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Mitochondrial genome of the Indian spot-billed duck and its phylogenetic and conservation implications

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The Indian spot-billed duck, *Anas poecilorhyncha* is a large dabbling and non-migratory breeding bird. The identification and phylogenetic relationship of *A. poecilorhyncha* remain uncertain due to the presence of overlapping meristic characters and hybridization with closely related species. Molecular data aids when there are challenges in morphological identification. However, genetic characterization of *A. poecilorhyncha* has been paid less attention. Apart from their functional and physiological role, mitochondrial genome can also be used for various purposes, including species identification, phylogenetic analysis, understanding the domestication history of species etc. Therefore, the present study aimed to sequence the mitochondrial genome of *A. poecilorhyncha* and its closely related domestic species *A. platyrhynchos* (mallard duck) to understand their mitochondrial genome structure and phylogenetic relationships. The length of mitochondrial genome of *A. poecilorhyncha* and *A. platyrhynchos* was 16,608 and 16,604 bp respectively. Mitochondrial genome contained 37 genes and a non-coding control region. Overall, the characteristics of mitochondrial genome of both species were found to be conserved. The phylogenetic tree exhibited seven major clades (A to G) with a high bootstrap support. Notably, the Indian *A. poecilorhyncha* population formed a distinct clade (C) whereas the *A. poecilorhyncha* that were probably sampled from China grouped along with *A. zonorhyncha* (clade B). Besides, one of the *A. poecilorhyncha* probably sampled from China was placed in the clade A, which predominantly consisted of *A. platyrhynchos*. It suggests that Indian *A. poecilorhyncha* population is genetically different from Chinese *A. poecilorhyncha* population. Further, it sheds light on the importance of conducting a comprehensive phylogenetic study on these species. The newly sequenced mitochondrial genome of *A. poecilorhyncha* and *A. platyrhynchos* would be useful not only to have a better understanding of the phylogeny and evolution of *Anas* species but also to help in the conservation of *A. poecilorhyncha* which is under constant threat from rapid urbanization, interspecific hybridization and other human activities.

Keywords Indian spot-billed duck, Mallard duck, *Anas*, Mitogenome, Phylogeny

The Indian spot-billed duck, *A. poecilorhyncha* belongs to the order Anseriformes and family *Anatidae*. It is a large dabbling non-migratory breeding bird inhabiting both inland and coastal wetlands such as ponds, lakes, rivers, marshes and estuaries. It is widely distributed in the Indian subcontinent. The genus *Anas* had several species but now contains about 36 species[1]. Recently, the genus *Anas* was divided into four distinct genera namely *Anas*, *Mareca*, *Spatula* and *Sibirionetta* based on a molecular study[2,3]. The other notable members of the genus *Anas* are Eastern spot-billed duck (*A. zonorhyncha*), Yellow-billed duck (*A. undulata*), Mallard duck (*A. platyrhynchos*), African black duck (*A. sparsa*), Laysan duck (*A. laysanensis*), Pacific black duck (*A. superciliosa*), Meller's duck (*A. melleri*), American black duck (*A. rubripes*), Hawaiian duck (*A. wyvilliana*), Cape

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teal duck (*A. capensis)*. The Eastern spot-billed duck and the Burmese spot-billed duck (*A. p. haringtoni*) were previously considered as subspecies of *A. poecilorhyncha*[4,5]. But, now the Eastern spot-billed duck is recognized as a separate species (*A. zonorhyncha)* based on the recent studies[1,5]. The genus *Anas* had undergone extensive taxonomic revision over a period of time. However, taxonomic uncertainty is still prevalent in the genus due to high morphological similarities among species.

Though IUCN (International Union for Conservation of Nature) has categorized *A. poecilorhyncha* as "Least Concern" species, the population size has decreased considerably in the last two decades due to poaching, habitat destruction and hybridization with other closely related species. Particularly, *A. poecilorhyncha* and *A. platyrhynchos* (domestic mallard duck) possess certain similar characteristics which allow them to interbreed naturally. Hybridization between *A. poecilorhyncha* and *A. platyrhynchos* represents a significant threat to biodiversity. It is very likely to cause negative consequences on the genetic integrity of *A. poecilorhyncha*. As a result, it could eventually become a threatened species. Thus, the conservation of *A. poecilorhyncha* is currently a matter of serious concern in India and elsewhere.

The advent of next-generation sequencing has enabled sequencing of the whole mitochondrial genome. Mitochondrial genome is a relatively small, circular and double stranded molecule. It is renowned for the remarkable variability, higher mutation rate and maternal inheritance. Hence, it has been widely used to unravel the genetic relationships, evolutionary history, gene flow, migration and maternal origin of species. It has also played prominent role in developing conservation and management strategies for numerous threatened species[6–10]. Most of the previous studies have used D-loop, cytochrome *b*, COI and other mitochondrial genes[11–13] to understand the phylogenetic relationship among *Anas* species. Single and combined gene markers have known shortfalls in the phylogenetic analysis. Therefore, the present study characterized the whole mitochondrial genome of *A. poecilorhyncha* and its closely related domestic duck *A. platyrhynchos* to understand their phylogenetic relationship as well as to elucidate the conservation programs for *A. poecilorhyncha*.

