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RESEARCH ARTICLE

Codon usage trend and codon context of mitochondrial ND genes among avians

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Abstract

Codon Usage Bias (CUB) refers to the non-random uses of synonymous codon that offers variable insights into an organism genetic architecture and evolutionary divergence. For this study, the avians mitochondrial ND genes were taken into consideration for their unique inheritance pattern and mutation rate. We analysed codon usage patterns to understand similarities, differences, and evolutionary forces shaping mitochondrial ND genes across avian species, representing the first systematic comparison among diverse avian lineages. Our analysis revealed variation in nucleotide composition, with a clear dominance of AT over GC content in all mitochondrial ND genes across avian species. The synonymous codon usage order (SCUO) values (0.26-0.39) suggested low codon usage bias in ND genes, while heat map analysis further revealed dissimilarities in codon usage across avian lineages. One of the remarkable findings in this study was the evolutionary disfavouring of the codons in ND3 (ACC, GCG) and ND6 (CAA) across different avian species. Neutrality plot analysis indicated that CUB of the avian mitochondrial ND genes was predominantly shaped by natural selection rather than mutation pressure. Evolutionary relationships among avian species of diverse and within taxonomic orders were supported by strong bootstrap values (98–100%), indicating stable evolutionary lineages.

Keywords: Codon Usage Trends; Respiratory Chain; CUB; ND genes; Avians; Mutation Pressure.

1. Introduction

Protein synthesis in mRNA is governed by triplet codons, where each codon corresponds to a specific amino acid. However, due to redundancy of genetic code, multiple codons often correspond to the same amino acid, also known as synonymous codons (Gustafsson et al., 2004; Oelschlaeger, 2024). In the vertebrate genetic code of mitochondria, 20 numbers of standard amino acids are represented by 60 sense codons and four stop codons namely TAA, AGG,AGA, TAG) during protein synthesis (Knight et al., 2001; Krüger et al., 2023). In protein-coding mRNAs, synonymous codons are not used uniformly to specify amino acids, resulting in a phenomenon known as codon usage bias (CUB) (Plotkin and Kudla, 2010). CUB is a unique characteristic in the genome of an organism which is specific to a species and may differ remarkably among varied organisms or within the genes of same organisms (Baeza et al., 2024; Behura and Severson, 2012; Prat et al., 2009a). Though mutations by the synonymous codon do not alter the structure of the protein properties but their usage may lead to translational efficiency of the protein and consequently affect in protein folding of the polypeptide chain (Buhr et al., 2016; O'Brien et al., 2014; Sander et al. 2014; Wu et al., 2024). Several factors, such as GC nucleotide composition at the 3rd position codons (Chen Lin et al., 2013; Tatarinova et al., 2010), expression level of gene (Blake et al., 2003; Zhao Fangzhou et al., 2021) and length of the gene (Duret and Mouchiroud, 1999; Khandia et al., 2022) influence the CUB patterns in different organisms.

CUB analysis has helped us in understanding the underlying genetics and evolution of an organism or gene at the molecular level (Herbert et al., 2024; Sorimachi, 2010). In addition, CUB is firmly associated with the mechanism of mRNA translation (Hanson and Coller, 2018; Hershberg and Petrov, 2008), protein secondary structure and function (Liu, 2020; Zhou et al., 2013), transgenic design (Carbone et al., 2003; Hemamalini et al., 2020; Parvathy et al., 2022),discovery of new gene (Murray et al., 1989; Wang and Hickey, 2007), identifying lateral transfer of gene and analysing the function of conserved gene expression (Lithwick and Margalit, 2005; Liu et al., 2021), as well as other biological functions (Dahal, 2025; Shabalina et al., 2013).

Several studies on CUB have been performed in nuclear genomes (Butt Azeem Mehmood et al., 2014) , but very few have been done on mitochondrial genomes of organisms(Uddin and Chakraborty, 2016). The study of the mitochondrial genome of an organism holds importance because of its small size and high mutation rate (Chial and Craig, 2008). Besides, the codes present in the genes of mitochondrial DNA vary in comparison to the nuclear DNA (Swire et al., 2005). Mitochondria is known as the cell's 'power house' as it produce 90% of total energy (ATP) needed by the body (via oxidative phosphorylation) and involves several complexes in the RC (respiratory chain) (Sharma et al., 2009). The complex I subunit of mitochondrial RC comprises of seven genes, which are collectively called ND genes, namely ND1, ND2, ND3, ND4, ND4L, ND5 and ND6 , which facilitate oxidative phosphorylation, electron transport and ATP generation (Wirth et al., 2016). In avian species, these genes support the intense energy requirements for flight and diverse ecological niches (Urantówka et al., 2020). These genes function as key molecular indicators for exploring avian evolutionary lineages, bioenergetic adaptation, and mitochondrial genomic variation (Noll et al., 2022).

The analysis of CUB assists in the comprehension of genetics and molecular biology of the genome. Several studies have been reported regarding the CUB of mitochondrial protein coding genes of other vertebrates, but so far, no analysis has been reported on

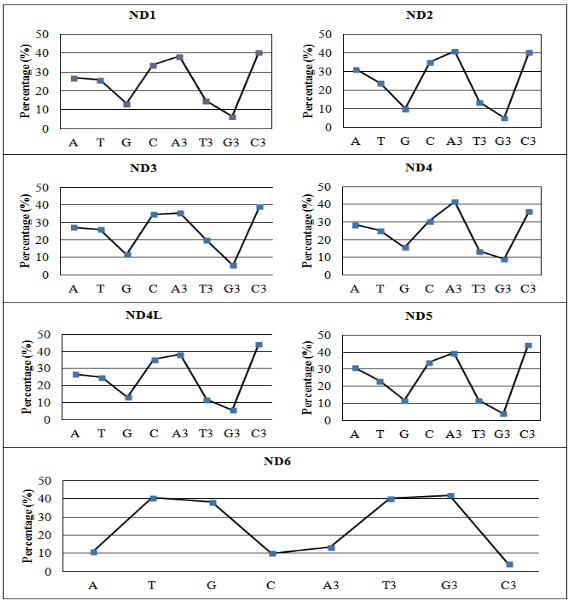


Figure 1. Overall nucleotide composition and its composition at 3rd codon position for mitochondrial ND genes among avian.

Table 1. Overall GC content and GC content at 1st, 2nd and 3rd position in for ND genes across birds.

