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Complete Mitochondrial Genome and Phylogenetic Analysis of Gruiformes and Charadriiformes

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ABSTRACT

In this study, we used the next-generation sequencing method to obtain mitochondrial DNA (mtDNA) of *Porzana paykullii*, *Rallus aquaticus* and *Gallirallus striatus* in Gruiformes, and *Hydrophasianus chirurgus* in Charadriiformes, after which we analysed and compared structure, phylogeny, and taxonomic origin of the Gruiformes and Charadriiformes. Based on sequencing, splicing, and annotating the mtDNA of four birds, the results showed that the lengths of mtDNA were 16,955 bp in *Porzana paykullii*, 17,149 bp in *Rallus aquaticus*, 17,647 bp in *Gallirallus striatus* and 16,855 bp in *Hydrophasianus chirurgus*, respectively. The base compositions were A > C > T > G in 73 species complete mitochondrial sequences in Gruiformes and Charadriiformes. The total AT content in the 73 species was larger than that of GC. The start codons in protein-coding genes (PCGs) included ATG, GTG, ATT, ATC and ATA, while its stop codons included TAA, TAG, AGG, AGA and the incomplete cipher T. In PCGs, the highest frequency of codon was CTA (Leu). The highest frequency of amino acids was Leu, whereas the lowest was Cys. In phylogenetic analyses, Gruiformes included Grui and Ralli, and Charadriformes included Charadrii, Lari and Scolopaci. The genus *Porzana* was closest to *Porphyrio*. *Gallirallus striatus* and *Lewinia muelleri* consisted a sister group, while *Rallus aquaticus* was a separate branch. *Hydrophasianus chirurgus* (Charadriiformes: Jacanidae) was closely related to Rostratulidae. According to the estimation of divergence time corrected by fossil records of related birds and compared with previous studies, the base divergence time of Gruiformes was 46.33 (58.46~25.60) Ma, the emergence time of the suborders Grui was about 17.62 (29.76~4.15) Ma, and the emergence time of the suborders of the Ralli was about 32.18 (46.17~19.69) Ma. The origin time of Charadriiformes was about 45.44 (58.21~24.67) Ma. The origin time of the Charadrii was 44.52 Ma (52.66 ~ 23.55 Ma). The divergence time of Charadrii, Lari and Scolopaci was about 33.46 (43.44~22.92) Ma, 29.10 (40.67~16.43) Ma and 27.06 (34.83 ~ 12.72) Ma, respectively.

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Key words
Next-generation sequencing, Mitochondrial genome, Classification status, Phylogenetic, Divergence time

INTRODUCTION

Aves originated in theropod out of the Jurassic and belongs to Vertebrata. The direct ancestor of Aves was the descendant of a small dinosaur (Ostrom and Mcintosh, 1967; Zheng, 2015) which was the most abundant class of tetrapod vertebrates (Zheng, 2015). In traditional taxonomy, Aves consists Archaeornithes and Neornithes (Gemmell *et al*., 1994; Jarvis *et al*., 2014; Prum *et al*., 2015; Zheng, 2015). Neornithes mainly consists of the Palaiognathae, which has no flying short wings, and all Neognathae that can fly. The study concluded that the Artistic depiction of asteroidal impact occurred approximately 66 million years (Ma) ago. This period serves as the boundary between cretaceous period and tertiary (paleogene) (k-pg) (Avise, 2004), during which a wide range of species extinction events took place, creating conditions for the differentiation of birds and the generation of new species (Jarvis *et al*., 2014; Prum *et al*., 2015). After the k-pg period, the ancestors of Neognathae

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birds differentiated and formed the largest group of existing birds, including Galloanseres and Neoaves (Jarvis *et al*., 2014; Prum *et al*., 2015; Zhang *et al*., 2017). Galloanseres are included in Galliformes and Anseriformes. Neoaves are included in Galliformes, Passeriformes, Charadriiformes and other more than 20 orders (Sibley *et al*., 1988; Zheng, 2012; Zheng, 2015).

In the face of such a large number of birds with complex kinship, research scholars were keen to explore the systematic classification of birds and the origin and evolution of birds (Zheng, 1979; Cracraft, 1981; Smith and Clarke, 2015). Early classification of bird systems were based on epigenetics, morphological features, geographical distribution, and behavioural differences, but the results were rather confusing (Jarvis *et al*., 2014; Prum *et al*., 2015). Especially the phylogenetic analysis for Gruiformes and Charadriiformes is more chaotic (Jarvis *et al*., 2014; Prum *et al*., 2015; García–R *et al*., 2014). The current research of Gruiformes and Charadriiformes mainly focus on macroecology, behavior and histomorphology (Zheng, 1979; Ripley and Beehler, 1985; Avise, 2004; Zheng, 2012; Smith and Clarke, 2015; Huang et al., 2017). The use of molecular means can more effectively solve controversial issues such as the origin and evolution of birds (Jarvis *et al*., 2014; Prum *et al*., 2015). According to traditional taxonomy, there are 143 bird species in Gruiformes, including 12 families and 40 genera (Ripley and Beehler, 1985). Most Gruiformes birds have a small distribution range, and many of them are endangered species, such as *Grus japonensis* and *Grus leucogeranus* of Gruidae (Ripley and Beehler, 1985). There are great differences in the body types of birds of Gruiformes, with larger body size of Gruidae species if compers with Rallidae (Zheng, 2012). Charadriformes is a complex group, mainly including small and medium-sized waders, curlew and gulls (Sibley *et al*., 1988; Zheng, 2012). Charadriiformes includes 8 suborders, 19 families, 94 genera and 384 bird species (Hu *et al*., 2017), which distribute on all continents, from the poles to the tropics (Sibley *et al*., 1988). Up to now, more than 300 species of mitochondrial genome sequence information of birds have been published in Genbank (Gao *et al*., 2009; Zhang, 2015; Cheng, 2017), among which 33 species are Gruiformes (García–R *et al*., 2014; Chen *et al*., 2017) and 34 species are Charadriiformes (Smith and Clarke, 2015; Hu *et al*., 2017).

There have also been a lot of studies on the molecular phylogenetic classification of Gruiformes and Charadriiformes (Fain *et al*., 2007; Smith and Clarke, 2015; Chen *et al*., 2017; Hu *et al*., 2017). But the phylogenetic tree constructed at present was not comprehensive enough to contain species, and the phylogenetic status of many species was controversial (Sibley *et al*., 1988; Chen *et al*., 2017). Ruan *et al*. (2012) used mitochondrial COI and Cytb genes of 15 Gruiformes species to construct phylogenetic trees, Fain *et al*. (2007) constructed phylogenetic trees and estimated analysis using genes such as Cytb, 12S rRNA and 16S rRNA of 26 Gruiformes species. The time of disagreement mainly includes the Gruidae, Psophiidae, Rallidae and Heliornithidae (Fain *et al*., 2007). Among them, the Gruidae and Psophiidae were sister group, while Rallidae and Heliornithidae formed sister group. It was believed that the divergence time of Rallidae was 21.8 Ma, Rallidae and Heliornithidae was 42.6 Ma. García–R *et al*. used 17 PCGs, rRNA and tRNA genes of 17 species mitochondria of Gruiformes to construct a phylogenetic tree and estimate the divergence time. It was believed that the divergence time of Rallidae and Heliornithidae was 52 Ma, and the results were more accurate and reliable (García–R *et al*., 2014). Gong *et al*. used a genome-wide sequence of 31 species of Gruiformes (excluding CR sequences) to construct a Bayesian inference (BI) tree. The results showed that Rallidae and Heliornithidae were a sister group, and Gruidae belonged to another branch, but did not estimated the divergence time of Gruiformes (Gong *et al*., 2017). Gong *et al*. (2017) was suggested that the genus Gallirallus were sister to Lewinia, and these groups in turn were sister Amaurornis and different from those of García–R (2014). The genus Fulica and the genus Gallinula were one branch, the genus Porphyrio and the genus Coturnicops belong to another branch. while the García–R (2014) considers the genus Porphyrio, genus Fulica and Coturnicops were a branch. While the genus Gallirallus and the genus Lewinia was another branch.

