND4L and ND4 on the H-strand, respectively. The overlapped sequences between genes were thought to be translated as a bicstron (Stewart and Beckenbach, 2005).

Table III. Characteristics of the mitochondrial genome of *T. merula.*

Gene	Position		Sizes		Codon			intergenic Nudeotide †	Strand ‡	A+T %
	From	To	Nudeotide (bp)	Amino acid	Anti-codons (tRNA)	Start	Stop*			
tRNA-Phe	1	68	68		GAA			0	H	51.5
12S ribosomal RNA	69	1052	984					-10	H	50.9
tRNA-Val	1043	1112	68		TAC			0	H	57.4
16S ribosomal RNA	1113	2713	1601					-3	H	54.9
tRNA-Leu	2711	2785	75		TAA			4	H	50.7
ND1	2790	3767	978	325		ATG	AGG	6	H	51.8
tRNA-Ile	3774	3845	72		GAT			6	H	55.6
tRNA-Gln	3852	3922	71		TTG			-1	L	64.8
tRNA-Met	3922	3990	69		CAT			0	H	47.8
ND2	3991	5029	1039	346		ATG	T--	0	H	51.6
tRNA-Trp	5030	5099	70		TCA			1	H	61.4
tRNA-Ala	5101	5169	69		TGC			4	L	60.9
tRNA-Asn	5174	5248	75		GTT			0	L	49.3
tRNA-Cys	5249	5315	67		GCA			0	L	52.2
tRNA-Tyr	5316	5385	71		GTA			1	L	59.2
COXI	5387	6937	1551	516		GTG	AGG	-9	H	50.8
tRNA-Ser	6929	7001	73		TGA			3	L	56.2
tRNA-Asp	7005	7074	70		GTC			10	H	60.0
COXII	7085	7768	684	227		ATG	TAA	1	H	52.0
tRNA-Lys	7770	7837	68		TTT			1	H	57.4
ATP8	7839	8006	168	55		ATG	TAA	-10	H	55.4
ATP6	7997	8680	684	227		ATG	TAA	5	H	51.5
COXIII	8686	9469	784	261		ATG	T--	0	H	48.9
tRNA-Gly	9470	9538	69		TCC			0	H	60.9
ND3	9539	9889	351	116		ATG	TAA	1	H	52.1
tRNA-Arg	9891	9960	70		TCG			1	H	60.0
ND4L	9962	10258	297	98		ATG	TAA	-7	H	49.8
ND4	10252	11629	1378	459		ATG	T--	0	H	52.2
tRNA-His	11630	11699	70		GTG			0	H	61.4
tRNA-Ser	11700	11765	66		GCT			-1	H	45.5
tRNA-Leu	11765	11835	71		TAG			0	H	59.2
ND5	11836	13653	1818	605		ATG	AGA	10	H	52.9
Cytb	13664	14806	1143					1	H	52.9
tRNA-Thr	14808	14876	69		TGT			8	H	66.7
tRNA-Pro	14885	14954	70		TGG			10	L	58.6
ND6	14965	15483	519	172		ATG	TAG	1	L	51.1
tRNA-Glu	15485	15556	72		TTC			0	L	47.2
D-loop	15557	16734	1180					0	H	54.9

* T represents incomplete stop codons. † Intergenic bp indicates gap nucleotides (positive value) or overlapped nucleotides (negative value) between two adjacent genes. ‡ H and L indicate genes transcribed on the heavy and light strands, respectively.

Table IV. Composition and skew values in *T. merula*.

Gene/region	Size (bp)	A (bp)	T (bp)	G (bp)	C (bp)	A %	T %	G %	C %	AT %	AT skew	GC skew
Whole genome	16734	4910	3905	2480	5439	29.34	23.34	14.82	32.50	52.68	0.11	-0.37
H-strand	14432	4211	3353	2173	4695	29.18	23.23	15.06	32.53	52.41	0.11	-0.37
L-strand	1087	390	193	150	354	35.88	17.76	13.80	32.57	53.63	0.34	-0.40
13 Protein-coding genes	11391	3095	2798	1657	3841	27.17	24.56	14.55	33.72	51.73	0.05	-0.40
1st	3797	1030	775	885	1107	27.13	20.41	23.31	29.15	47.54	0.14	-0.11
2nd	3797	692	1524	494	1087	18.22	40.14	13.01	28.63	58.36	-0.38	-0.38
3th	3797	1373	499	278	1647	36.16	13.14	7.32	43.38	49.30	0.47	-0.71
tRNA genes	1543	495	377	275	396	32.08	24.43	17.82	25.66	56.51	0.14	-0.18
rRNA genes	2583	856	523	531	673	33.14	20.25	20.56	26.05	53.39	0.24	-0.12
Control Region	1180	302	346	165	367	25.59	29.32	13.98	31.10	54.92	-0.07	-0.38