	GC	GC1	GC2	GC3
ND1	47.09	52.84	41.52	46.89
ND2	45.15	45.41	44.53	45.52
ND3	46.56	51.00	44.18	44.50
ND4	46.35	51.32	42.69	45.04
ND4l	48.54	51.23	44.51	49.90
ND5	45.96	46.79	42.61	48.49
ND6	48.35	56.85	42.04	46.16

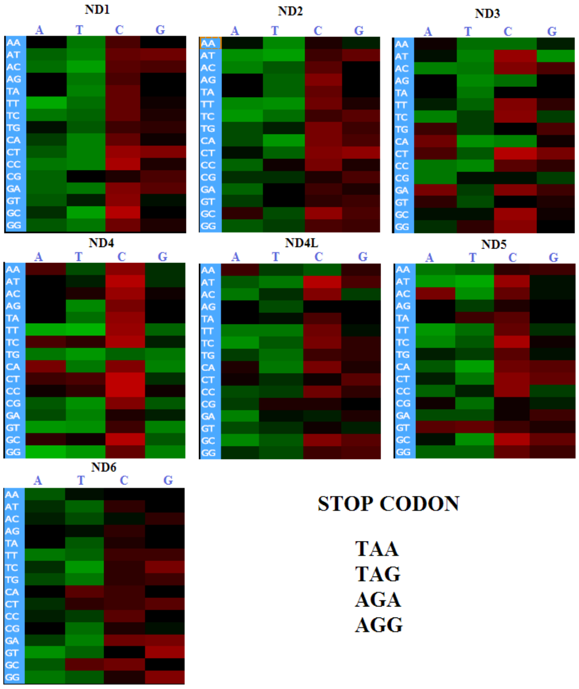


Figure 2. Correlation between codon usage and GC3 in different ND genes of avian. (Green, red and black color indicates negative, positive and no correlation, respectively.)

the codon usage of mitochondrial ND genes among avian. This research gives an insight into the pattern of codon usage, selection pressure, phylogenetic relationship and structural diversity among avian species based on mitochondrial ND genes. The findings of the study provide information on molecular biology and genetics, which will help in comprehending the evolutionary relationship among varied avian species.

2. Methodology

2.1 Sequence retrieval

The protein coding sequences of 100 avian bird species of different order and their mitochondrial ND genes (ND1, ND2, ND3, ND4, ND4L, ND5, ND6) which are available in GenBank database of NCBI (http://www.ncbi.nlm.nih.gov/) was retrieved. The sequences selected in our analysis were multiples of three with proper start and stop codons irrespective of any canonical bases (N).The names of avian species of different orders along with their NCBI accession number are reported in S1.

2.2. Nucleotide composition analysis

The nucleotide composition considered in our analysis were the overall percentage of A, T, G and C across the sequences retrieved and also at the third position of codons i.e. A3, T3, G3, C3. Further, GC bias at different positions 1, 2 and 3 were considered. The analysis was carried out using the genetic code of vertebrate mitochondria *i.e.* translational table 2 of NCBI.

2.3. Relative synonymous codon usage (RSCU)

RSCU value was calculated as the observed frequency of a particular codon to its expected frequency for a certain amino acid within a family of degenerate codons.

Mathematically,

$$RSCU = \frac{g_{ij}}{\sum_{j}^{ni} gij} ni$$

here,
g_{ij} represents the rate of occurrence of the i^{th} codon for the j^{th} amino acid and n_i is the total number of synonymous codons in the degenerate codon family (Sharp et al., 1988).

2.4. Synonymous codon usage order (SCUO)

The prediction rate of predetermined synonymous usage of codons that encode an amino acid is measured analytically by SCUO. The SCUO value varies from zero (indicating no biases) to one (indicating high biases). SCUO is measured to predict the expression of gene and the rate of CUB throughout the sequence (Angellotti et al., 2007, Wan et al., 2003).

2.5. Correspondence analysis (CoA)

CoA is multifaceted and designed for the prediction of major variation trends in the codon usage of *ND* genes across the avian species. In this plot RSCU values of 60 codons are scattered across axis1 and axis2 with its trends (Shields and Sharp, 1987). Here, each cds was distributed in 60 dimensional vectors. PAST software was used to construct the analysis of CoA.

2.6. Neutrality plot analysis

The neutrality plot analysis was applied to assess how mutation pressure and natural selection interact in shaping codon usage bias (CUB). The neutrality plot was constructed by plotting GC content at the first and second codon positions (GC12) along the *y*-axis against GC content at the third codon position (GC3) along the *x*-axis. A regression slope approaching zero, accompanied by a narrow GC3 distribution, reflects the stronger influence of natural selection, whereas a slope nearing one with a broad GC3 distribution highlights the predominance of mutation pressure in shaping codon usage bias (CUB), indicating neutrality (Sueoka, 1988).

2.7. Codon context analysis

Table 2. Correlation between overall nucleotide composition (%) and its composition at 3rd codon position in different species of avian for ND genes.

		A3	T3	G3	C3	GC3
ND1	A	0.951**	0.186	-0.521**	-0.632**	-0.762**
	T	0.099	0.967**	-0.267*	-0.733**	-0.744**
	G	-0.263*	-0.461**	0.898**	0.142	0.498*
	C	-0.591**	-0.654**	0.051	0.963**	0.851**
	GC	-0.631**	-0.789**	0.497**	0.887**	0.972**
ND2	A	0.928**	0.104	-0.609**	-0.529**	-0.759**
	T	0.070	0.937**	-0.097	-0.738**	-0.698**
	G	-0.550**	-0.185	0.916**	0.110	0.534**
	C	-0.437**	-0.709**	-0.074	0.960**	0.814**
	GC	-0.665**	-0.670**	0.468**	0.827**	0.955**
ND3	A	0.546**	-0.260**	-0.543**	-0.175	-0.417**
	T	-0.471**	0.698**	0.258**	-0.671**	-0.599**
	G	0.452**	-.583**	0.383**	0.221*	0.399**
	C	-0.483**	0.160	-0.045	0.502**	0.510**
	GC	-0.096	-0.278**	0.235*	0.593**	0.728**
ND4	A	0.568**	-0.653**	-0.957**	0.818**	0.136
	T	-0.271**	0.826**	0.935**	-0.958**	-0.545**
	G	-0.374**	0.724**	0.994**	-0.935**	-0.365**
	C	0.248*	-0.783**	-.973**	.980**	0.524**
	GC	-0.657**	-0.207*	0.210*	0.117	0.740**
ND4l	A	0.913**	-0.179	-0.452**	-.0488**	-0.651**
	T	0.020	0.822**	-0.220*	-0.545**	-0.589**
	G	-0.087	-0.225*	0.800**	-0.177	0.230*
	C	-0.679**	-0.317**	0.064	0.866**	0.798**
	GC	-0.716**	-0.435**	0.498**	0.757**	0.911**
ND5	A	0.692**	-0.136	-0.663**	0.102	-0.385**
	T	-0.053	0.843**	0.397**	-0.710**	-0.597**
	G	-0.073	0.181	0.908**	-0.617**	-0.083
	C	-0.334**	-0.557**	-0.647**	0.904**	0.653**
	GC	-0.627**	-0.601**	0.293**	0.522**	0.894**
ND6	A	0.933**	0-.220*	-0.606**	-0.041	-0.546**
	T	-0.245*	0.872**	-0.261**	-0.591**	-0.502**
	G	-0.580**	-0.432**	0.912**	-0.008	0.789**
	C	0.088	-0.475**	-0.101	0.856**	0.310**
	GC	-0.406**	-0.683**	0.666**	0.593**	0.855**

(**represents significant at p<0.01 and *p<0.05)

Table 3. Correlation coefficients between SCUO and various GC contents for mitochondrial ND genes.