Materials and methods

Sample collection and sequencing

The blood samples (3 nos.) of *A. poecilorhyncha* were collected from Jaipur Zoo, Rajasthan, India. Whereas, the blood samples (3 nos.) of domestic *A. platyrhynchos* were collected from a poultry farm at Thrissur, Kerala, India. Around 0.5 ml of blood was collected from the wing/leg vein of each duck using BD Vacutainer K2EDTA Plus tubes which contains EDTA as an anticoagulant. The collected blood samples were stored at -80 °C until DNA extraction. Genomic DNA was extracted from the blood samples using phenol-chloroform method[14]. Amplification of whole mitochondrial genome was performed using long range PCR with the following two overlapping primer sets: (P241 5'-GCGAGTCTGAACTGGTCTCA -3', P242 5'-AGGTGTAGGGCGATGTTT TG-3'; P243 5'-GGCCTCCGAACCATATGTAA-3', P244 5'-AGGGCTGGGTTGAGAGATTT-3'). The primers were designed based on a mitochondrial genome sequence (GenBank Accession No. EU755253) using Primer3 softwere[15]. Subsequently, genomic libraries were prepared using the PCR amplicons and sequenced on Illumina HiSeq 2500 at AgriGenome Labs, Cochin[7].

Data analysis

Cutadapt[16] was used for removing adapter sequences from the reads. Further, Sickle[17] and FastUniq[18] were used to remove low quality and duplicate reads respectively. The resulting high quality reads were mapped to the respective reference mitochondrial genome (*A. poecilorhyncha*: KF156760; *A. platyrhynchos*: KX592536) with BWA-MEM[19]. The mitochondrial genome annotation was performed on MITOS web server[20]. Mitochondrial genome map was illustrated using OGDRAW web server[21]. tRNAscan-SE web server[22] was utilized to predict the secondary structure of tRNAs. The MEGA[23] was used to examine the relative synonymous codon usage (RSCU) of each codon. The phylogenetic tree was constructed following the maximum parsimony method using MEGA[23] with 2000 bootstrap value.

Results and discussion

Mitochondrial genome organization

The whole mitochondrial genome was sequenced in three samples for each species using the Illumina HiSeq 2500 sequencing platform. In total, 3,279,072 and 2,920,272 raw sequencing reads were generated for *A. poecilorhyncha* and *A. platyrhynchos* respectively (Table 1). Mitochondrial genome length was 16,608 bp in *A. poecilorhyncha* and 16,604 bp in *A. platyrhynchos,* which were consistent with their closest evolutionary relative species as well as other duck species[24–27]. Although both species showed only four base pair difference in size, they exhibited 141 variable sites. The *A. poecilorhyncha* had three haplotypes resulting from 13 variable sites. Similarly, *A. platyrhynchos* showed three haplotypes resulting from 3 variable sites. Therefore, there was no significant difference in the haplotype diversity (1.0000 ± 0.2722) between the species. The average nucleotide composition of *A. poecilorhyncha* was A 29.1%, C 32.9%, G 15.8%, and T 22.2% while *A. platyrhynchos* possessed 29.2% A, 32.8% C, 15.8% G, and 22.2% T. Both species showed a circular, double-stranded mitochondrial genome which includes 22 tRNA genes, 13 protein-coding genes, 2 rRNA genes, and a noncoding control region (Table 2). Further, both species had the same gene order and arrangement (Fig. 1), which was found to be consistent with other avian and vertebrate species[7–9].

Protein coding genes (PCGs)

The mitochondrial genome of both species contained 13 PCGs which accounted for 68% of the total mitochondrial genome. The number of PCGs in the mitochondrial genome of both species was found to be typical of a duck, as well as most other birds[9,25–28]. The AT content of the PCGs was almost same for both species (Fig. 2) which was

Sample ID	Species	Read orientation	Number of raw reads	% GC	% Q > 30
SB01	*A. poecilorhyncha*	R1	482924	48.77	92.92
		R2	482924	48.71	79.73
SB02		R1	502317	48.62	92.92
		R2	502317	48.57	82.65
SB03		R1	654295	48.85	92.35
		R2	654295	48.77	70.27
MA01	*A. platyrhynchos*	R1	773177	48.87	95.08
		R2	773177	48.94	95.32
MA02		R1	141502	48.60	96.00
		R2	141502	48.66	91.00
MA03		R1	545457	49.09	95.80
		R2	545457	49.15	95.08

Table 1. Characteristics of mitochondrial genome sequencing data of *A. poecilorhyncha* and *A. platyrhynchos*.

in agreement with other closely related species of birds[27,29,30]. As with other duck mitochondrial genomes[25,27], the heavy strand encoded six NADH genes (*nad1, nad2, nad3, nad4, nad4L,* and *nad5*), three cytochrome *c* oxidase genes (*cox1, cox2,* and *cox3*), two ATPase genes (*atp8* and *atp6*), and a cytochrome *b* gene (*cob*) while the light strand encoded *nad6* gene. The length of the PCGs significantly varied across 13 genes, with *nad5* (1,824 bp) being the longest and *atp8* (168 bp) being the shortest. In both species, ATG was the initiation codon for 10 of the 13 PCGs, except for *cox1*, *cox2* and *nad5* genes with GTG. In total, there were 3,802 codons in both species. The most frequently used codon was CCU while the least frequently used codon was GCG. The relative synonymous codon usage (RSCU) displayed that both species have the same codon family as well as identical RSCU features (Fig. 3).