Another 9 bp long overlapping motif (CAAGAAAGG) was detected between COXI and tRNA$^{Ser\ (UCN)}$ in mitogenomes of *T. merula*, which was also present in other Passeriformes (Fig. 3). The sequenced motifs between ATP8 and ATP6, between ND4L and ND4, and between COXI and tRNA$^{Ser\ (UCN)}$ were relatively conserved in the Passeriformes species after mitogenomic comparisons. We found that gene overlap regions are ubiquitous in eukaryotic mitochondria. The existence of gene overlap regions enables limited genomic sequences to encode more genetic information, which is very economical and effective for the transmission of genetic information of species.

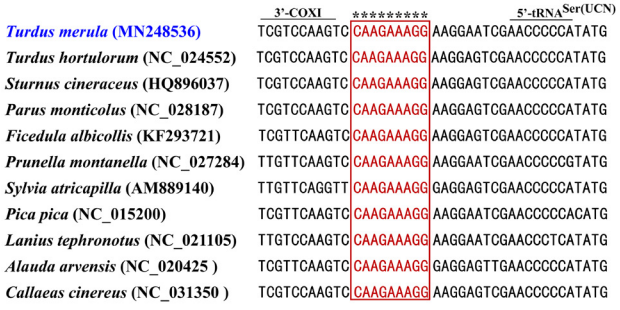

Fig. 3. Sequence alignment of the space region between COXI and tRNA$^{Ser\ (UCN)}$ of passerine birds. The boxed nucleotides indicate the 'CAAGAAAGG ' highly conserved motif. "ATGAACCTAA" between ATP8 and ATP6, and "ATGCTAA" between ND4L and ND4, and "CAAGAAAGG".

Protein-coding genes

All the 13 protein-coding genes found in other animals were also presented in *T. merula*, including 7 NADH dehydrogenase subunits (ND1-6, ND4L), 3 cytochrome c oxidase subunits (COXI-III), 1 Cytb, 2 ATPase subunits (ATP6, ATP8), and one cytochrome b gene (Cytb). The 13 mitochondrial protein-coding genes were 11,394 bp in length, accounting for 68.09% of the entire mitogenome sequence. The base composition of 13 PCGs were shown in Table IV. The A+T content of the 13 PCGs was 51.67% and the AT skew (0.10) of PCGs was slightly positive, while the GC skew (-0.45) was strongly negative. Contents with A+T in the second position were slightly higher than G+C while those in the first and third positions were on the contrary (Table IV). Two start codons (ATG, GTG) (ATG account for 92.31% of all initial codons), four stop codons of three (TAA, AGG, TAG), and the single incomplete stop codon (T) were used for initiating and terminating the coding of mitochondrial 13 PCGs. Only one protein-coding gene (COXI) utilized GTG as the start codon and all the others used ATG as standard start codon. TAA was the most frequent termination codon and seven protein-coding genes (COXII, ATP8, ATP6, ND3, ND4L, ND5, Cytb) ended with it; whereas ND1 and COXI ended with AGG and ND6 with TAG, which were also found in other Turdidae birds (Li *et al*., 2016; Zhang *et al*., 2018). The uncanonical T termination codon was used in the other three PCGs (ND2, COXIII, and ND4), which may be completed by poly-adenylation of the 3'-end of the mRNA after transcription (Boore, 1999; Yoon *et al*., 2013). In addition, the codon usage was shown in Table V and Figure 4. Encoding 3,797 amino acids (excluding stop codons), the most frequent amino acids in the 13 PCGs of *T. merula* were Leucine (19.44%), then Isoleucine (11.43%), and the next Alanine (9.15%). The highly abundant like this were similar to mitochondrial proteins of other birds (Liu *et al*., 2015, 2016; Song *et al*., 2016; Yong *et al*., 2015). According to relative synonymous codon usage shown in Table IV and Figure 5, the RSCU values of UUC, CUC,

CUA, AUC, and other 26 codons were great than or equal to 1, they were called preference codons of the *T. merula* mitochondrial genome.