There were also reports on mitochondrial genome sequencing and phylogenetic development in Charadriiformes birds. Based on 12 PCGs markers, Hu *et al*. (2017) constructed BI and Maximum Likelihood (ML) trees of 40 species to classify Charadriiformes into Charadrii, Lari and Scolopaci, which was more comprehensive, but no divergence time estimate for Charadriiformes. Cheng *et al*. (2017) studied phylogenetic trees of 18 Charadriiformes birds, and also divided Charadriiformes into the same three suborders. The phylogenetic tree supports the Lari as a suborder, belonging to Charadriiformes. While the suborder of Scolopaci was the newly differentiated Charadriiformes and included Jacanidae and Scolopacidae (Cheng, 2017). It was believed that Charadrii were included Recurvirostridea and Charadriidae, while *Recurvirostra avosetta* and *Haematopus ostralegus* was belonged to Recurvirostridea (Chen, 2003). At present, there are few studies on the divergence time of the Charadriiformes. Fain et al. (2007) used the genes of Cytb, 12S rRNA and 16S rRNA of 26 species to construct phylogenetic trees

and estimate the divergence time. The results indicated that the base divergence time of Charadriiformes was 74.3 Ma.

Combined with the previous phylogenetic analysis of Gruiformes and Charadriiformes. It was found that due to the lack of molecular data, phylogenetic analysis of Rallidae of Gruiformes was relatively chaotic, the accuracy of estimation of divergence time of Gruiformes was not enough. And the systematic analysis of the divergence time of Charadriiformes was not carried out. Therefore, it was necessary to make a more accurate analysis of phylogenetic development and divergence time of Gruiformes and Charadriiformes. So as to provide favourable reference and sufficient molecular evidence for the improvement of their classification system. This study has collected samples of Gruiformes and Charadriiformes and performed mitochondrial sequencing. Including three species *Porzana paykullii*, *Rallus aquaticus* and *Gallirallus striatus* from Gruiformes, and another bird *Hydrophasianus chirurgus* from Charadriiformes (Taylor and van Perlo, 1998; Manson and Goldizen, 2000). Combined with mtDNA sequences of four species and data of Gruiformes and Charadriiformes retrieved from GenBank, data of mitochondrial genome of 73 bird species were collected (Table I). In addition, the structure and base ratio of the mitochondrial genome of 73 species of birds of Gruiformes and Charadriiformes were compared, and the use types of starting and ending codons as well as the use frequency of codons and amino acids were compared. Phylogenetic tree was constructed by collecting and sorting mitochondrial genome sequences of Gruiformes and Charadriiformes, analysing the taxonomic status of the four species sequenced in this study, and exploring the relationship between the species of Gruiformes and Charadriiformes. Combined with the known fossil record of birds and the previous research results, this paper estimated the divergence time between the species of Gruiformes and Charadriiformes. So as to provide more molecular data for the study of evolutionary species, conservation aspects and phylogenetic relationships of birds.

MATERIALS AND METHODS

Ethics statement

The sample collection was strictly conducted under national ethical guidelines (Regulations for Administration of Affairs Concerning Experimental Animals, China, 1988) for animal husbandry and humane treatment.

Sample collection and DNA extraction

In this study, blood samples from 4 bird species without mitochondrial complete genome sequencing were collected nationwide from Gruiformes and Charadriiformes. Gruiformes including *Porzana paykullii*, *Rallus aquaticus* and *Gallirallus striatus*. The sampling points were Bengbu Anhui, province, Lianyungang Jiangsu, province and Liuzhou, Guangxi province, respectively. Another one species blood samples of *Hydrophasianus chirurgus* of Charadriiformes collected in Mianyang, Sichuan province. The blood was stored in a 1 ml centrifuge tube, and 20 µL of anticoagulant (0.5% sodium heparin) was added and stored in a refrigerator at – 20 °C. Total DNA was extracted using phenol/chloroform and examined on 1.0% agarose/TBE gel and used as template for PCR reactions.

DNA extraction and sequencing

The mtDNA of four species of birds was extracted and sequenced. First, the blood samples were digested. 10 µL of the sample blood was placed in a 1 mL centrifuge tube, and 375 µL of TE buffer, 20 µL of 10% SDS solution and 5 µL of 20 mg/L protease were added. The total volume of K was 400 µL of mixed digest. Mix the digestive juice, and then bathe in a water bath at 55-65 °C for 10 to 14 hours. Mix the digestive juice at intervals until the blood tissue was completely digested and dissolved.

The total DNA from birds was extracted by phenolic extraction: the cells were first broken up by protease K and SDS, and the proteins were digested. Then the supernatant was extracted by phenolic and phenol-chlorine. And the supernatant liquid was taken after high speed centrifugation (Chen *et al*., 2017). The centrifuged DNA was washed two to three times with 70% ethanol, dried, dissolved in 100 µL of sterilized double distilled water, and stored in a refrigerator at 4 °C until use. Take 4 µL of total DNA solution, mix with 1 µL of 6×Loading Buffer, and then spot it in agarose gel well. After 120V of constant pressure electrophoresis for 30 minutes, observe total DNA in UV detector or portable UV lamp. Extract the results. The complete sequence of the mitochondrial genome of the three species were sequenced by Beijing Jinnuo Ruijieji Technology Co. Ltd. and the CR partial sequence results of common pheasant and leeches were incomplete and corrected by one generation sequencing. Primers were designed based on the alignment of complete mtDNA sequences of *Gallinula chloropus* and *Rallina eurizonoides* by using Primer 5.00 (PREMIER Biosoft International) (Chen *et al*., 2017) and shown in Supplementary Table I. The complete mitochondrial genome sequence of four species were deposited in GenBank with accession numbers MG200164, MH229988, MH219930 and MH219929, respectively (Table I and Supplementary Tables II, III, IV and V).

Data analysis

Data acquisition and analysis

Mitochondrial complete genomes of Gruiformes and

Table I. The mitochondrial genome of Gruiformes and Charadriiformes.

Order	Family	Genus	Species	All(bp)	PCGs(bp)	GeneBank
Gruiformes	Gruidae	*Anthropoides*	*Anthropoides paradiseus*	16696	11381	NC_020572
			A. virgo	16541	11381	NC_020573
		Balearica	*B. pavonina*	16786	11375	NC_020570
			B. regulorum	16802	11375	NC_020569
			Grus carunculatus	16677	11381	NC_020571
		Grus	*G. americana*	16651	11381	NC_020576
			G. antigone	16549	11381	NC_020581
			G. canadensis	16697	11381	NC_020582
			G. grus	16649	11381	NC_020577
			G. japonensis	16715	11381	NC_020575
			G. leucogeranus	16688	11381	NC_020574
			G. monacha	16650	11381	NC_020578
			G. nigricollis	16646	11381	NC_020579
			G. rubicunda	16693	11381	NC_020580
			G. vipio	16678	11381	NC_021368
	Rallidae	*Amaurornis*	*Amaurornis akool*	16950	11296	NC_023982
			A. phoenicurus	17213	11368	NC_024593
		Coturnicops	*Coturnicops exquisitus*	17136	11368	NC_012143
		Eulabeornis	*Eulabeornis castaneoventris*	17339	11368	NC_025501
		Fulica	*Fulica atra*	17029	11368	NC_025500
		Gallicrex	*Gallicrex cinerea*	17184	11377	NC_028408
		Gallinula	*Gallinula chloropus*	17027	11365	NC_015236
		Gallirallus	*Gallirallus australis*	17464	11368	KF701060
			G. okinawae	18404	11368	NC_012140
			G. philippensis	17359	11368	NC_025507
			G. striatus	17647	11368	MH219930
		Lewinia	*Lewinia muelleri*	17273	11368	NC_025502
		Porphyrio	*Porphyrio hochstetteri*	16988	11368	NC_010092
			P. porphyrio	17020	11368	NC_025508
		Porzana	*Porzana fusca*	16935	11368	KY009736
			P. paykullii	16955	11368	MG200164
			P. pusilla	16978	11368	KY009737
		Rallus	*Rallus aquaticus*	17149	11368	MH229988
		Rallina	*Rallina eurizonoides*	16942	11377	NC_012142
		Sarothrura	*Sarothrura ayresi*	16767	11362	NC_034316
	Rhynochetidae	*Rhynochetos*	*Rhynochetos jubatus*	16937	11396	NC_010091
	Heliornithidae	*Heliornis*	*Heliornis fulica*	17008	11367	NC_025499
	Otididae	*Otis*	*Otis tarda*	16849	11397	NC_014046
Charadriiformes	Laridae	*Larus*	*Larus crassirostris*	16701	11397	KM507782
			L. dominicanus	16746	11399	AY293619
			L. vegae	16379	11397	NC_029383
			Ichthyaetus relictus	16586	11399	KC760146
		Chroicocephalus	*L. brunnicephalus*	16769	11399	JX155863
			L. ridibundus	16807	11391	KM577662
			L. saundersi	16724	11379	JQ071443
		Gelochelidon	*Gelochelidon nilotica*	16748	11397	NC_036344