Ribosomal RNA and transfer RNA

The mitochondrial genome of *A. poecilorhyncha* and *A. platyrhynchos* encoded two ribosomal RNA subunits, 12S rRNA and 16S rRNA which were located between the *trnF* and *trnL2* genes, with *trnV* separating them (Fig. 1). The 12S rRNA was 986 bp in both species, while the 16S rRNA had an additional nucleotide in *A. poecilorhyncha* (1603 bp). Both subunits were identical to those found in other duck species[25,27]. Besides, there were 22 tRNA genes in the mitochondrial genomes of both species which ranged between the length of 66 and 76 bp. Out of the 22 tRNA genes, 14 tRNA genes (*trnF, trnV, trnL2, trnI, trnM, trnW, trnD, trnK, trnG, trnR, trnH, trnS1, trnL1,* and *trnT*) encoded on the heavy strand showed a typical and conserved order as observed in other ducks and vertebrate species[7–9,25,27]. The closer examination of tRNA secondary structures showed that all the tRNA genes displayed the cloverleaf secondary structure except *trnS1*. Notably, the dihydrouridine stem and loop were absent in *trnS1* (Figs. 4, 5) which is similar to that of birds in general[9,29,30].

Control region

The control region also known as "D-loop" is the longest non-coding region in the mitochondrial genome, which plays a pivotal role in the regulatory functions of replication and transcription[31]. Control region was placed between the tRNAs *trnF* and *trnE*. The length of the control region varied between *A. poecilorhyncha* (1,052 bp) and *A. platyrhynchos* (1,049 bp). The comparison of control region revealed 28 variable sites between the species. Of the 28 variable sites, five mutations were observed within *A. poecilorhyncha* population whereas a single mutation was observed within *A. platyrhynchos* population. The AT content was found to be 53.0% in *A. poecilorhyncha* and 53.1% in *A. platyrhynchos*. Four and two copies of palindromic sequence motifs 'TACAT' related to termination of heavy strand replication were observed in *A. poecilorhyncha* and *A. platyrhynchos* respectively as reported in other species[32,33]. Furthermore, poly-C region was found at the start of the control region in both species. Similar findings have also been observed in other avian mitochondrial genomes[34,35]. The results showed that the size, AT content, and copy numbers of the palindromic sequence motifs of the control region are variable among duck species.

Phylogenetic analysis

The genus *Anas* formerly had several species but now contains about 36 species[1]. The classification of the genus *Anas* has been evaluated repeatedly using different methods which leads to several rearrangements in the genus. Recently, based on a molecular study the genus was divided into four distinct genera namely *Anas*, *Mareca*, *Spatula* and *Sibirionetta*[2,3]. However, the phylogenetic relationships among *Anas* species have been considerably debated since most of the previous findings were based on a few samples and genetic markers. In order to resolve the phylogenetic relationship among *Anas* species, we constructed a maximum parsimony phylogenetic tree using the whole mitochondrial genome sequences of six *Anas* species and five former *Anas* species. The phylogenetic tree was rooted using a mitochondrial genome sequence of domestic chicken. Additional mitochondrial genome sequences were retrieved from GenBank. The resulting phylogenetic tree was well resolved and showcased seven major clades A, B, C, D, E, F and G for 11 duck species (Fig. 6). In agreement with previous studies[1,2,27,36], *A. poecilorhyncha, A. zonorhyncha* and *A. platyrhynchos* were recovered as sister

Genes	Start		Stop		Strand	Size (bp)		IGS (bp)
	A. poec	A. plat	A. poec	A. plat		A. poec	A. platy	
trnF	1		70		H	70		0
rrnS	71		1055		H	985		0
trnV	1056		1126		H	71		0
rrnL	1127		2729	2728	H	1603	1602	0
trnL2	2730	2729	2803	2802	H	74		4
nad1	2808	2807	3785	3784	H	978		-2
trnI	3784	3783	3855	3854	H	72		7
trnQ	3863	3862	3933	3932	L	71		-1
trnM	3933	3932	4001	4000	H	69		0
nad2	4002	4001	5040	5039	H	1039		0
trnW	5041	5040	5116	5115	H	76		3
trnA	5120	5119	5188	5187	L	69		2
trnN	5191	5190	5263	5262	L	73		0
trnC	5264	5263	5329	5328	L	66		0
trnY	5330	5329	5400	5399	L	71		1
cox1	5402	5401	6952	6951	H	1551		-9
trnS2	6944	6943	7016	7015	L	73		2
trnD	7019	7018	7087	7086	H	69		1
cox2	7089	7088	7775	7774	H	687		1
trnK	7777	7776	7845	7844	H	69		1
atp8	7847	7846	8014	8013	H	168		-10
atp6	8005	8004	8688	8687	H	684		-1
cox3	8688	8687	9471	9470	H	784		0
trnG	9472	9471	9540	9539	H	69		0
nad3	9541	9540	9892	9891/9892	H	352	352/353	0/1
trnR	9894	9893	9963	9962	H	70		0
nad4l	9964	9963	10260	10259	H	297		-7
nad4	10254	10253	11631	11630	H	1378		0
trnH	11632	11631	11700	11699	H	69		0
trnS1	11701	11700	11766	11765	H	66		-1
trnL1	11766	11765	11836	11835	H	71		0
nad5	11837	11836	13660	13659	H	1824		-1
cob	13660	13659	14802	14801	H	1143		2
trnT	14805	14804	14873	14872	H	69		10
trnP	14884	14883	14953	14952	L	70		10
nad6	14964	14963	15485	15484	L	522		0
trnE	15486	15485	15556	15555	L	71		0
Control region	15557	15556	16608	16604	H	1052	1049	0