Table V. Codon usage in *T. merula* mitochondrial protein-coding genes.

Codon	Count	RSCU	%
UUU(F)	41	0.39	1.08%
UUC(F)	171	1.61	4.50%
UUA(L)	47	0.41	1.24%
UUG(L)	24	0.21	0.63%
CUU(L)	55	0.49	1.45%
CUC(L)	185	1.63	4.87%
CUA(L)	317	2.8	8.35%
CUG(L)	52	0.46	1.37%
AUU(I)	64	0.48	1.69%
AUC(I)	224	1.68	5.90%
AUA(I)	112	0.84	2.95%
AUG(M)	42	1	1.11%
GUU(V)	40	0.84	1.05%
GUC(V)	60	1.26	1.58%
GUA(V)	65	1.37	1.71%
GUG(V)	25	0.53	0.66%
UCU(S)	36	0.74	0.95%
UCC(S)	102	2.1	2.69%
UCA(S)	84	1.73	2.21%
UCG(S)	10	0.21	0.26%
CCU(P)	27	0.48	0.71%
CCC(P)	101	1.8	2.66%
CCA(P)	88	1.56	2.32%
CCG(P)	9	0.16	0.24%
ACU(T)	52	0.67	1.37%
ACC(T)	142	1.83	3.74%
ACA(T)	113	1.46	2.98%
ACG(T)	3	0.04	0.08%
GCU(A)	56	0.7	1.47%
GCC(A)	160	2	4.21%
GCA(A)	97	1.21	2.55%
GCG(A)	7	0.09	0.18%
UAU(Y)	33	0.59	0.87%
UAC(Y)	79	1.41	2.08%
UAA(*)	6	1.71	0.16%
UAG(*)	1	0.29	0.03%
CAU(H)	20	0.37	0.53%
CAC(H)	87	1.63	2.29%
CAA(Q)	88	1.85	2.32%
CAG(Q)	7	0.15	0.18%
AAU(N)	22	0.34	0.58%
AAC(N)	108	1.66	2.84%
AAA(K)	82	1.91	2.16%
AAG(K)	4	0.09	0.11%
GAU(D)	11	0.33	0.29%
GAC(D)	56	1.67	1.47%
GAA(E)	67	1.52	1.76%
GAG(E)	21	0.48	0.55%
UGU(C)	7	0.41	0.18%
UGC(C)	27	1.59	0.71%
UGA(W)	95	1.78	2.50%
UGG(W)	12	0.22	0.32%
CGU(R)	8	0.65	0.21%
CGC(R)	17	1.38	0.45%
CGA(R)	37	3	0.97%
CGG(R)	9	0.73	0.24%
AGU(S)	8	0.16	0.21%
AGC(S)	51	1.05	1.34%
AGA(R)	1	0.08	0.03%
AGG(R)	2	0.16	0.05%
GGU(G)	19	0.35	0.50%
GGC(G)	77	1.4	2.03%
GGA(G)	74	1.35	1.95%
GGG(G)	50	0.91	1.32%

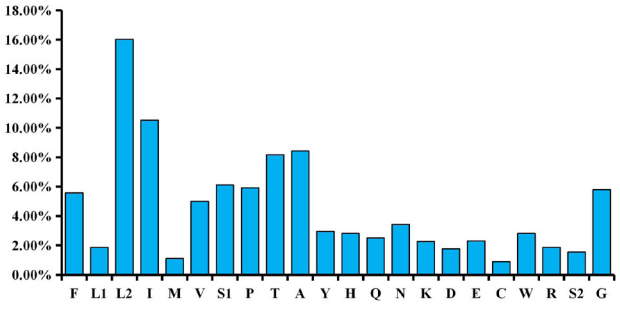

Fig. 4. Percentages of amino acid usage in mitochondrial genomes of *T. merula*. A. Codon distribution in *T. merula* mitogenome. Codon families were provided on the X axis. Each amino acid is represented by the one-letter abbreviation. Note that leucine and serine are each coded by two different genetic codons, and listed separately.