Sternidae	*Sterna*	*Sterna albifrons*	16357	11397	KT350612
		S. hirundo	16707	11397	NC_036345
Alcidae	*Synthliboramphus*	*Synthliboramphus antiquus*	16730	11402	AP009042
		S. wumizusume	16714	11397	KT592378
	Pinguinus	*Pinguinus impennis*	16784	11397	NC_031347
Stercorariidae	*Stercorarius*	*Stercorarius maccormicki*	16669	11403	KM401546
Scolopacidae	*Eurynorhynchus*	*Eurynorhynchus pygmeus*	16707	11397	KP742478
	Arenaria	*Arenaria interpres*	16725	11399	AY074885
	Tringa	*Tringa erythropus*	16683	11397	NC_030585
		T. ochropus	16906	11397	KX668223
		T. semipalmata	16603	11399	NC_036016
	Xenus	*Xenus cinereus*	16817	11397	KX644890
	Gallinago	*Gallinago stenura*	16899	11400	KY056596
	Scolopax	*Scolopax rusticola*	16984	11391	KM434134
	Numenius	*Numenius phaeopus*	17091	11396	KP308149
	Limosa	*Limosa lapponica*	16732	11399	KX371106
Jacanidae	*Jacana*	*Jacana jacana*	16975	11394	KJ631049
		J. spinosa	17063	11397	KJ631048
	Hydrophasianus	*Hydrophasianus chirurgus*	16855	11397	MH219929
Charadriidae	*Vanellus*	*Vanellus cinereus*	17074	11399	KM404175
		V. vanellus	16795	11397	KM577158
	Pluvialis	*Pluvialis fulva*	16854	11391	KX639757
	Charadrius	*Charadrius placidus*	16895	11397	KY419888
Recurvirostridae	*Recurvirostra*	*Recurvirostra avosetta*	16897	11397	KP757766
	Himantopus	*Himantopus himantopus*	17378	11397	NC_035423
Haematopodidae	*Haematopus*	*Haematopus ater*	16791	11400	AY074886
		H. ostralegus	16798	11394	NC_034237

Charadriiformes species were retrieved and downloaded from GenBank, including 35 Gruiformes species and 34 Charadriiformes species (Table I). Combined with the mitochondrial complete genomes of 4 birds determined in this study (Fig. 1), a total of 73 mitochondrial complete genomes of birds were obtained. We firstly used Clustal X (Thompson *et al*., 1997) and MEGA5.0 (Tamura *et al*., 2011) and DNASTAR (Burland, 2000) to compare the mitochondrial genome sequences of 73 birds and analysed the base content, start codon and termination of the complete genome and PCGs. Then we also analysed the codon usage and synonymous codon usage of Gruiformes and Charadriiformes PCGs. The calculation formula for base skew was AT skew = (A-T) / (A + T), GC skew = (G-C) / (G + C) (Perna and Kocher, 1995). We used Adobe Illustrator CS6 and OriginPro 8 (Wass, 2008) to draw processing diagrams.

Analysis of phylogeny

A total of 73 birds mitochondrial genome sequences were collected in Gruiformes and Charadriiformes, and the PCG and rRNA genes of 15 species of Charadriiformes were searched in GeneBank (Supplementary Table VI). The sequences of 12 PCGs, 12S rRNA and 16S rRNA genes of mitochondria of 88 species. Both 38 species in 19 genera, 5 families of Gruiformes, and 50 species in 34 genera, 17 families of Charadriiformes were sequenced and what were perform phylogenetic analysis. The construction of the phylogenetic tree requires the use of DAMBE (Xia, 2001; Xia and Lemey, 2009) software to verify the saturation of the selected sequence. The use of the obtained gene sequence substitution does not reach saturation before the phylogenetic analysis can be continued.

ML and BI trees were constructed by using the optimal parameter model selected by MrModelTest3.06 software (Klaus-J *et al*., 2004) and PAUP 4.0b10 software (Swofford, 2003) with Akaike information Criterion (AIC) to select the model with the minimum statistical value. Among them, the BI trees of Gruiformes and Charadriiformes birds were constructed using MrBayes 3.1.2 software (Huelsenbeck and Ronquist, 2001). In the process of BI tree construction, four Monte Carlo Markov chains (MCMC) were established. Start with random

number as the starting tree, run 3,000,000 generations in total, and sample once every 100 generations. After discarding the 25% of burning-in samples, the consistent tree can be constructed according to the remaining 75% samples, and the Posterior Probability (PP) of the BI tree can be calculated finally. The ML tree was constructed by using the RaxML software to run the files in Phy format (Stamatakis *et al*., 2008; Stamatakis, 2014), selected the best model for MrModelTest3.06 software filter, and set the ML thorough bootstrap mode, bootstrap reps to 50 and running 10000 times to get the ML tree. Finally, Treeview32 (Saldanha, 2004) and Figtree 1.4.2 (Rambaut, 2014) software were used to open and annotate the evolutionary tree diagram. The genetic evolution and phylogenetic relationship of different species in the two orders were analysed and the taxonomic status of some birds was discussed.

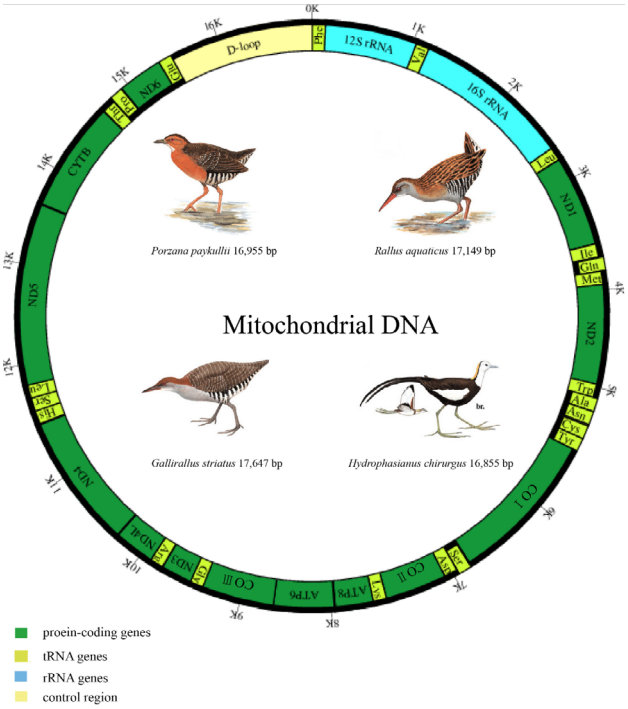

Fig. 1. Mitochondrial genomes of four species.