Table 2. Characteristic features of mitochondrial genome of *A. poecilorhyncha* and A. *platyrhynchos*.

species which indicated that *A. poecilorhyncha, A. platyrhynchos,* and *A. zonorhyncha* are genetically closer to each other. However, mitochondrial genome sequences of *A. poecilorhyncha* generated from our study formed a separate clade (C) from other *A. poecilorhyncha* that were probably sampled from China. Whereas the Chinese *A. poecilorhyncha* was close to *A. zonorhyncha* (B). This suggests that Indian population of *A. poecilorhyncha* is genetically different from Chinese *A. poecilorhyncha* population. Besides, one of the *A. poecilorhyncha* probably sampled from China was placed in the clade A which predominantly consisted of *A. platyrhynchos.* This might be a hybrid but contains *A. platyrhynchos* alike mitochondrial genome or due to misidentification of the sample. The ambiguity noticed in the phylogenetic tree necessitates to conduct further comprehensive taxonomic and phylogenetic studies on these species. Therefore, we emphasize to analyse more samples from the regions where both species co-exist to have an improved understanding of the phylogenetic relationship and natural hybridization among duck species. Besides, distinct clades were obtained for the genera *Mareca, Spatula* and *Sibirionetta* which support the separation of these genera from the genus *Anas* as proposed by Gonzalez et al.[2].

Conclusions

Considering the distribution, diversity, and threats, molecular characterization of *A. poecilorhyncha* is vital for its conservation. Therefore, present study sequenced the whole mitochondrial genome of *A. poecilorhyncha*

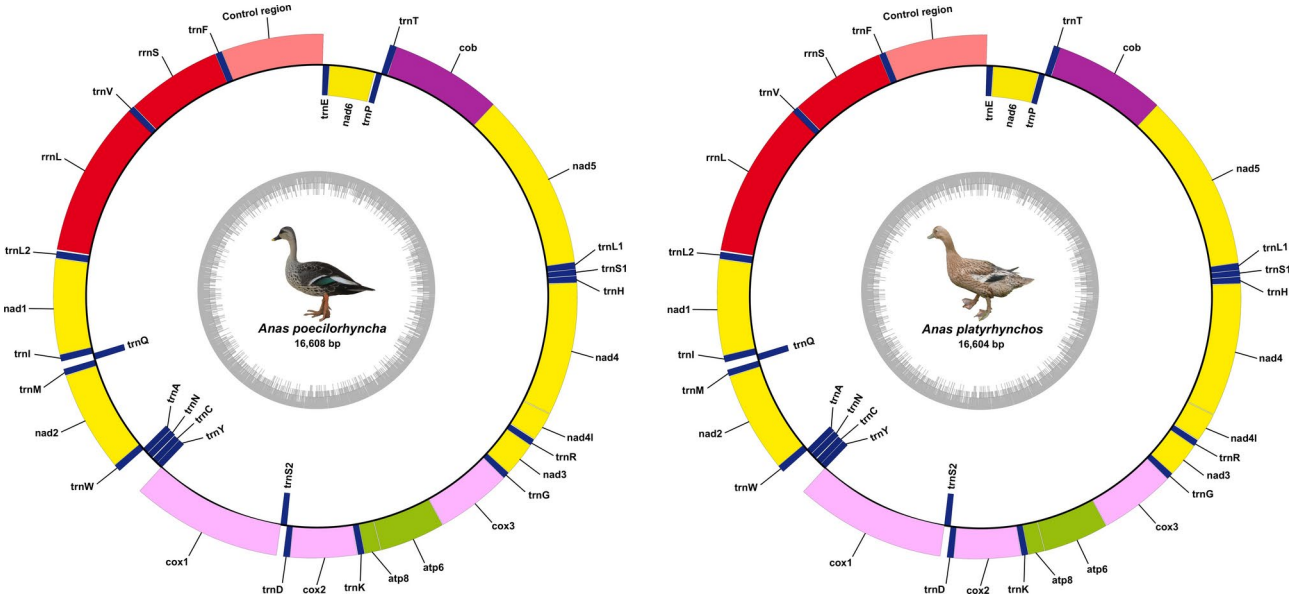

Fig. 1. Schematic representation of mitochondrial genome of *A. poecilorhyncha* and *A. platyrhynchos*. Genes encoded on the H strand are mapped outside the outer circle whereas genes encoded on the L strand are mapped inside the outer circle. The inner circle represents the GC content.

and its closely related domestic duck *A. platyrhynchos*. The characteristics of mitochondrial genomes of the two species were similar to each other but varied by 141 mutations. The phylogenetic analysis showcased close genetic relationship of *A. poecilorhyncha* and *A. platyrhynchos*. Further, it suggested that Indian population of *A. poecilorhyncha* might be genetically different from Chinese *A. poecilorhyncha* population. The management and conservation programs require the correct identification of species and their hybrids in natural habitats. The genetic variation observed between the two species could be used as a potential marker for the identification of both species which can lead towards the development of strategies for the conservation and breeding program of *A. poecilorhyncha*. The sequences generated in the study would serve as valuable resources to enable further studies in related species.