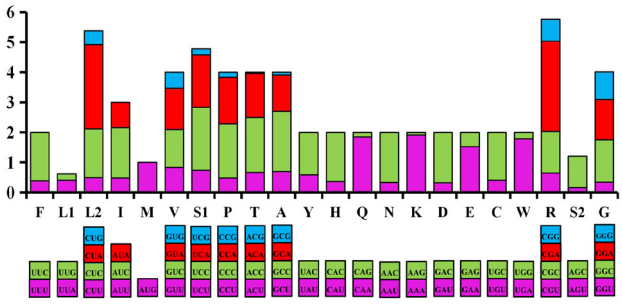

Fig. 5. Relative Synonymous Codon Usage (RSCU) in *T. merula* mitogenome. Codon families were provided on the X axis and the RSCU values on the Y axis.

Ribosomal RNA and transfer RNA genes

The 12S rRNA was 984bp and the 16S rRNA was 1601bp in length, which were located between $tRNA^{Phe}$ and $tRNA^{Leu\ (UUR)}$ genes, and separated by $tRNA^{Val}$ gene, as other avian rRNA genes (Yoon *et al*., 2015). The base composition of the two rRNA gene sequences are shown in Table V. The content of A+T (53.4%) was higher than that of C+G (46.6%).

Like most *Turdus* mtDNA, scattering throughout the mitogenome, the typical set of 22 tRNA genes was identified, ranging from 66 bp ($tRNA^{Ser(AGY)}$) to 75 bp ($tRNA^{Leu}$ and $tRNA^{Asn}$) in size (Table V). Among those tRNAs, fourteen tRNA were encoded on the H-strand and eight on the L-strand. Their secondary clover-leaf structures were predicted by tRNAscan-SE Search Server and presented in Figure 2. Only one ($tRNA^{Ser(AGY)}$) of them can not fold into the canonical cloverleaf secondary structure, whose dihydroxyuridine arm is absent. These features are common in most metazoans mitogenomes (Ohtsuki *et al*., 2002; Yoon *et al*., 2015). Furthermore, except the $tRNA^{Ser(AGY)}$, the other tRNA genes each have a 7bp length on the amino acid acceptor arm and the anticodon loop; the length of the DHU arm is 3 to 4bp, the anticodon arm is 3 to 5bp, the TψC arm was 4 to 5bp; the DHU ring, variable ring, and T ring vary greatly. This may be one of the main reasons for the variation of the tRNA length. The sequence and structure of anticodon, amino acceptor, and TψC arms were highly conserved, while the structure of the variable loop was highly diverse with obvious indels polymorphisms (Chen *et al*., 2018a, b). In addition to the typical A-U and G-C pairing, there were 30 swinging mismatched base pairs (G-U) in the mitochondrial genome of tRNA secondary structure of *T. merula*, most of which were found in the amino acid acceptor arm (12 places) and the anticodon arm (9 places). In addition, the remaining contained 4 places in DHU arm and 5 places in the TψC arm, and there are also some other mismatched bases, such as U-U, C-C, A-A and A-C (Fig. 6). In most arthropod mtDNA, these mismatches might be corrected by RNA editing, so that they can not lead to obstruction in amino acid transportation (Yokobori and Pääbo, 1995; Dang *et al*., 2008; Liu *et al*., 2012). At the same time, Varani and Mcclain (2000) believed that the unmatched base pair G-U can play an important role in the stability of tRNA secondary structure.