Analysis of divergence time

In this study, the divergence time was calculated based on the ML tree topology constructed by mitochondrial genome, and the accurate fossil record was used as the calibration point. The earliest fossilized Galliformes was recorded as *Gallinuloides wyomingensis* (Avise, 2004) with a minimum time of 51.58 Ma (Graybeal, 1998; Prum *et al*., 2015), and the stem phenisciform *Waimanu* (Slack *et al*., 2006) with a normal distribution of 61.5–65.5 Mya. Combining the fossil record and comparing the results of Jarvis *et al*. (2014) and Prum *et al*. (2015), the calibration point of the Neognathae was set as 70.00 Ma, and Galloanseres and Neoaves calibration point was 65 Ma (Jarvis *et al*., 2014; Prum *et al*., 2015) and set the standard deviation of 0.5. BEAST 1.4.6 was the software that can build phylogenetic trees and estimate the time of species divergence (Drummond and Rambaut, 2007). BEAST 1.4.6 software was used to establish MCMC to estimate the bifurcation time of species. Yule Prior was selected in the branching evolution rate (reference?). The data calculation model setting was consistent with the phylogenetic tree model. MCMC operation parameters were as follows that length of chain was 8 × 106 generations, once every 200 generations, and burn-in 10% of the samples. Tree Annotator 1.6.1 (Drummond and Rambaut, 2007) software was used to construct the tree with the maximum branch credibility. The running results were analysed by Tracer v 1.4 (Rambaut and Drummond, 2007), and then Fig Tree 1.4.2 (Rambaut, 2014) was used to open the evolutionary tree with divergence time and 95% of the highest posterior density (HPD).

RESULTS

Genome organization and arrangement

The mitochondrial genome sequences of 38 species (19 genera, 5 families) of Gruiformes and 35 species (35 genera, 9 families) of Charadriformes were analysed in this study (Table I). The structure and gene arrangement of mitochondrial genomes of Gruiformes and Charadriiformes were basically the same, including 37 coding genes and one CR, and the content range of each base was almost the same, and the fluctuation range was less than 3% (Fig. 2).

The mean base composition of mitochondrial genome of Gruiformes was T (23.92±0.56%), C (30.74±0.71%), A (31.82±0.64%) and G (13.52±0.34%). The AT content ranged from 54.70% to 57.53%, with an average value of 55.74 ± 0.70%, higher than GC content (Fig. 2). The sequence of base richness of mitochondrial genome was mostly A>C>T>G, and only *Balearica pavonina*, *Rhynochetos jubatus* and *Otis tarda* had base contents of C>A>T>G. The mean AT skew was 0.14±0.02, and GC skew was -0.39±0.02, indicating that the nucleotide composition of mitochondrial complete genome of Gruiformes had a slight specific bias towards A and C (Fig. 3).

The average base composition of mitochondrial genome of Charadriiformes was T (24.61 ± 0.76%), C (30.48 ± 0.69%), A (31.20 ± 0.47%) and G (13.72 ± 0.38%). The AT contents ranged from 54.39% to 58.35%, with an average value of 55.81 ± 0.86%, slightly higher than that of the mitochondrial whole genome

of Gruiformes. The sequence of base abundance of mitochondrial genome was mostly A > C > T > G (Fig. 2). As for *Larus crassirostris*, *Larus dominicanus*, *Ichthyaetus relictus*, *Larus brininucephalus*, *Larus ridibundus*, *Larus saundersi, Arenaria interpres*, *and Pluvialis fulva* have a base content of C > A > T > G. The mean deviation of AT was 0.12 ± 0.02, and the mean deviation of GC was -0.38 ± 0.01, indicating that the nucleotide composition of the mitochondrial whole genome of Charadriiformes birds was slightly biased towards A and C (Fig. 3).

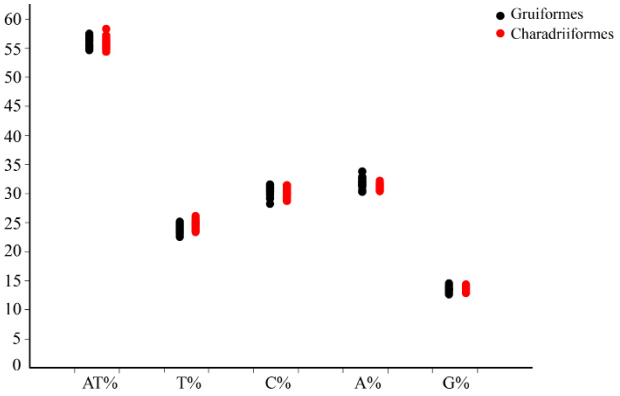

Fig. 2. Analysis of base content of complete mitochondrial genome of Gruiformes and Charadriiformes.

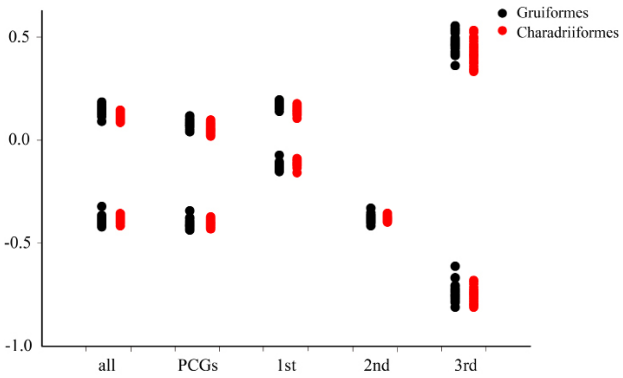

Fig. 3. The mitochondrial genome base AT skew and GC skew of Gruiformes and Charadriiformes.

Analysis of PCGs

The structure of PCGs of Gruiformes and Charadriiformes were very conservative, including 7 NADH reductase genes, 3 cytochrome oxidase genes, 2 ATP synthase genes (ATPase8 and ATPase6) and a cytochrome oxidase b gene. Among the Gruiformes, the shortest PCGs was *Amaurornis akool* (11,296 bp), while the longest was *Otis tarda* (11,397 bp), and the shortest PCGs of Charadriiformes was the *Larus saundersi* (11,379 bp), whereas the longest PCGs was *Stercorarius maccormicki* (11,403 bp) (Table I).

Sequence analysis of PCGs

The average base composition of Gruiformes PCGs was T (25.25 ± 0.55%), C (31.60 ± 0.80%), A (29.82 ± 0.68%) and G (13.33 ± 0.36%). The AT content ranged from 53.60% to 57.09%, and the average value was (55.07 ± 0.82%) higher than the GC content (Fig. 4). The AT skew was 0.08 ± 0.02, and the GC skew was − 0.41 ± 0.02, indicating that the nucleotide composition of Gruiformes PCGs has a slight specific bias for A and C (Fig. 3). The AT content of the first codon of PCGs of Gruiformes was 50.51 ± 0.59%, where the AT skew was 0.17 ± 0.01, and the GC skew was −0.13 ± 0.01 The second codon AT content was 58.37 ± 0.18%, where AT skew was −0.37 ± 0.01 and GC skew was −0.39 ± 0.01. Third codon AT content was 56.33 ± 2.02%, where AT skew was 0.47 ± 0.04 and GC skew was −0.74 ± 0.04 (Fig. 3). The AT content of the third codon of PCGs of Gruiformes fluctuated the most, and the AC content of the first and third codon bases was more than the TG content, while the second codon base consisted of the TC content more than the AG content.