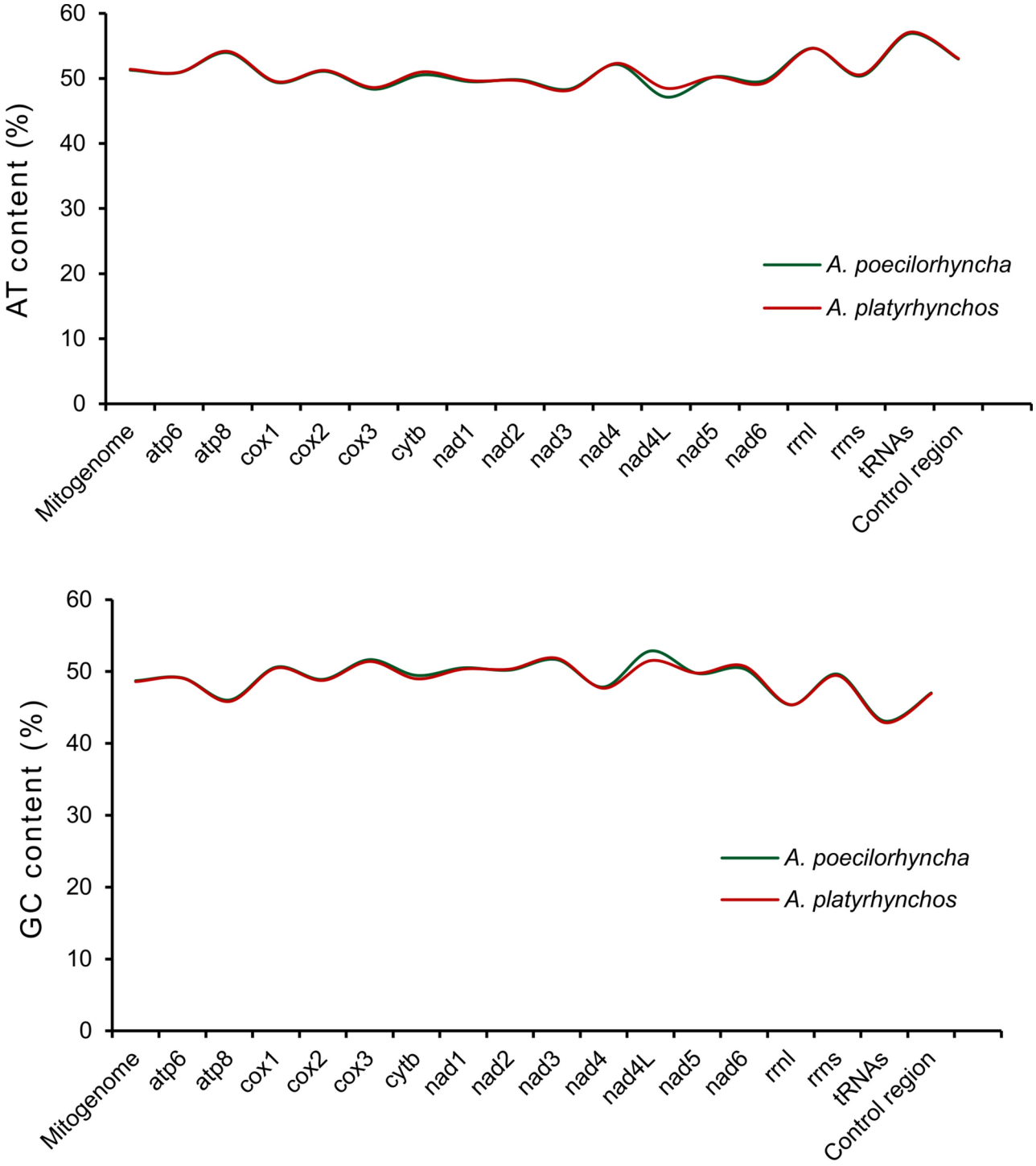

Fig. 2. The average AT and GC content of the mitochondrial genome of *A. poecilorhyncha* and *A. platyrhynchos*.