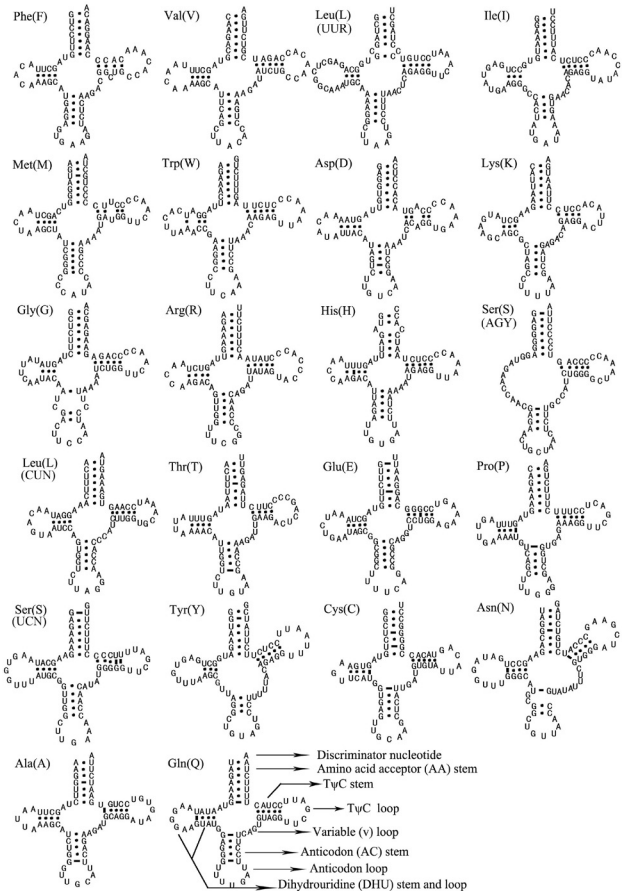

Fig. 6. Predicted secondary structures for the 22 tRNA genes of *T. merula*.

Non-coding regions

The non-coding region of *T. merula* includ a 1,180 bp long control region (D-loop) and a few intergenic spacers. As is the case in most avian mitogenomes, the D-loop region is located between $tRNA^{Glu}$ and $tRNA^{Phe}$ on H strand. The nucleotide composition of the *T. merula* CR is 25.5% A, 29.4% T, 31.0% C, and 14.0% G, with a distinct bias against G. The AT-skew is slightly negative (-0.07) while the GC-skew is strongly positive (0.38). Comparing the conserved motifs with those of other avian CRs and according to the basis of the variability (Anderson *et al*., 1981; Brown *et al*., 1986; Doda *et al*.,

1981), *T. merula* CR could be divided into three domains (Fig. 7): (i) the extended termination-associated sequence motif (ETAS) domain, which is associated with the termination of the newly synthesized H-strand during replication (Ryu and Hwang, 2012; Sbisa *et al*., 1997; Yoon *et al*., 2015); (ii) the central domain (CD); (iii) the conserved sequence block (CSB) domain. Locating between the 5'-end of the CR and the beginning of the F-box in the central domain, the ETAS domain contains conserved sequence blocks (CSB1-like) and two Extended termination associated motifs (ETAS1-2). Futhermroe closer to the 5'-end of the CR, a cytosine stretches sequence (CTCCACCCCCCCCCTTCCCCCCC) was found, which also exists in many bird species (Randi and Lucchini, 1998; Ritchie and Lambert, 2000; Yoon *et al*., 2015). The central domain contains six highly conserved sequence blocks (F-box, E-box, D-box, C-box, b-box and B-box). The F-box is at the upstream of the CD region while the B-box at downstream. CSB region is known to contain three conserved regions (CSB1, CSB2, CSB3) in the mitochondrial of vertebrates (Walberg and Clayton, 1981), but there is only one CSB1 and a bi-directional transcriptional promoter (HSP/LSP box) in *T. merula* of this region. Gene duplications, rearrangements and missing may lead to gene order changes. Generally, there are two types of arrangements between Cytb and 12S rRNA genes of mitochondrial organisation: (i) $tRNA^{Thr}$/ $tRNA^{Pro}$/NADH6/$tRNA^{Glu}$/CR/$tRNA^{Phe}$; (ii) $tRNA^{Thr}$/ CR/ $tRNA^{Pro}$/NADH6/$tRNA^{Glu}$/ NC/$tRNA^{Phe}$ and the *T. merula* follow the first pattern.

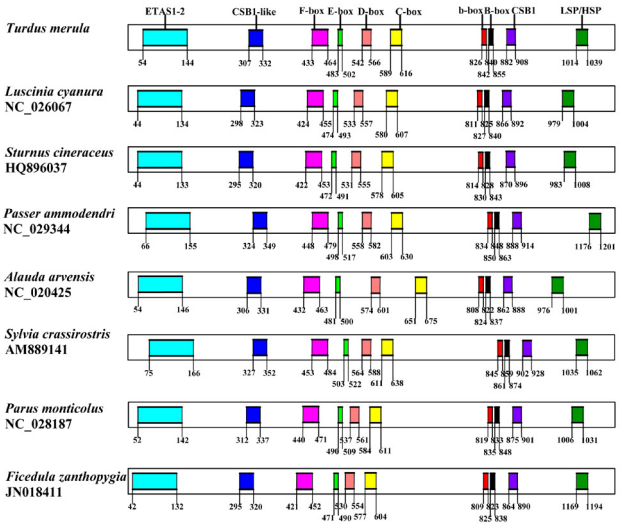

Fig. 7. The structure of the control region of *T. merula* in this study. The number in rectangle frames denotes the numerical value of the boundary. Sequential features are indicated above the graph.