		GC	GC1	GC2	GC3
ND1	SCUO r	0.407**	0.491**	-0.130	0.337**
	p	000	000	0.198	0.001
ND2	SCUO r	0.309**	0.213*	0.210*	0.285**
	p	.002	0.033	0.036	0.004
ND3	SCUO r	0.281**	0.638**	-0.662**	0.413**
	p	.005	.000	.000	.000
ND4	SCUO r	-0.019	-0.609**	-0.495**	0.509**
	p	0.853	.000	.000	.000
ND4l	SCUO r	0.294**	0.073	-0.267**	0.380**
	p	0.003	0.468	.007	.000
ND5	SCUO r	0.239*	-0.005	-0.321**	0.411**
	p	.017	0.964	0.001	.000
ND6	SCUO r	-0.298**	-0.469**	-0.383**	-0.028
	p	0.003	.000	.000	0.785

(**represents significant at p<0.01 and *p<0.05)

Table 4. Correlation between SCUO and properties of protein.

		Protein length	GRAVY	hydrophilicity	aromaticity
ND1	SCUO r				
	p				
ND2	SCUO r	0.035	0.031	0.089	-0.196
	p	0.727	0.762	0.379	0.051
ND3	SCUO r	-0.529**	0.797**	-0.703**	0.502**
	p	0.000	0.000	0.000	0.000
ND4	SCUO r	-0.343**	0.612**	0.498**	-0.841**
	p	0.000	0.000	0.000	0.000
ND4l	SCUO r	0.274**	0.219*	-0.266**	-0.092
	p	0.006	0.029	0.008	0.363
ND5	SCUO r	0.192	0.288**	-0.154	-0.218*
	p	0.055	0.004	0.126	0.029
ND6	SCUO r	0.261**	0.434**	-0.372**	0.263**
	p	0.009	0.000	0.000	0.008

(**represents significant at p<0.01 and *p<0.05)

Figure 3. Hierarchical clustering of RSCU values of each codon among avian for ND genes. (Red colour indicates over-represented, light green indicates under-represented, green colour indicates less frequently used while dark green and black colour indicates more frequently used.)

This method was applied to identify the occurrence of paired codons across the protein-coding mitochondrial avian ND genes. Analysis of codon context was performed using ANACONDAV2.0 software which utilizes python-based scripts for codon context analysis (Moura et al., 2007).

2.8. Statistical analysis

All compositional features and indices related to CUB were estimated with a Nucleotide composition and RSCU values of each codon was calculated using CAIcal server (http://genomes.urv.es/CAIcal)(Puigbò et al., 2008). Analyses of correlation between related indices were established using IBM SPSS version 27.0.

3. Result

3.1. Compositional features of ND genes among avian

In the genome, the non-random codon preference can be affected by its compositional constraints (Jenkins and Holmes, 2003). Hence, we examined the compositional characteristics of these varied species belong to different order of avian for the mitochondrial ND gene (Figure 1). The overall composition of nucleotide was analysed in coding regions of ND gene and observed that the share of cytosine (C) was the highest while the guanine (G) being the lowest with exception to ND6 gene. However, in ND6 gene, the thymine (T) showed the highest contribution, whereas cytosine (C) contributed the least. Besides, at the 3rdcodon position of the nucleotide, cytosine (C) was the highest (except ND2, ND4 and ND6 gene), while guanine (G) being the lowest (except in ND6 gene). Similarly, nucleotide at the 3rd codon position in ND2 and ND4 genes, the highest contribution of adenine (A) was observed while the lowest contribution of guanine (G) was observed inND6 gene across the CDs of different bird species. This finding suggested the variance of percentage in different nucleotide compositions among ND genes. Moreover, the overall GC contents was determined to be lesser in percentage (i.e. less than 50%) in all ND genes, which further suggested the AT richness of the genes as shown in Table 1. The proportion of GC nucleotides at the first codon position (GC1) was above 50% (except in ND2 and ND5), while at the second codon position (GC2) and third codon position (GC3), the proportion was below 50% (Table 1). Further, we performed t-test analysis between overall GC and different position of GC contents to check variation among them for all ND genes (S2). Notably, a significant difference was identified between overall GC and second and third codon position (GC2 and GC3) in ND1 but no significant difference in ND2 gene. Conversely, in ND3 significant difference was observed between overall GC and 1st position of GC1 while significant difference with all position of GC content and overall GC was observed in ND4, ND4l, ND5 and ND6.

3.2. Codon usage of ND genes among avian

The overall SCUO values in different species of avian were as follows: ND1 (0.26), ND2 (0.29), ND3 (0.35), ND4 (0.29), ND4l (0.39), ND5 (0.32) and ND6 (0.39). The results revealed that a weak CUB subsist in ND genes of different bird species, which could contribute to maintain a stable level (Zhang et al., 2013).

3.3. Relationship of codon usage bias with GC3 in avian ND genes

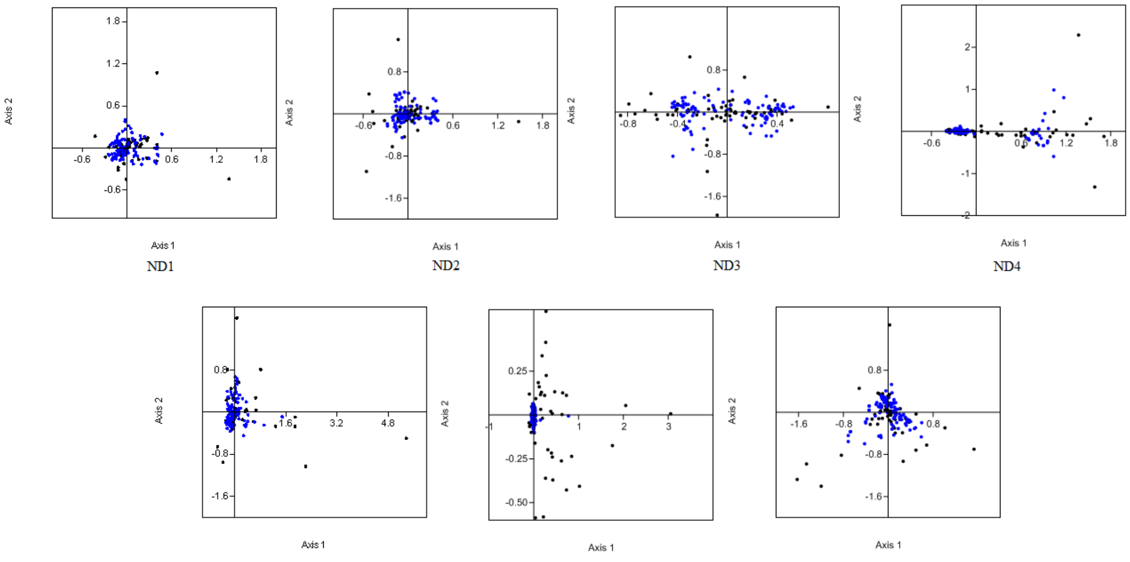
To elucidate the association of GC3 and codon usage, correlation analysis was performed (Figure 2). From the Figure 2, it was clearly observed that nearly all the AT-ending codons exhibit negative correlation (green colour) suggesting that codon usage would decline with the rise of GC3 while the GC-ending codons exhibit positive correlation (red colour)suggesting the codon usage would increase with the rise of GC3 and this result supported the previous findings (Palidwor et al., 2010).