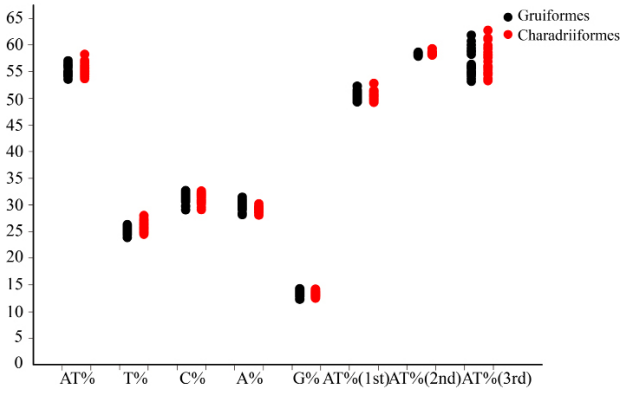

Fig. 4. Analysis of base content of mitochondrial PCGs of Gruiformes and Charadriiformes.

The base composition of Charadriiformes PCGs was T (26.13 ± 0.91%), C (31.36 ± 0.86%), A (29.15 ± 0.51%) and G (13.35 ± 0.40%). The AT content ranged from 53.75% to 58.31%, and the average value was 55.28 ± 0.99%, which was slightly higher than the AT content of Gruiformes PCGs (Fig. 4). The AT skew was 0.05 ± 0.02 and the GC skew was − 0.40 ± 0.02, indicating that the nucleotide composition of Charadriiformes PCGs has a slight specific bias for A and C. The first codon AT content of Charadriiformes PCGs was 50.55 ± 0.72%, where the AT skew was 0.14 ± 0.01 and the GC skew was − 0.11 ± 0.01. The second codon AT content was 58.45 ± 0.23%,

where the AT skew was − 0.37 ± 0.01 and the average value of GC skew was − 0.39 ± 0.01. The AT content of the third codon was 56.85 ± 2.35%, where the AT skew was 0.42 ± 0.05 and the GC skew was − 0.75 ± 0.04. 3.3) (Fig. 3). The AT content of the third codon of the PCGs of Charadriiformes fluctuated the most, and the AC content of the first and third codon bases was more than the TG content, while the second codon base consisted of the TC content more than the AG content.

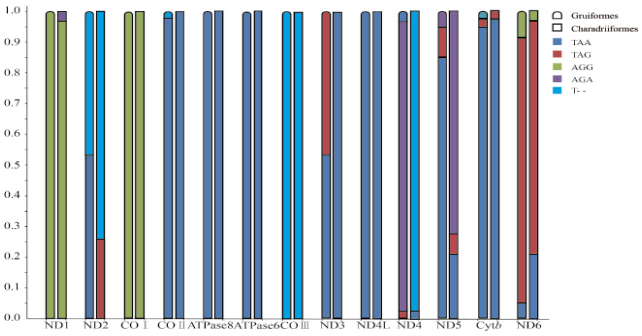

Fig. 5. The relative usage of stop codon of Gruiformes and Charadriiformes.

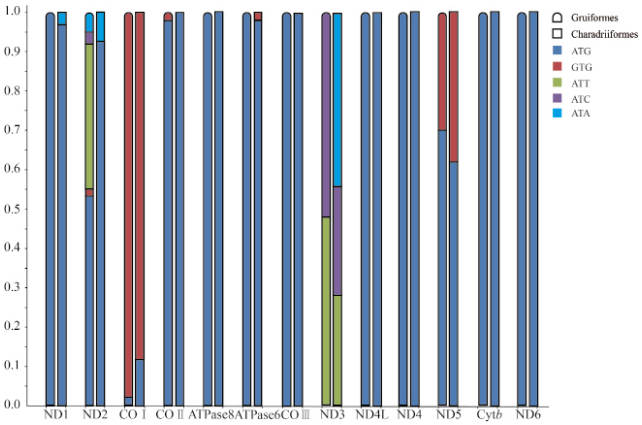

Fig. 6. The relative usage of start codon of Gruiformes and Charadriiformes.

Codon usage of PCGs

Most of Gruiformes and Charadriiformes PCGs were used ATG, GTG, ATT, ATC and ATA as the start codon, while TAA, TAG, AGG, AGA and incomplete codon T were used as the stop codons (Fig. 5 and Fig. 6). Incomplete stop codons were frequently found in the COIII gene in birds and can be complemented by the acidification of adenosine or poly (A) at the mRNA3' during transcriptional processing Stop codon (TAA). The initiation codons of Gruiformes and Charadriiformes ATPase8, COIII, Nd4L, Nd4, Nd6 and Cytb of Gruiformes and Charadriiformes were ATG, whereas the codons of COI and Nd5 were ATG and GTG. The starting codons of Gruiformes and Charadriiformes other PCGs genes were somewhat different. The stop codons of the mitochondrial genome of Gruiformes and Charadriiformes were more complicated. Among them, only the stop codons of ATPase8, ATPase6 and Nd4L were TAA, and the stop codons of COI were AGG. The stop codons of Gruiformes and Charadriiformes the other 9 PCGs were different.

In the PCGs encoding process of Gruiformes, the four codons with the highest frequency average were CTA (Leu), ATC (Ile), ACA (Thr) and TTC (Phe). The average number of occurrences of these four codons was separately 312.80, 201.00, 155.80, and 155.30, while the average value of the Synonymous Codon Usage (RSCU) was 2.85, 1.36, 1.71, and 1.45, respectively. In the PCGs encoding process of Charadriiformes, codons having a relatively high frequency average were CTA (Leu), ATC (Ile), TTC (Phe) and ACA (Thr). The average number of occurrences of these five codons was separately 301.20, 200.90, 160.60, and 149.50, while the average RSCU was 2.73, 1.38, 1.44, and 1.69, respectively. By counting the frequency of amino acid use of Gruiformes PCGs, we found that the highest frequency of use was Leu (17.44%) whereas the lowest was Cys (0.70%). The results for Charadriiformes PCGs were similar, with the highest average frequency of use being Leu (17.48%) and the lowest being Cys (0.76%) (Fig. 7).

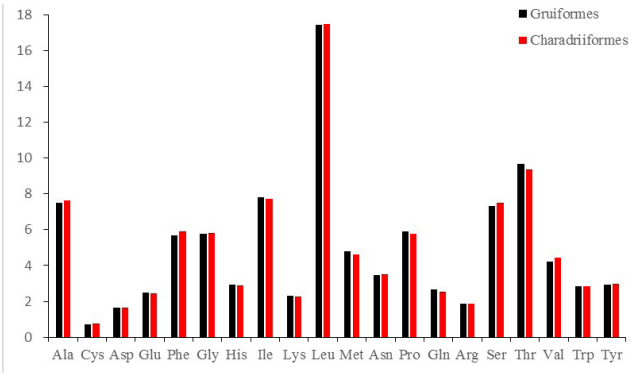

Fig. 7. Frequency of mitochondrial PCGs amino acids of Gruiformes and Charadriiformes.