Molecular phylogenetic analyses

The bayesian inference (BI) and maximum likelihood (ML) phylogenetic trees, which are based on the concatenated 13 PCGs of mitochondrial gene sequences of 78 passeriformes species and two outgroup species (*Menura novaehollandiae* and *Climacteris picumnus*), were reconstructed. In our study, the concatenated PCG data set of the mitogenome sequences included 11,382 nucleotide positions, including 5,229 conserved sites, 6,153 variable sites, and 5,577 potentially parsimony-informative sites. The phylogenetic relationship among these species shows the tree topologies of ML and BI were similar, except for several Turdidae species (*T. migratorius, T. rufiventris* and *T. merula*) in relationships or placements (Fig. 8).

Our phylogenetic trees strongly support the monophyly of Passeriformes group, as suggested by a previous study (Sibley and Ahlquist, 1992). The topological structure of the phylogeny of passerines allowed Sibley and Ahlquist (1992) to verify the traditional division of passerines into two suborders, the Suboscines and Oscines forming two obvious monophyletic lineages. The Oscines can be further split into two suborders: Corvida and Passerida, and Passerida can be divided into three superfamilies: the Muscicapoidea, the Sylvioidea, and the Passeroidea (Sibley and Ahlquist, 1992; Zhang *et al*., 2018). Although Passeroidea is a sister taxon of Muscicapoidea and Sylvioidea, Muscicapoidea and Sylvioidea are more closely related to each other. This relationship is also inconsistent in previous studies (Barker *et al*., 2002, 2004; Marshall *et al*., 2013; Zhang *et al*., 2018). Furthermore, corvida and passerida cluster on a large branch, and form a sister classification relation. *Terpsiphone atrocaudata* (Monarchidae) is located at the base of the Corvidae and Laniidae clade, while *Regulus calendula* and *R. regulus* (Regulidae) combine into a clade at the base of the Passerida group (Fig. 8). The two species were previously placed in the family Sylviidae, which is inconsistent with the results of our phylogenetic tree, and it has been previously reported (Keith, 2014). The systematic evolutionary relationships of two species representing the Regulidae in Passerida belong to other taxonomic groups because of the high support value of nodes (1.00/975) (Fig. 8). However based on the phylogeny reasoning of our study, there may be a clear statement when the sampled taxa and molecular markers are added. *Hyliota flavigaster* (Hyliotidae) forms a sister group with the family Paridae at the base of this family. One obvious result of the Fuchs *et al*. (2006) and Johansson *et al*. (2008) studies was the identification of the presumptive sylvioid genus *Hyliota* as another deeply-diverging member of the Passerida with unclear affinities. In line with our phylogenetic tree, *H. flavigaster* has a

distant relationship with the family Sylviidae, which is different to the previous study and considered it to family Sylviidae (Keith, 2014). Our data suggest that it should be classified into the superfamily Sylvioidea.