3.4. Patterns of ND genes codon usage among avian

The patterns of synonymous codon usage in mitochondrial-ND gene among different species of avian can be understood by calculating and comparing the frequency of relative synonymous codon usage (RSCU) value of individual codons in each CDs (Behura and Severson, 2012). The heat map was produced by basing on RSCU values of each codon and average value of each codon taken from 100 different species of each gene. In Figure 3, red colour indicates over-represented, light green indicates under-represented, green represents less frequently used while dark green and black indicate more frequently used codons. Heat map analysis revealed different codon usage patterns among ND genes and it might correlate with the different subunit of complex I. However, one of the interesting findings was that nature disfavoured CAA codon in ND6 gene, ACG and GCG codons in ND3 gene among different species. The codon CGG was over-represented and had highest RSCU value in ND6 gene while it was under-represented in other genes. The overall results of RSCU and nucleotide composition suggested that compositional properties under the presence of mutation pressure control the selection of preferred codon in Mitochondrial ND genes among different species of avian (Behura and Severson, 2012).

3.5. Selection pressure on ND genes' codon use bias among avian

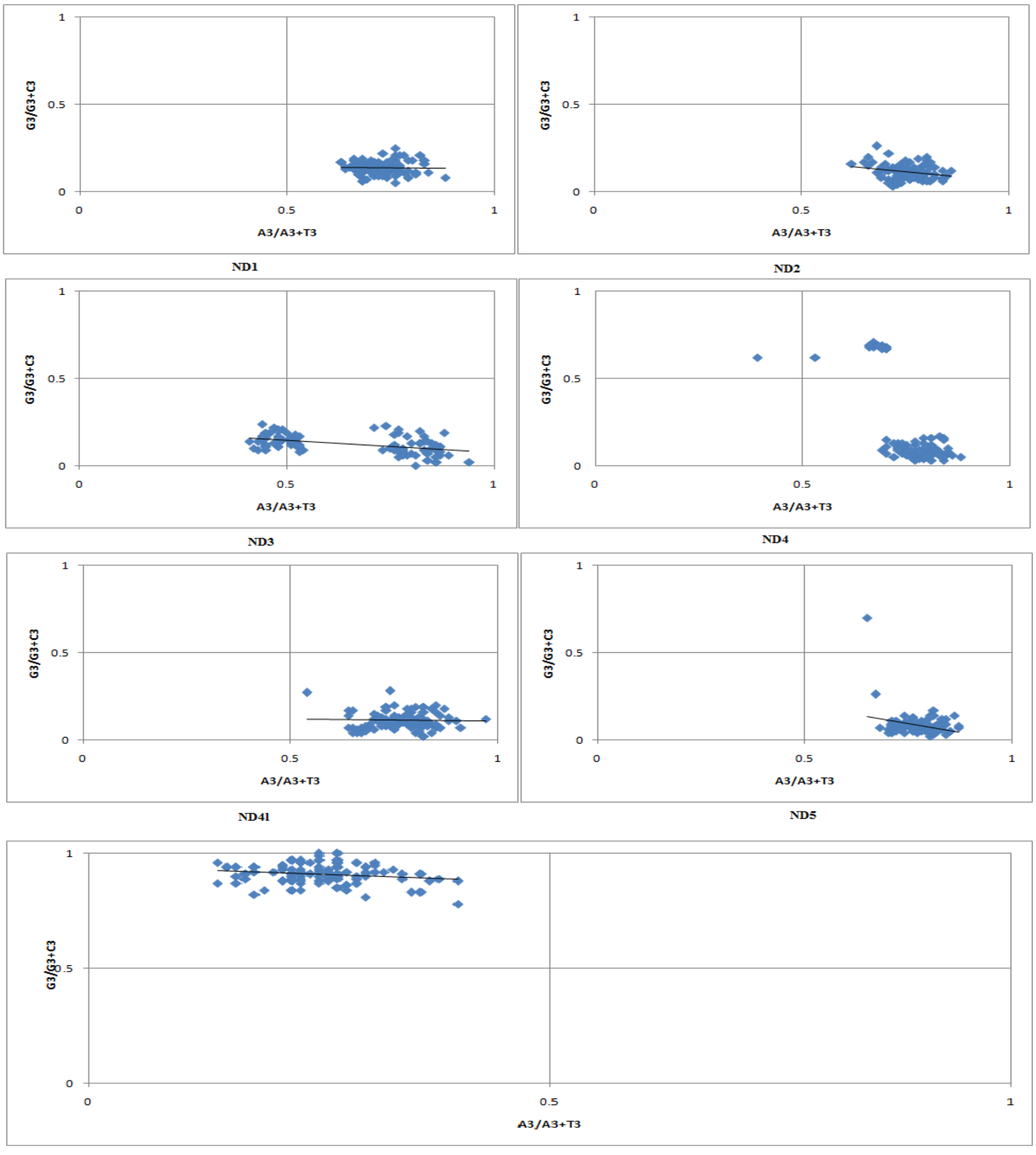
The CUB of a gene or genomes of organism are believed to be controlled by two forces namely natural selection and mutation pressure (Tatarinova et al., 2010). Hence, to assess the extent of impact on the ND genes codon usage bias among avian, correlation analysis was carried out to examine the nucleotide composition at the third codon position and overall composition of nucleotide (Table 2) which revealed significant correlation. Nevertheless, correlation analysis of SCUO with GC content at different codon



ND1 ND2 ND3 ND4

ND4l ND5 ND6

Figure 4. Correspondence analysis of synonymous codon usage towards the codonsin ND genesacross avian



ND1 ND2

ND3 ND4

ND4l ND5

ND6

Figure 5. Parity plot analysis of synonymous codon usage towards the codonsin ND genesacross avian