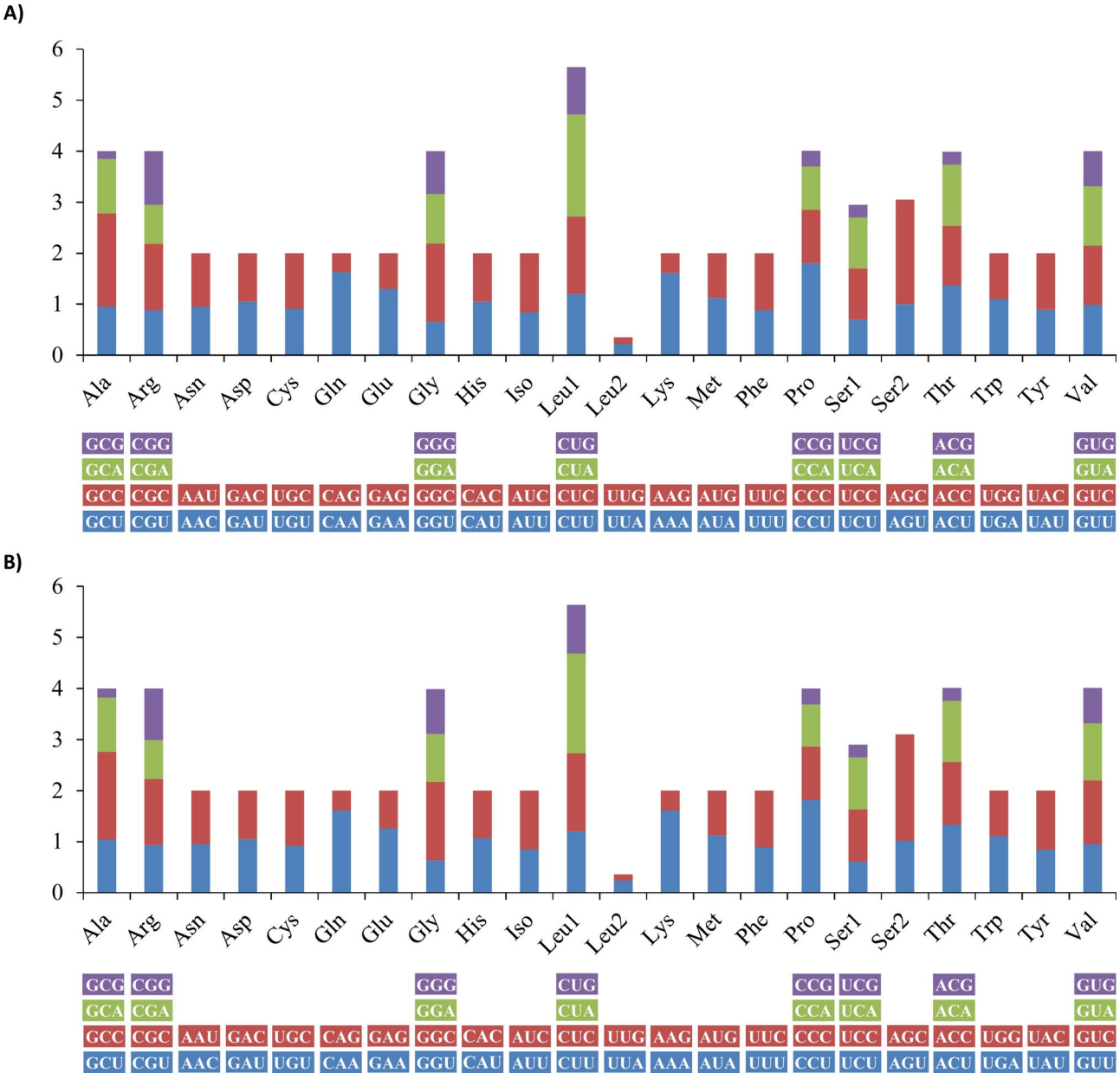

Fig. 3. Relative synonymous codon usage (RSCU) of the mitochondrial genome of *A. poecilorhyncha* (**A**) and *A. platyrhynchos* (**B**). Codons are given on the X-axis whereas RSCU values are given on the Y-axis.