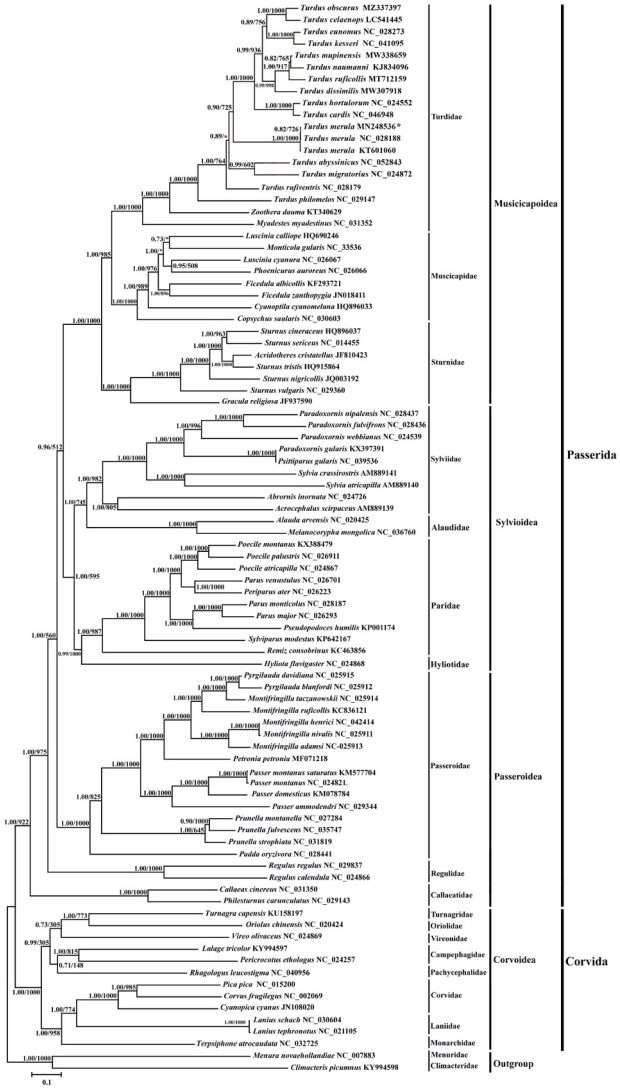

Fig. 8. Inferred phylogenetic relationships based on 13 protein-coding genes with Bayesian inference (BI) and maximum likelihood (ML), respectively. *Menura novaehollandiae* (NC_007883) and *Climacteris picumnus* (KY994598) were used as outgroups. Bayesian posterior probability (BPP) and Bootstrap value (BP) of each node are shown in turn, such as BPP/BP 1.00/1000. The asterisk indicates that the BP value is less than 500. Sequences were analysed under the GTR+I+G model, based on the AIC criterion.

The phylogenetic analysis based on the 13 PCGs of the mitochondrial genome suggests that the species of genus *Paradoxornis* should not be placed in the superfamily Muscicapoidea or family Muscicapidae (Fig. 8). Based on our phylogeny, we propose that genus *Paradoxornis* should be placed in the superfamily Sylvioidea or the family Sylviidae. Moreover, the systematic taxonomic status of the *P. humilis* (Paridae) has long been controversial. The species is classified into the family Corvidae based on skeletal characteristics and taxonomic data, while Hope (1989) considered *P. humilis* not to be placed in Corvidae. Then, James *et al*. (2003) obtained similar results with different methods, namely, the skeletal characteristics, nuclear gene (*c-myc*), and mitochondrial gene (*Cytb*). Subsequently, some researchers (Ericson and Johansson, 2003; Johansson *et al*., 2008; Treplin *et al*., 2008) strongly supported that this family should be separated from all other Sylvioidea, and independently form a taxonomic group, which is consistent with previous studies (Alström *et al*. 2006). In our mitochondrial genome analysis, the *P. humilis* is nested within other species of the family Paridae (Fig. 8), and should belong to this Sylvioidea. However, this relationship is inconsistent with previous research and proposed that it should be placed within the Muscicapoidea (Ericson *et al*., 2003; Johansson *et al*., 2008; Treplin *et al*., 2008; Zhang *et al*., 2018). Furthermore Marshall *et al*. (2013) suggested that the family Paridae represented by *P. humilis* should be separated from Sylvioidea and moved to the superfamily Muscicapoidea. However our phylogenetic data, similar to Zhang *et al*. (2018), show that this family belongs to Sylvioidea and confirmed that *P. humilis* was included in the Paridae.

Within the taxa of the superfamily Muscicapoidea, Sangster *et al*. (2010) and Zhang *et al*. (2018) strongly suggest that Muscicapidae is not monophyletic, and exists extensive paraphyly at the family and genus levels within this group. If some species are not classified into other families, such as *Paradoxornis*, *Luscinia cyanura,* and *Monticola gularis*, our taxonomic relationships are similar to previous studies (Sangster et al., 2010; Zhang *et al*., 2018). Based on the topology tree we reconstructed, we suggest that the genus *Paradoxornis* needs to be classified as Sylviidae, while the two species (*Luscinia cyanura* and *Monticola gularis*) are placed in the family Muscicapidae (the specific classification is shown in Fig. 8). The formation of the sister taxa of these two families, Muscicapidae and Turdidae, indicates that they are closely related, and then they form the superfamily Muscicapoidea together with the Sturnidae families (Fig. 8).