Analysis of phylogeny and divergence time

By arranging the gene sequences of 12 PCGs, 12S rRNA and 16S rRNA of mitochondria (Table I; Supplementary Table VI), the base substitution saturation was detected based on the F84 model in the DAMBE software. We also analysed the ratio of nucleotide conversion number (×s) and nucleotide transversion number (Δv) to the genetic distance of nucleotide sequences of different species. The results showed that the sequence ratio was increased with the increase of genetic distance. The conversion and

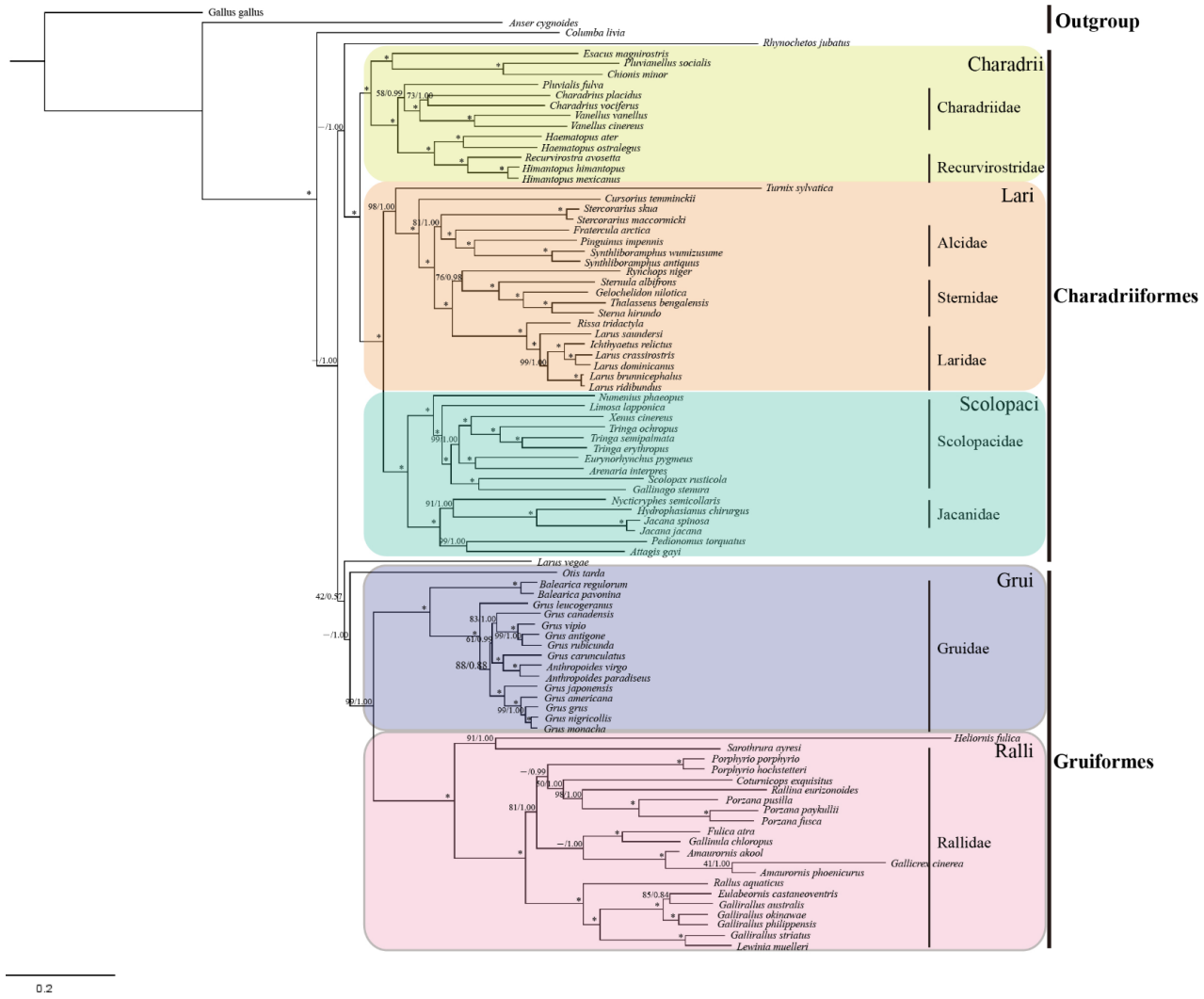

Fig. 8. BI and ML trees constructed based on 12 PCGs and 2 rRNA genes of Gruiformes and Charadriiformes. *Numbers* represent bootstrap values (MP/ML) and only those > 40% are shown. *Asterisks* indicate posterior probabilities of 100%.

transversion of nucleotides increased linearly (Supplementary Fig. 1), which was a linear regression model. The results also indicated that the substitution of gene sequences was not saturated, so phylogenetic analysis could continue. The sequence was screened by Modeltest and Mrmodeltest. The best model was GTR+G+I (-lnL = 322120.5625, K = 10, AIC = 644261.1250) which was then used to construct BI and ML trees. Since the constructed BI and ML tree topologies were identical, only the ML tree (the number on the branch represents the support rate of the BI and ML trees) was presented to show the phylogenetic relationship between Gruiformes and Charadriiformes.

The selected model (GTR+G+I) was the same as the phylogenetic analysis of the divergence Gruiformes and Charadriiformes, and based on 12 PCGs, 12S rRNA and 16S rRNA gene of 88 species mitochondria sequence of Gruiformes and Charadriiformes (Fig. 8 and Fig. 9). The estimating of the divergence time was performed ML tree harvested from BEAST software. The results showed that the differentiation of Neognathae was about 70.22 (71.21 ~ 69.22) Ma (Fig. 9), belonging to the Late Cretaceous period. The boundary between the Cretaceous and Tertiary (Paleo) strata (K-Pg) was about 66 Ma (Jarvis *et al*., 2014; Prum *et al*., 2015), and the species of the Neoaves was beginning to differentiate at this time, with a divergence time of 64.84 (65.83 ~ 63.82) Ma (Fig. 9). The birds of Galloanseres were emerged slightly earlier than the birds of Neoaves, and the time of divergence was 65.91 (66.89 ~ 64.94) Ma. This result was basically consistent with the results of studies by Jarvis *et al*. (2014) and Prum *et al*. (2015).