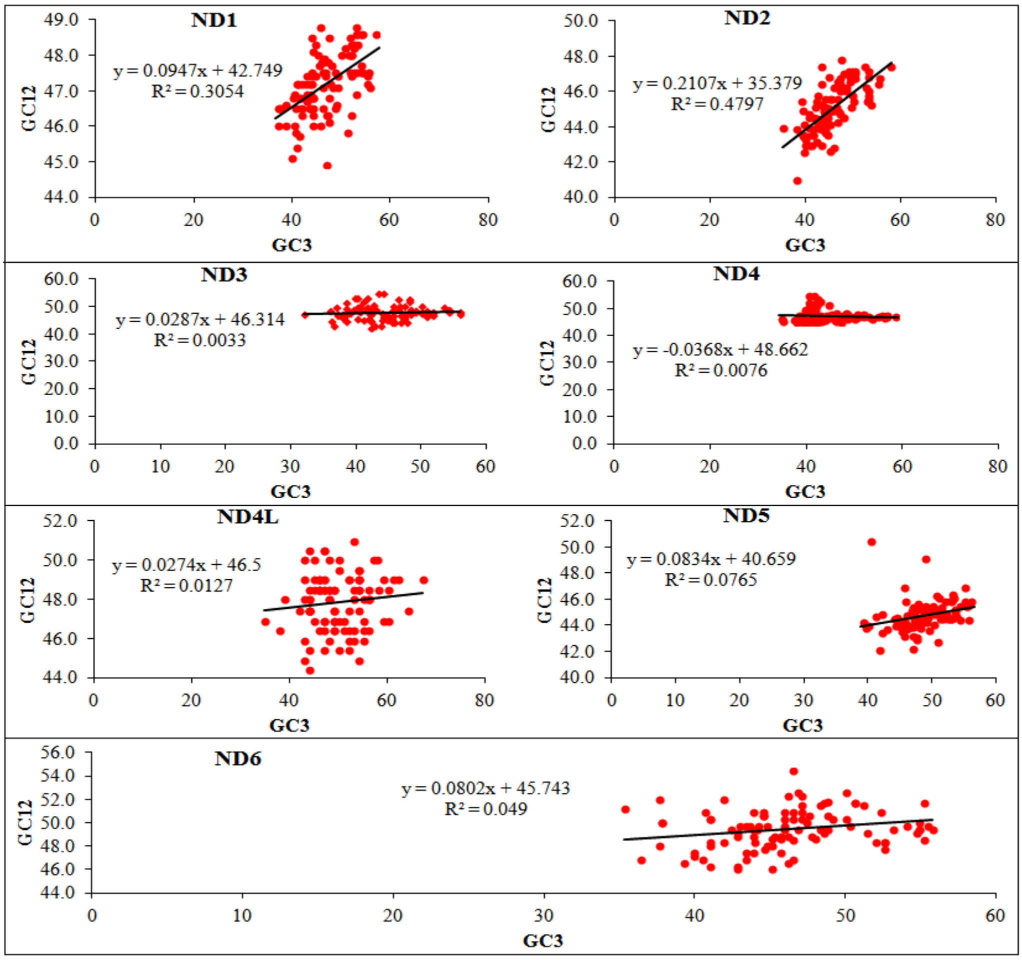


Figure 6. Neutrality plot of mitochondrial ND genes among avian

Table 5. Correlation between SCUO and various skews

		GC skew	AT skew	Purine skew	pyrimidine skew	keto skew	amino skew
ND1	SCUO r	-0.300**	0.796**	0.122	-0.673**	-0.200*	-0.348**
	p	0.002	0.0001	0.225	0.0001	0.046	0.000
ND2	SCUO r	-0.578**	0.669**	0.326**	-0.682**	-0.284**	0.022
	p	0.000	0.000	0.001	0.000	0.004	0.829
ND3	SCUO r	0.387**	0.673**	-0.257**	-0.419**	0.184	-0.603**
	p	0.000	0.000	0.010	0.000	0.066	0.000
ND4	SCUO r	-0.914**	0.932**	0.904**	-0.929**	-0.890**	0.864**
	p	0.000	0.000	0.000	0.000	0.000	0.000
ND4l	SCUO r	-0.282**	0.603**	0.209*	-0.589**	-0.080	-0.262**
	p	0.004	0.000	0.037	0.000	0.431	0.008
ND5	SCUO r	-0.484**	0.573**	0.420**	-0.610**	-0.392**	0.149
	p	0.000	0.000	0.000	0.000	0.000	0.138
ND6	SCUO r	0.698**	-0.671**	-0.488**	0.729**	0.192	0.319**
	p	0.000	0.000	0.000	0.000	0.056	0.001

(** represents significant at $p<0.01$ and *$p<0.05$)

positions (Table 3) showed highly significant correlation between them. The overall result revealed that presence of either mutation pressure or natural selection affects the ND genes codon usage resulting from compositional bias (Butt Azeem Mehmood et al., 2014; Zhang et al., 2013).

3.6. Correspondence analysis (CoA) of ND genes among avian

We performed correspondence analysis using the frequency of synonymous codon usage (RSCU) value to examine the principal axis in the heterogeneity of codon usage patterns across the coding regions of ND genes among avian (Figure 4). We observed difference in distribution of points in these plots, suggesting the heterogeneity in the codon usage among ND genes. Moreover, nearly all the codons were located close to the axes with a narrow distribution which indicate that base composition mutation bias might correlate to the codon usage bias (Figure 4). A very few genes showed distinct distribution, which again indicated the role of some other factors like natural selection in codon usage bias of complex I genes in the RC among avian (Jia et al., 2015; Uddin et al., 2016; Wei Lei et al., 2014a; Zhao Yongchao et al., 2016).

3.7. Analysis of Parity plot among ND genes of avian

The values of AT bias (A3/A3+T3) were used for the *x*-axis and GC bias (G3/G3+C3) for the *y*-axis to perform parity plot analysis across the coding regions of ND genes among different avian species. According to this analysis, proportionate distribution of AT and/or GC suggests that mutation bias is solely responsible for

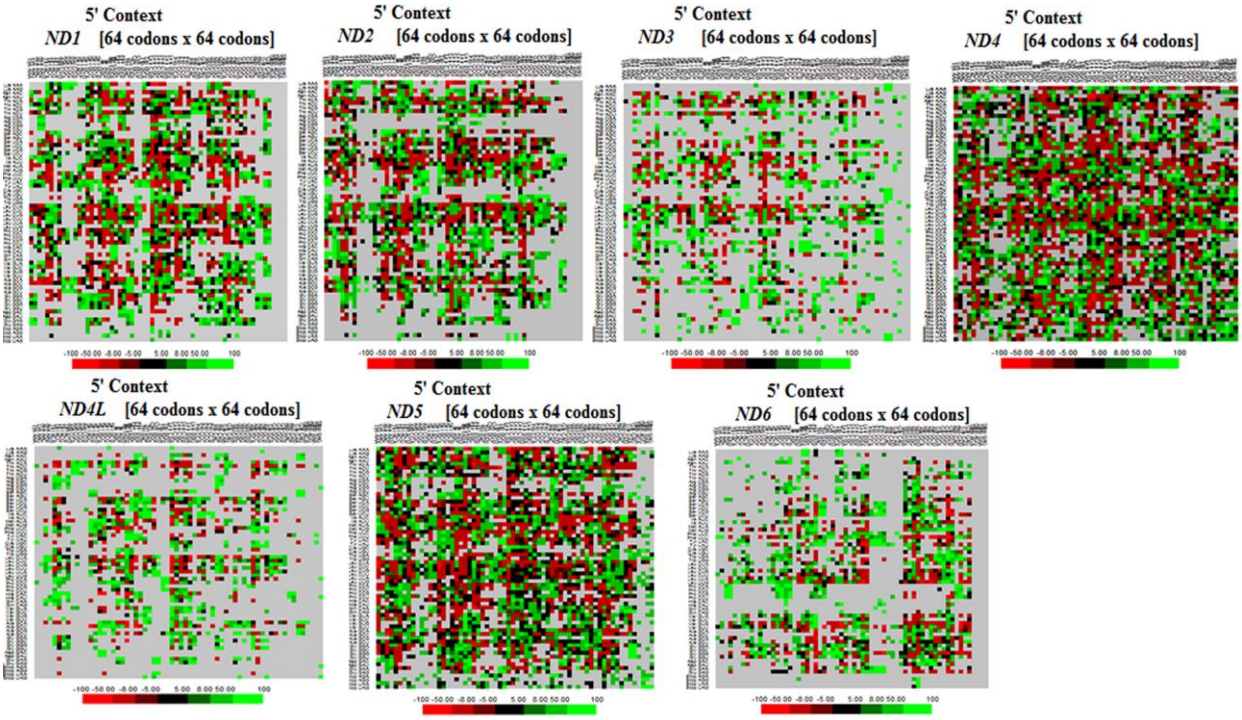


Figure 7. Codon context patterns of ND genes among avian. (The row represents the 5'codons and the column as 3' codons. The highest number of codon contexts are represented by green colour and the lowest number of codon context as red colour.)