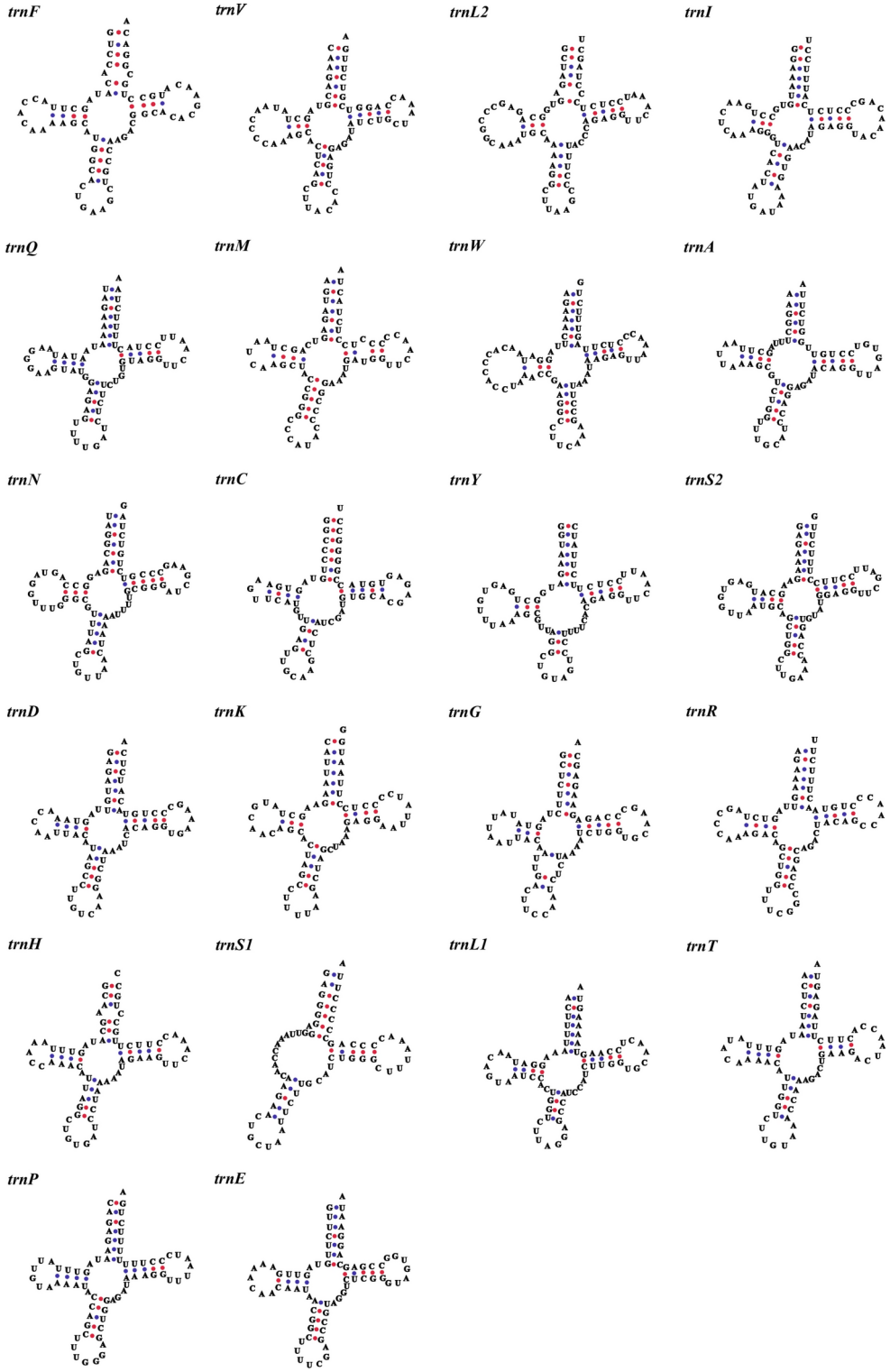

Fig. 4. Mitochondrial tRNAs secondary structures of *A. poecilorhyncha*.

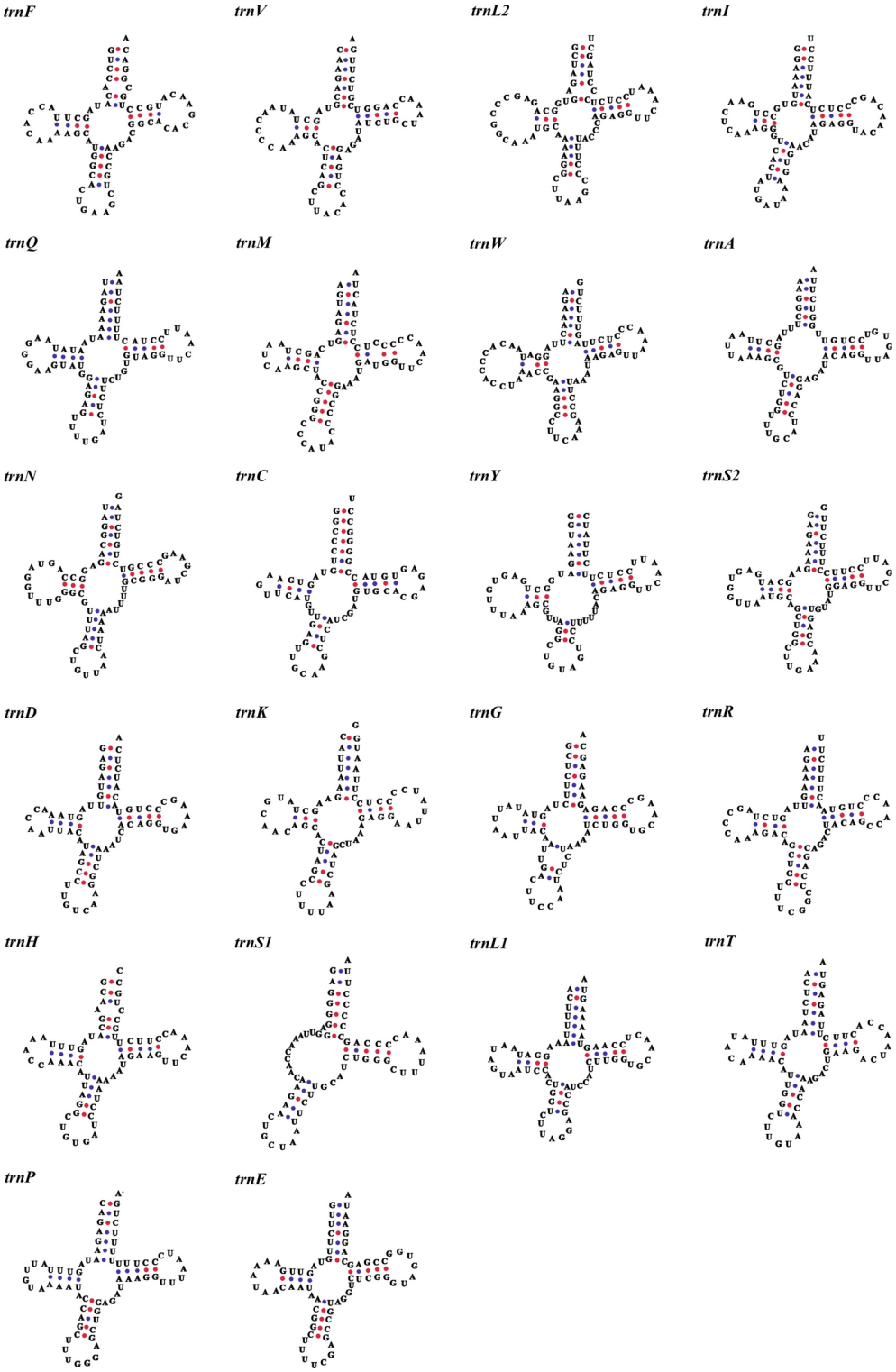

Fig. 5. Mitochondrial tRNAs secondary structures of *A. platyrhynchos*.