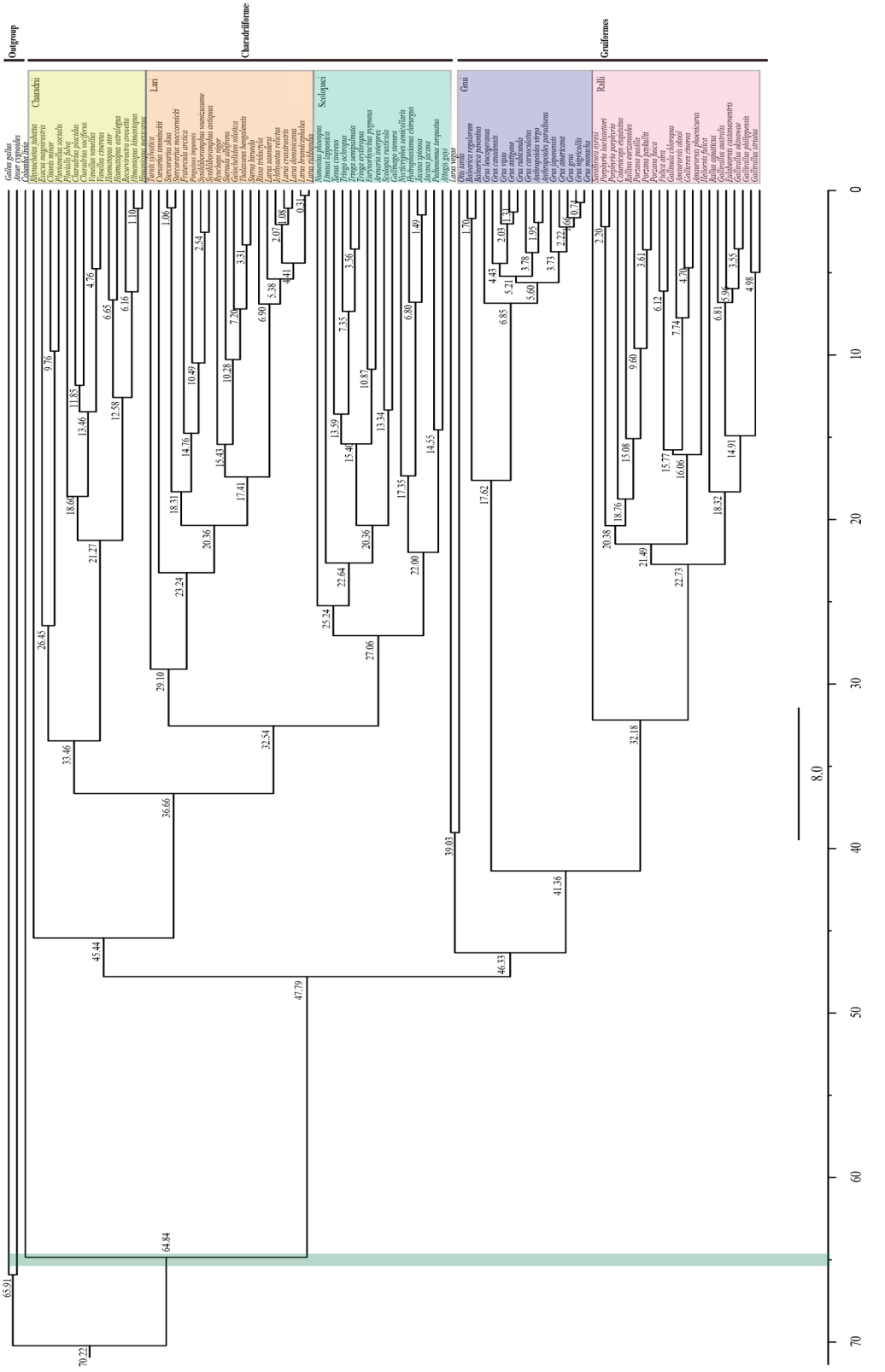

Fig. 9. Analysis of divergence time based on 12 PCGs and 2 rRNAs genes of Gruiformes and Charadriiformes.

Phylogeny and divergent time of Gruiformes

In phylogenetic analyses, Gruiformes include Grui and Ralli, Gruidae and Otididae were included in the branch of Grui, while Rallidae and Heliornithidae were included in the branch of Ralli (Fig. 8). Rhynochetidae was a separate one and had a long relationship with other Gruiformes birds. Grui branches were dominated by Gruidae, the genus *Grus* and genus *Bugeranus* belonged to one branch, while the genus *Balearica* was the other. Ralli were dominated by Rallidae, among which *Porzana fusca*, *Porzana paykullii*, *Porzana pusilla* sequenced in this study and all belonged to the genus *Porzana*. The genus *Porzana* and genus *Porphyrio* belonged to one branch, and then the genus *Amaurornis*, genus *Fulica* and genus *Gallinula* belong to another branch (81% support for ML trees and 1.00 support for BI trees). In addition, *Gallicrex cinerea* was also included in the genus *Amaurornis*. *Gallirallus striatus* was sister to *Lewinia muelleri*, and this groups belonged to a branch with the genus *Gallirallus*. Whereas *Rallus aquaticus* belonged to the genus Rallus for another branch. (100% support for ML trees and 1.00 support for BI trees) (Fig. 8). In addition, *Heliornis fulica* and *Sarothrura ayresi* were a sister group and were separated from other birds of Rallidae to form a single one (100% support for ML trees and 1 support for BI trees).

The basal divergence time of Gruiformes was 46.33 (58.46 ~ 25.60) Ma, and the divergence time of Grui and Ralli was 41.36 (53.10 ~ 24.18) Ma (Fig. 9). The divergence time of Grui was about 17.62 (29.76 ~ 4.15) Ma, and the divergence time of Ralli was about 32.18 (46.17 ~ 19.69) Ma. The divergence time of *Porzana fusca*, *Porzana paykullii*, *Porzana pusilla* of the genus *Porzana* was 9.60 (14.81 ~ 5.83) Ma, which were sequenced in this study. The divergence time of *Rallus aquaticus* of the genus *Rallus* was 18.32 (24.99 ~ 8.98) Ma. The divergence time of *Gallirallus striatus* of the genus *Gallirallus* was 4.98 (8.87 ~ 1.93) Ma.

Phylogeny and divergence time of Charadriiformes

In this study, Charadriformes included Charadrii, Lari and Scolopaci (Fig. 8). The branch of Charadrii mainly included Charadriidae and Recurvirostridea. The branch of Lari mainly included Alcidae, Sternidae and Laridae. The branch of Scolopaci mainly included Jacanidae and Scolopacidae. While *Larus vegae* was a separate branch. Among Charadrii, Charadriidae, Haematopodidae and Recurvirostridea were one branch (100% support for ML trees and 1.00 support for BI trees). Chionididae and Burhinidae was a sister group (100% support for ML trees and 1.00 support for BI trees). In the Lari, Laridae, Sternidae and Rhynchopidae belonged to a branch (100% support for ML trees and 1.00 support for BI trees), and Alcidae and Stercorariidae was a sister group (81% support for ML trees and 1.00 support for BI trees). While Glareolidae and Turnicidae were in separate branches. In Scolopaci, Scolopacidae belonged to a branch (100% support for ML trees and 1.00 support for BI trees), while the relative relationship between Jacanidae, Rostratulidae, Pedionomidae and Thinocoridae was close (100% support for ML trees and 1.00 support for BI trees). In addition, *Gelochelidon nilotica* in Laridae was closed relationship with Sternidae (100% support for ML trees and 1.00 support for BI trees). *Pluvianellus socialis* in Charadriidae and *Chionis minor* was a sister group (100% support for ML trees and 1.00 support for BI trees) and closed relationship with Chionididae.

The basal divergence time of Charadriiformes was 45.44 (58.21 ~ 24.67) Ma, the divergence time of Charadrii, Lari and Scolopaci was about 33.46 (43.44 ~ 22.92) Ma, 29.10 (40.67 ~ 16.43) Ma and 27.06 (34.83 ~ 12.72) Ma, respectively (Fig. 9). The divergence time of Charadriidae and Scolopacidae was 21.27 (33.19 ~ 8.54) Ma and the divergence time of Laridae and Sternidae was 17.41 (24.29 ~ 6.78) Ma, while the divergence time of Alcidae and Stercorariidae was 18.31 (27.29 ~ 7.10) Ma. The divergence time of Scolopacidae was 25.24 (30.96 ~ 13.38) Ma, and the divergence time of Jacanidae was 6.80 (11.87 ~ 3.39) Ma (Fig. 9).

DISCUSSION

Complete genome sequence

Studies have shown that the length of the mitochondrial genome of birds was 16,300 bp to 23,500 bp (Gao *et al*., 2009; Zheng, 2015), and in this study the shortest mitochondrial genome of Gruiformes was *Anthropoides virgo* (16,541 bp), while the longest was *Gallirallus okinawae*. (18,404 bp). The shortest mitochondrial genome of Charadriiformes was *Sternula albifrons* (16,357 bp), whereas the longest was *Himantopus himantopus* (17,378 bp). The structure and gene arrangement of the mitochondrial genome of Gruiformes and Charadriiformes were basically the same as those of most birds (Gao *et al*., 2009; Zheng, 2015), also including 37 coding genes and a CR (Zheng, 2015). The AT content of the mitochondrial genome sequence of Gruiformes and Charadriiformes mitochondria were greater than the GC content, and the highest AT content was *Gallinago stenura*, which reached 58.35% and was higher than the AT content of the general birds (50.5% to 57.7%) (Gao *et al*., 2009; Cheng *et al*., 2017). PCGs of Gruiformes and Charadriiformes mitochondria were very conservative, and their structure and arrangement were consistent with previous studies of bird mitochondrial PCGs (Gao *et al*., 2009; Zheng, 2015). Studies have shown that most birds have a first codon AT content of 47.50% to 52.10%, a

second codon AT content of 50.0% to 60.0%, and a third codon AT content of 42.1% to 62.0% (Gao *et al*., 2009). The results of the AT content of each codon of PCGs in Gruiformes and Charadriiformes were similar to this result, and the AT content of the third codon fluctuated the most. Because the first and second codons face greater selection pressures, while the third codon and non-coding regions were subject to the least selection pressure. This difference can itself cause an imbalance in base distribution (Zhong *et al*., 2002). Moreover, the restriction of amino acids on bases and the frequency of corresponding codon usage were also an important reason for the local base imbalance in the genome (Frank and Lobry, 1999). This group of genomic local bases was uneven, showing differences in nucleotide base composition in different regions (Belozersky and Spirin, 1958). Compared with the base composition of the first two digits of the codon, the third base of the codon has a higher mutation rate (Daubin and Perrière, 2003). Whether this situation was the result of choice or neutral change was still inconclusive (Necşulea and Lobry, 2006). In addition, differences in AT and GC content were also considered to be closely related to genomic features such as repetitive element distribution, methylation patterns, and gene density. Most of the PCGs of the genus and the genus PCGs use ATG, GTG, ATT, ATC and ATA as the start codon, and TAA, TAG, AGG, AGA and incomplete codon T as stop codons. In the process of coding mitochondrial proteins in the order of Gruiformes and Charadriiformes, the codons with the highest frequency average were CTA (Leu), and the highest frequency of amino acid use was also Leu. The frequency of amino acid use was similar to that of other birds, such as *Emberiza chrysophrys* (Ren *et al*., 2014), but it was very different from Invertebrates, which may be related to the function of mitochondria and the utilization efficiency of mitochondria in different species (Zhao *et al*., 2018).