CUB, while disproportionate distribution implied that natural selection & mutation pressure affect CUB of gene (Sueoka Noboru, 1995). In our study, we noted that AT and GC were not utilized proportionally in complex I genes of RC among avian (Figure 5) and there by indicating the impact of both natural selection and mutation pressure assisted to the CUB in the coding regions of ND genes among avian(Chen Haixia et al., 2014).

3.8. Analysis of neutrality plot among ND genes of avian

Neutrality plot analysis gives the clue for correlating GC3 and the combined average of GC content at codon positions GC1 and GC2 (GC12) and can also be employed to examine the balance between mutation and selection pressure on the bias of codon usage among ND gene. In this plot, the mutation against selection can cause a broad range of GC3 distribution and regression line slope approaching one. Moreover, natural selection is presumed to be the major contributing factor to the CUB, when a concentrate distribution of GC3 with regression line slope close to zero is found (Yang et al., 2014). We observed diagonal distribution of few points and concentrate distribution of GC3 which suggests that either selection or mutation might affect on the CUB of ND genes in avian (Uddin et al., 2017). Neutrality plot of GC12 of GC3 was drawn (Figure 6) to evaluate the degree of selection force towards the CUB of ND genes among avian. The regression coefficient of different ND genes were estimated as ND1 (0.0947), ND2 (0.2107), ND3 (0.0287), ND4 (0.0368), ND4l (0.0274), ND5 (0.0834) and ND6 (0.0802) and the result revealed the value less than 0.5 *i.e.* approaching towards zero, suggesting the major control of natural selection towards CUB over mutation pressure in mitochondrial complex I genes of RC among avian (He et al. ,2016: Wei Lei et al., 2014a; Zhao Yongchao et al., 2016).

3.9. Codon context patterns among ND genes of avian

The codon context patterns were evaluated across the protein-coding ND genes of the selected avian species. The relative high frequency and common feature of codon context patterns were observed in ND1, ND2, ND4 and ND5 genes, while low frequency of codon context in ND3, ND4L and ND6 genes (Figure 7).

3.8. Association between SCUO and protein properties

Researcher reported that properties of proteins like hydrophobicity and aromaticity contribution affecting CUB of genes (Sablok et al., 2011). Significant correlation was detected among protein lengthin ND genes and various factors including SCUO, GRAVY, protein length, hydrophilicity and aromaticity with the exceptionof ND6 and ND5gene (with protein length and hydrophilicity) (Table 4). Our results recommended that hydrophobicity and aromaticity could play a part in the CUB of ND genes among avian (Uddin and Chakraborty, 2016).

3.9. Relationship between SCUO and skewness

Transcription process is correlated with the composition of nucleotide skewness (Beletskii and Bhagwat, 2001).The compositional properties, including GC, AT, keto, amino, purine, and pyrimidine skews, were calculated for complex I genes of avian. We performed correlation analysis between SCUO and various skews to point out the impact of skews on codon usage bias of the gene. In our study, a highly significant correlation was detected between SCUO and various skews (Table 5), which implied that skewness might have influenced the codon usage bias of complex I genes of RC among avian (Uddin and Chakraborty, 2015).

3.8. Phylogenetic analysis

The maximum-likelihood method was employed to construct the phylogenetic tree based on nucleotide sequence alignment of the protein coding genes of mitochondrial ND1 among different orders of avian species (Figure 8). For phylogenetic analysis, we selected the mitochondrial ND1 gene because it is a well-established marker in avian evolutionary studies, providing an ideal balance between conserved regions that enable reliable sequence alignment and variable sites that offer sufficient phylogenetic resolution across taxonomic levels (García-Moreno et al., 2003; Uddin et al., 2018).The tree depicts the evolutionary relationships with 98% of bootstrap support confirming significant clades among different species of avian belong to diverse orders. Moreover, we also constructed phylogenetic tree between different species of avian belong to same order and represented in Figure 9.These trees revealed major clades among closely related avian species belong to same order with 100% bootstrap values confirming high reliable patterns of branch.

4. Discussion

This study explained the compositional features and codon usage trend in the coding region of the mitochondrial ND genes among avian. The first complex of respiratory chain is represented by the mitochondrial ND genes and engaged in manufacturing of energy (ATP). The phenomenon of CUB is a complicated evolutionary process which varies from minor (prokaryotes) to higher (eukaryotes) organism. The most accepted theories of CUB in different organisms were reported as theory of neutral and selection–mutation–drift. According to theory of selection-mutation drift, there should be a balanced through the combined influence of natural selection, mutation pressure, and genetic drift, which determines the CUB of different organisms. Neutral theory on the other hand states that mutation in the wobble base is neutral resulting from random use of synonymous codons. The completion of genome sequencing in multiple organisms shed a light on the above mentioned theories which were found to be inadequate to determine the CUB origin (Yang et al., 2014). Several factors like gene length, hydrophobicity, aromaticity, recombination rate, extent of gene expression, environmental stress also acts as a key factor in the CUB of different organisms (Berg, 1996; Orešič et al., 2003; Prat et al., 2009b; Romero et al., 2000).