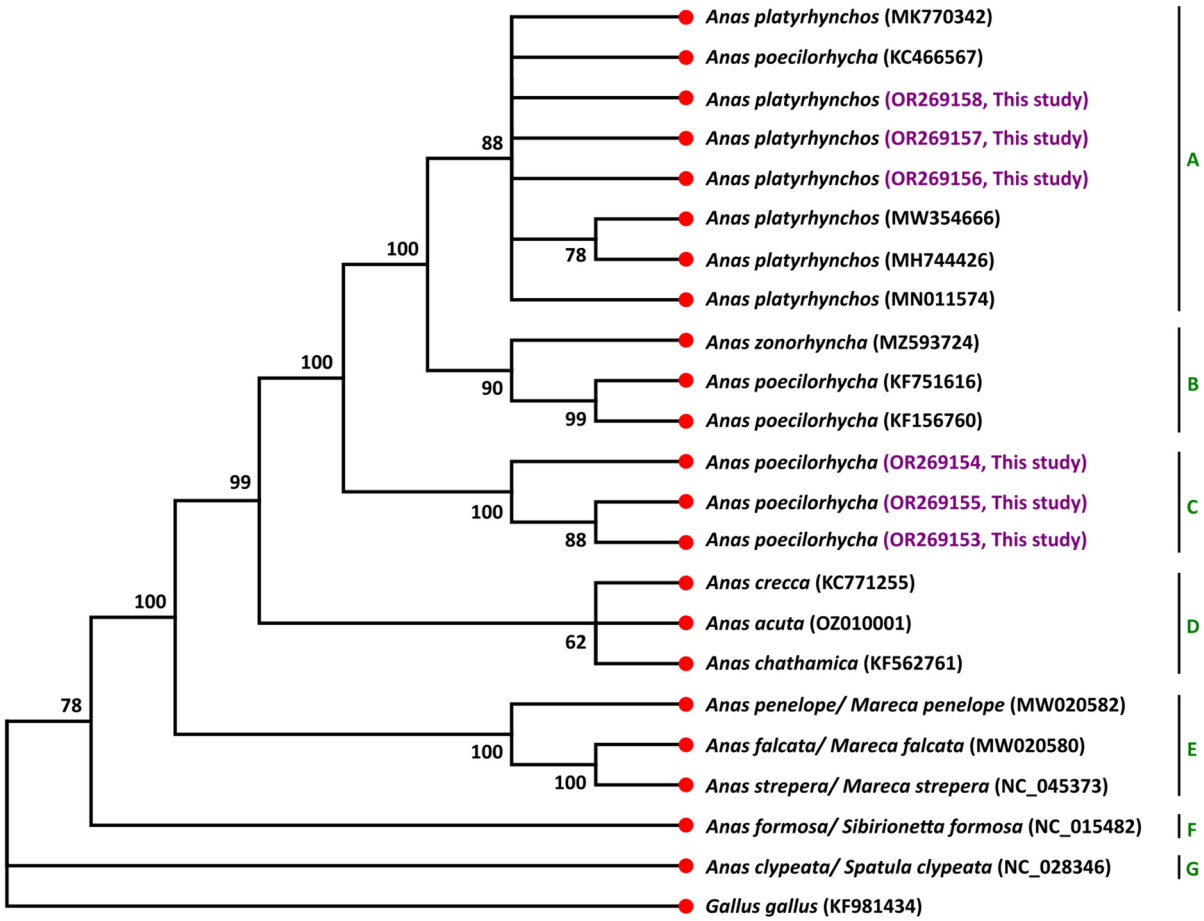

Fig. 6. Phylogenetic tree of the whole mitochondrial genome of 11 duck species. The phylogenetic tree was constructed by maximum parsimony method with 2000 bootstrap replicates. Additional mitochondrial genome sequences were downloaded from GenBank. The accession number of the sequences used for tree construction is listed near the species name.

Data availability

The sequencing data has been deposited in GenBank with the following accession numbers OR269153-OR269158.

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Author contributions

MN conceived and supervised the study. VRP, KB, and ST performed the experiments. MN and RK performed the data analyses. GM and ASR contributed to the data analyses. MN, VRP, AG, ACS, AP and MSM wrote the manuscript. All authors read and approved the final manuscript.

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Declarations

Competing interests

The authors declare no competing interests.

Ethical approval

Animal experiments were approved by the Institutional Animal Ethics Committee of the Mangalore University. The samples were collected with necessary permissions from the respective state forest departments/zoo authorities. Further, the samples were collected by qualified veterinary doctors with consent of the duck owners. The sample collection procedures complied with the present laws on animal welfare and research in India. This study is in accordance with ARRIVE guidelines.

Additional information

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