In the family Turdidae, *M. myadestinus* was basal position of the other Turdidae species, indicating that its differentiation is relatively primitive. And *T. merula* form a big branch with *T. obscurus*, *T. celaenops*, *T. eunomus*, *T. kesseri*, *T. mupinensis*, *T. naumanni*, *T.*

ruficollis, *T. dissimilis*, *T. cardis* and *T. hortulorum*, which indicates that they are closely related (Fig. 8). The species evolutionary relationship of genus *Turdus* we constructed is consistent with that of Xu *et al*. (2010), but different from that of Peng *et al*. (2016) (*T. merula*, *T. migratorius*; *T. naumanni*, *T. hortulorum*), Batista *et al*. (2020) (*T. migratorius*; *T. rufiventris*; *T. eunomus*; *T. naumanni*; *T. obscurus*; *T. hortulorum*; *T. merula*), Nylander *et al*. (2008) (*T. naumanni*; *T. ruficollis*; *T. kesseri*; *T. obscurus*; *T. celaenops*; *T. cardis*; *T. hortulorum*; *T. dissimilis*; *T. migratorius*; *T. rufiventris*; *T. merula*; *T. abyssinicus*; *T. mupinensis*) and Nagy *et al*. (2019) (*T. naumanni*; *T. ruficollis*; *T. obscurus*; *T. kesseri*; *T. cardis*; *T. hortulorum*; *T. dissimilis*; *T. merula*; *T. rufiventris*; *T. migratorius*). However in *T. merula*, *T. migratorius*, *T. mupinensis* and *T. rufiventris* there are some differences in the topological structure among different researchers. Hence, we suggest that the specific systematic taxonomic relationships of these species should be further analyzed and discussed. It may be possible that such a taxonomic status could be recovered using a more comprehensive taxa sampling of complete mtDNA genomes and supplementary nuclear gene molecular markers.

With regard to the New Zealand wattlebirds (*Philesturnus carunculatus* and *Callaeas cinereus*), our phylogenetic trees are strongly supported as a monophyletic group with both nuclear gene and mitochondrial DNA sequence data (Shepherd and Lambert, 2007; Gibb *et al*., 2015). Furthermore, the position of the New Zealand wattlebird taxa within the Oscines, as determined in our study (i.e. embodied in the oscines but excluded from the Passerida and Corvoidea), was consistent with the placement of *P. carunculatus* in previous studies (Barker *et al*., 2004; Shepherd and Lambert, 2007; Gibb *et al*., 2015). However, in our system topology, there are different classification arrangements (Fig. 8). For example, *P. carunculatus* and *C. cinereus* congregate in the same branch and are closely related to the Passerida, and they are located at the base of the order. Therefore, it is suggested that the two species should be classified into the order Passerida.

CONCLUSIONS

In this study, the complete mitochondrial genome of *T. merula* was determined and analyzed, and it is similar to other Passeriformes with many significant features. The motifs obtained by sequence comparison, "ATGAACCTAA" between ATP8 and ATP6, and "ATGCTAA" between ND4L and ND4, and "CAAGAAAGG" between COXI and $tRNA^{Ser(UCN)}$ were highly conservative in Passeriformes species. In the current research, the phylogenetic relationships based on nucleotide sequences of 13 PCGs showed that the phylogeny analyses of Passerida were conducted with the clear support of dividing the group into three superfamilies: the Muscicapoidea, the Sylvioidea, and the Passeroidea, and Passeroidea is a sister taxon for Muscicapoidea and Sylvioidea. We suggest that the genus *Paradoxornis* will be classified as Sylviidae, while these two species (*Luscinia cyanura* and *Monticola gularis*) are placed in the family Muscicapidae. Furthermore, Turdidae formed a sister group with Muscicapidae, and then they form the superfamily Muscicapoidea together with the Sturnidae families. The relationships between some species of the order Passeriformes may remain difficult to resolve despite an effort to collect additional characters and samples for phylogenetic analysis and our results could be useful in future research on population genetic structure, phylogeny, and conservation genetics.

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IBR approval

The animal handling procedures conformed with the China Animal Welfare guidelines and have been approved by the Ethic and Animal Welfare Committee and the Animal Protection and Use Commission of Mianyang Normal University (MNU: 19ZBJC-01).

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Ethical statement

This study was carried out in accordance with the guidelines of Animal Care and Use Committee at the Mianyang Normal University. Efforts were taken to minimize bird suffering by administering anesthesia. The study did not involve endangered or protected species.

Statement of conflict of interest

The authors have declared no conflict of interest.

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