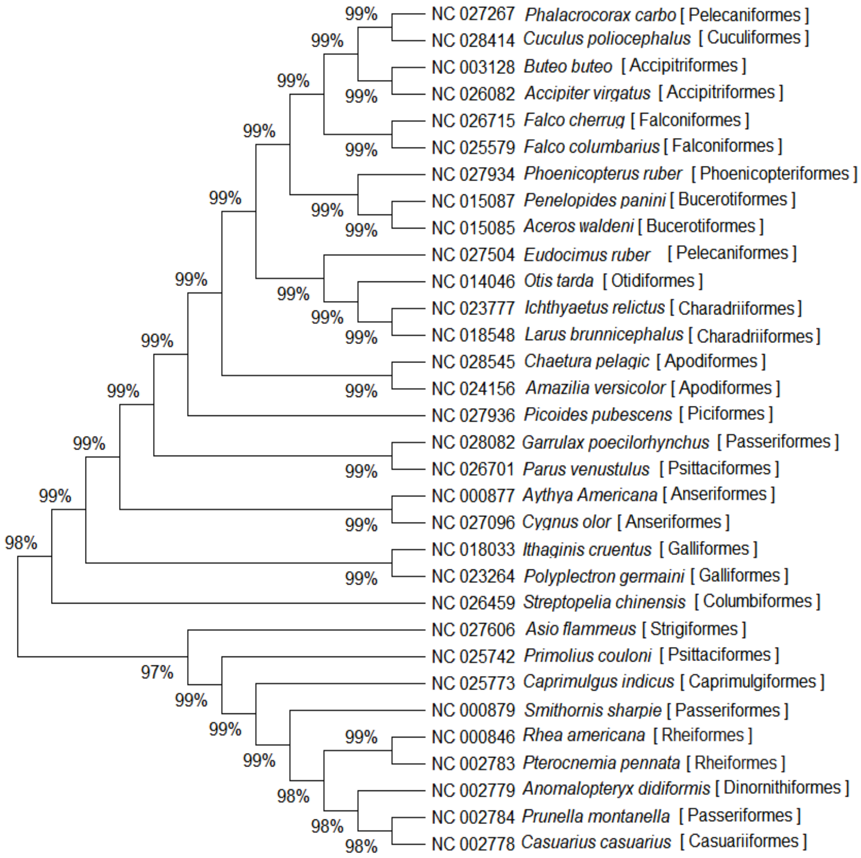


Figure 8. Phylogenetic tree was constructed based on nucleotide sequence alignment of the protein coding genes of mitochondrial ND1 among different orders of bird species. . The phylogeny was inferred using the Maximum Likelihood method and Tamura-Nei (1993) model. Evolutionary analyses were conducted in MEGA12.

Directional measure of CUB is represented by SCUO while the non-directional measure of CUB is represented by effective number of codon (ENC). In our study, SCUO values for ND genes were established to be low, which suggested low CUB in these genes (Uddin et al., 2019). Previously, our study showed similar result in other mitochondrial genes in different vertebrates. The average ENC values (Mean ±SD) of ND1 genes among the species of pisces, aves and mammals were 40.86±3.79,40.49±1.90, 38.20±2.67respectively (Uddin et al., 2017). However, the values of ENC for COI genes were calculated as 47.9, 40.9, 46.1 and that of COII genes were 42.3, 37.2, 41.2. Moreover, ENC for COIII genes were found as 47.0, 44.1 and 48.0 among pisces, aves and mammals respectively (Uddin et al., 2019).

Compositional constraints generally influence the codon usage patterns in the genome of an organism (Bernardi, 1986). As the wobble hypothesis depicts that the third position of codon always varies among organisms, so the nucleotide composition at the third position of codon is always a good index for measuring the degree of base composition bias. Within the genome of an organism, the preference for A or T ending codon is displayed by the genes is rich in AT regions while the preference for G or C ending codon is displayed by GC rich regions (Mirsafian et al., 2014). The overall nucleotide composition analysis for the mitochondrial ND1 genes yielded higher AT content in comparison to GC. However, our previous study on ND1 & ND2 gene reported the occurrence of high frequency of nucleotide A at 3rd position of codon whereas the frequency of the nucleotide G was the lowest among fishes, avian and mammals (Uddin et al., 2015). A major determinant influencing codon usage patterns could be the background nucleotide composition of genes or genomes of organisms. In our previous study, we observed that in case of aves and pisces the frequency of nucleotide C was greater in percentage while for mammals the frequency of A nucleotide was greater and G was found to be lowes. Moreover, we discovered that in ND1 gene, the nucleobase A3 and C3 at the third codon position was highest while G3 being the lowest (Uddin et al., 2017). It was reported in some earlier studies that GC3 (Guanine and Cytosine at 3rd position) of codon might be an important indicator to get to know the extent of base compositional bias (Shen et al., 2015). Besides this, Tatarinovaet, al., (2010) reported that genes with elevated GC3 content might be prone to methylation as compared to gene with lower content of GC3. Codon usage bias across the genomes of several organisms may arise from the equilibrium between mutation pressure and translational/natural selection (Butt et al., 2016). Study in mitochondrial genomes of ribbon worm stated that mutation pressure is the major influencing factor of the CUB (Chen

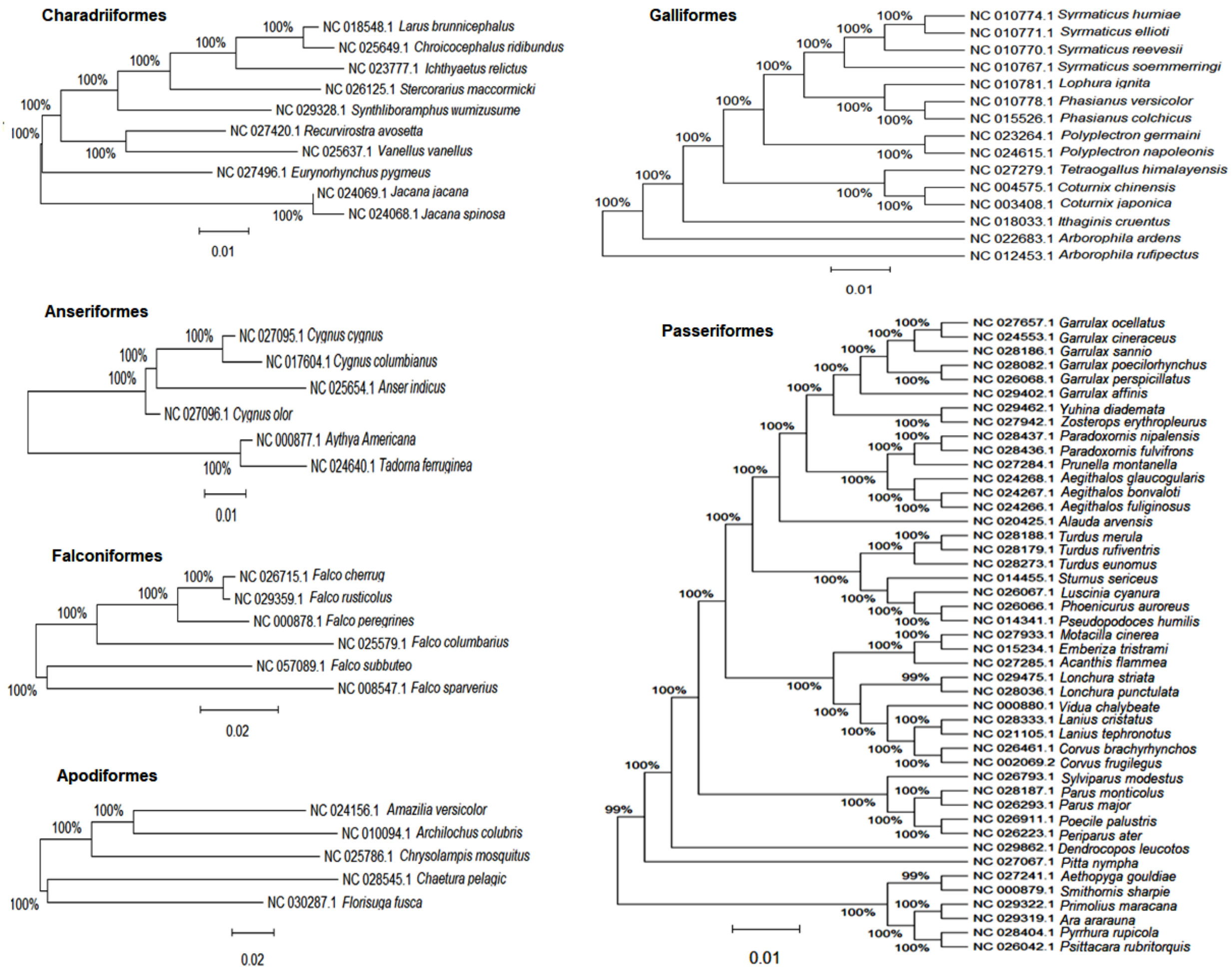


Figure 9. Phylogenetic tree between different species of avian belong to same order. Evolutionary analyses were conducted in MEGA12.