Phylogeny and divergence time

At present, the classification of the species of Gruiformes and Charadriiformes was based on the characteristics of the bird's morphology, sound, and tracheal evolution (Taylor and van Perlo, 1998; Yang and Wang, 2004; Zheng, 2012), as well as the molecular phylogenetic analysis (Ruan *et al*., 2012). The species of Gruiformes and Charadriiformes were relatively clear in the classification level of the family, but they were very complicated and controversial at the classification level of the genus (García–R *et al*., 2014; Hu *et al*., 2017).

Most of the studies have concluded that Rallidae and Heliornithidae were closely related, while Gruidae belongs to another branch (Fain *et al*., 2007; García–R *et al*., 2014). The classification of birds in Rallidae was more complicated. The genus *Grus* and the genus *Bugeranus* of Gruidae was sister group, while the genus *Balearica* was another branch (Yang and Wang, 2004). In this study, the evolutionary analysis of Gruidae in Gruiformes was generally consistent with previous studies (Yang and Wang, 2004; Gong *et al*., 2017), but the genetic relationship between the species of Rallidae was slightly different (García–R *et al*., 2014; Chen *et al*., 2017; Gong *et al*., 2017). For the classification of Rallidae birds, the results of García–R *et al*. (2014) were consistent with this study, and they were believed that the genus *Porphyrio*, the genus *Amaurornis*, the genus *Fulica* and the genus *Gallinula* were belong to a branch. The genus *Gallirallus* and the genus *Lewinia* was more closely related and belonged to another branch. Gong *et al*. (2017) thought that the genus *Gallirallus* was closely related to the genus *Lewinia*, and then that with the genus *Amaurornis*, *Fulica* and *Gallinula* were belonged to branch, while the genus *Porphyrio* and *Coturnicops* belonged to another branch. The differences in these classifications were related to different molecular markers. García–R *et al*. (2014) used 12 PCGs, tRNA and rRNA gene sequences to construct phylogenetic trees, while Gong *et al*. (2017) used the complete sequences of mitochondrial genome (The CR sequence was removed) to construct a phylogenetic tree. Therefore, the accuracy of the phylogenetic tree can only be compared by the support rate and the posterior probability, and there was no more acceptable definition (Zheng, 2015). However, the species data contained in this study was more comprehensive, and the sequence information also contains a higher posterior probability value, which has a higher degree of credibility. In addition, according to the morphological classification, *Gallicrex cinerea* was considered to be a bird of the genus *Gallicrex*, and *Gallirallus striatus* belonged to the genus *Gallirallus* (Zheng, 2012). However, this study believes that *Gallicrex cinerea* and *Amaurornis phoenicurus* were sisters and should be classified as the genus *Amaurornis*. In addition, *Gallirallus striatus* and *Lewinia muelleri* belong to sister group, preferring the genus *Lewinia*. Regarding the classification of the genus *Gallicrex*, Ruan *et al*. (2012) and Gong *et al*. (2017) also believed that it should be classified as the genus *Amaurornis*, rather than the traditionally believed the genus *Gallicrex*. The classification results of *Gallirallus striatus* need further research and demonstration.

According to the analysis of this study, the divergence time of Neoaves was 64.84 (65.83 ~ 63.82) Ma, while the divergence time of Galloanseres was 65.91 (66.89 ~ 64.94) Ma and diverged about the same time as Neoaves. This result was basically consistent with the findings of Jarvis *et al*. (2014) and Prum *et al*. (2015). The basal divergence time of Gruiformes was 46.33 (58.46 ~ 25.60) Ma, which was less than that of Jarvis *et al*. (2014) (65.00 Ma) and Fain *et al*. (2007) (66.40 Ma). The diverging time of 41.36 (53.10

~ 24.18) Ma of Ralli was similar to that of García–R *et al*. (2014) (40.10 Ma), and was consistent with the fossil record of two ancient birds of Ralli (Mayr, 2009; García–R *et al*., 2014). The reasons for the differences in the divergence time of Gruiformes include the difference in the amount of data used and the reference point used for the estimation of the divergence time. Prum *et al*. (2015) and Jarvis *et al*. (2014) used relatively little data on Gruiformes, and even used only one species instead of the whole order to estimate the time difference, which may lead to some deviation in their results. Fain *et al*. (2007) used the fossil records of Rallidae and Heliornithidae and compared the results of previous studies (Krajewski and Fetzner, 1994; Houde, 2009), and set 43 Ma as the reference base point for the estimation of Rallidae and Heliornithidae as the reference base point, which was not authoritative and accurate enough (García–R *et al*., 2014).

The evolutionary analysis results of Charadriiformes were basically consistent with previous studies (Smith and Clarke, 2015; Cheng, 2017; Hu *et al*., 2017), including the three branches of Charadrii, Lari and Scolopaci. Lari and Scolopaci form a sister group, while Charadrii was a separate branche. Among them, *Hydrophasianus chirurgus* of Jacanidae, which was closely related to Rostratulidae. Cheng *et al*. (2017) studied the phylogenetic tree of 18 species of Charadriiforme, which were also divided into Charadrii, Lari and Scolopaci, and analysed the phylogenetic evolution of 12 species of Charadriiformes, then concluded that Recurvirostridea and Charadriidae belonged to Charadrii (Chen, 2003). The results of the evolution analysis of Charadriiformes were basically the same as those of the traditional classification, but there were differences between *Gelochelidon nilotica* and *Pluvianellus socialis*. Traditionally, *Gelochelidon nilotica* belonged to Laridae, and *Pluvianellus socialis* belonged to Charadriidae (Zheng, 2012). However, this study suggested that *Gelochelidon nilotica* belonged to Sternidae as a sister group to *Thalasseus bengalensis* and *Sterna hirundo*. *Pluvianellus socialis* were sisters of *Chionis minor*, belonging to Chionididae. There were no other molecular methods for classifying the two species, and the results need to be further confirmed.

The estimated divergence time for Charadriiformes was 45.44 (58.21 ~ 24.67) Ma, lower than that of Jarvis *et al*. (2014) (65 Ma) and Fain *et al*. (2007) (74.3 Ma), but consistent with that of Prum *et al*. (2015) (55 Ma). According to the time difference of each suborder, Charadrii species first began to differentiate, followed by Lari, and finally Scolopaci, which also confirmed the prediction of Chen *et al*. (2003) on the differentiation of the suborder of Charadriiformes. This study ensured accurate and reliable estimation of the divergence time of Gruiformes and Charadriiformes species through fossil record calibration (Prum *et al*., 2015) and sufficient molecular data support

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Supplementary material

There is supplementary material associated with this article. Access the material online at: https://dx.doi.org/10.17582/journal.pjz/20190603010623

Conflict of interest statement

The authors report no conflicts of interest and were alone responsible for the content and writing of the paper.

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