Haixia et al., 2014) and the similar finding was also reported in *Bombyx mori* (Wei et al., 2014b). Therefore, evaluate the contribution of mutation pressure and natural selection in the coding sequences of mitochondrial ND gene among avian, we did several analyses in a stepwise manner. Correlation analysis was carried out between the nucleotide composition of overall genes and the corresponding composition of codons at the 3rd position.

The results of this analysis revealed significant positive correlation exists between 3rd position of codon and overall composition of nucleotide. We therefore, hypothesized that those compositional constraints, which were resulted from the combined effects of natural selection and mutation pressure on the codon usage patterns of the mitochondrial ND gene among avian.

We further calculated RSCU values of all the 60 codons for ND genes among avian. We then elucidated over-represented and under-represented codons for each of these genes. From our observation, we noticed that CAA codon was absent in ND6 gene, while, ACG and GCG codons were absent in ND3 gene. The CGG codon was identified as over-represented in ND6 gene while the same was detected as under-represented in other genes. In our earlier study of pisces, we found that in MT-ND1 gene, the frequently used codons were 27 (A- ending 13 and C-ending 14). Similar pattern was noted in aves with 28 codons (A-ending 13 and C-ending 15) as well as in mammals (14 A and 14 C-ending). In some species, these codons were used as frequent codons while in some other species they acted as rare codons or vice-versa. However, in this previous work of pisces, aves and mammals, out of 60 codons, only 6 codons which includes CGA, CTA, CAA, TGA, GCC and AAA were predicted as over-represented (RSCU>1.6),and the under-represented codons (RSCU<0.6) were estimated as CAG, TGG, TCG, AGT, TTG, TGT, CCG, CAT, ACG, AAG, GTG, GGT,GCG, and GAG (Uddin et al., 2017). While, from our previous work on CO genes, the TCA codon (encodes Serine) was observed as over-represented in pisces, avian and mammals for COI gene but in case of COII gene, the same was detected as over-represented only in avian and mammals excluding the pisces. The CAA codon (encodes glutamine) which was over-represented for COI gene in avian and mammals, whereas it showed overrepresentation across pisces, avian and mammals in COII and COIII gene. Similarly, for the COI gene, the TGC codon (encodes cysteine) was observed as over-represented only in pisces and avian. Moreover, the three codons which include CGA, AAA, and CTA (encodes arginine, lysine and leucine amino acid respectively) were over-represented throughout the genes of COI, COII and COIII in all three groups. Besides, TGA encoding tryptophan amino acid was over-represented in pisces, avian and mammals for COI, COII. However, this pattern was not observed in the COIII genes of pisces (Uddin et al., 2019).

The correspondence analysis (COA) which was based on the values of RSCU was performed to investigate the major trends in codon usage variation. The results indicated that the principal axis (f_1) accounted for major variation (%) than all other axes within the gene set. The positions of most of the codons among bird species were found to be closer to the axes with a very concentrate distribution, which suggested that mutation bias might play a pivotal role in shaping the codon usage patterns of mitochondrial ND genes among avian. However, discrete distribution of few codon indicating that the factors like natural selection have played a key role in codon usage of genes involved. The result was similar to our earlier findings on ND1 and ND2 genes (Uddin et al., 2015) as well as the findings on human serotonin receptor gene family (Dass and Sudandiradoss, 2012).

Further to comprehend the influence of natural selection and mutation pressure in the CUB of ND1 genes among avian, neutrality plot analysis was carried out. Alternation of base in third position of codon generally leads to synonymous substitution of codons while changes in first and second base positions might relate to non-synonymous substitutions. Non-synonymous alterations are less preferred in nature for the conservation of gene function. Hypothetically, mutation should occur randomly in absence of any external force. The preference of base in a codon might be varied in presence of selection pressure. On analysis of neutrality between GC12 on GC3 contents, the impact of natural selection against mutational bias can be predicted across the gene. Here, the regression coefficient value of the plot depicts the condition of equilibrium between mutation-selection pressure (Sueoka, 1988). In this plot, there exists a significant correlation between GC12 and GC3 content when a gene is placed in the slope of unity, which indicates that the gene may be evolving under neutral mutations driven by random selective forces. However, if the gene is subject to directional mutation pressure, it would shift below the slope of unity, positioning closer to the X-axis and farther from the Y-axis. Thus, a regression line with a slope less than 1 suggests non-neutral mutation pressure influencing codon usage among different genes of the same genome (Necsulea and Lobry, 2006; Sueoka and Kawanishi, 2000). The neutrality plot demonstrated the equilibrium coefficient between mutation and selection pressure. Again in our analysis, several points aligned along the diagonal, with GC3 values confined to a narrow range, indicating the combined influence of mutation pressure and natural selection (Sueoka, 1988). Furthermore, the regression coefficient was used to quantify the extent of natural selection and mutation pressure. In the mitochondrial ND gene, the regression coefficient of GC12 against GC3 was found to be less than 0.5, which further suggested that natural selection played a more dominant role than mutation pressure in the CUB of Mitochondrial ND genes among avian. The result was similar in our earlier study on ND1 and ND2 gene among fishes, avian and mammals (Uddin et al., 2015).Our earlier work for ND1 gene showed the regression coefficient between GC12 on GC3 was 0.109 in pisces, 0.0809 in aves and 0.079 in mammals which suggested the higher value of relative constraint than that of the relative neutrality. This revealed the dominant role of selective pressure over mutation for the mitochondrial ND1 gene in pisces, aves and mammals (Uddin et al., 2017). In CO genes, the slopes of the regression line *i,e*., regression coefficient for COI, COII and COIII gene in pisces were 0.044, 0.05 and 0.052 respectively. Similarly, in aves the regression coefficients were 0.044, 0.035 and 0.148 while in mammals the regression coefficients were 0.031, 0.116 and 0.051 for COI, COII and COIII gene respectively. Therefore, the major role of natural selection over mutation pressure in the codon usage of CO genes among pisces, aves and mammals was reported (Uddin et al., 2019) supporting our present work.

Furthermore, a phylogenetic tree was generated using the maximum-likelihood method based on protein coding genes of mitochondrial ND1 among different orders of bird species. The tree clearly depicts the older order Casuariiformes and Rheiformes along with the extinct Dinornithiformes cluster with a boot strap value of 98-99 percent. Interestingly, the bird species *Prunellamontanella* of Passeriformes clustered same clad with *Casuariuscasuarius* of Casuariformes. The placement of waterbird lineages such as Anseriformes (e.g., *Aythyaamericana*, *Cygnusolor*) at the base of the tree suggests that they diverged early from the rest of the Neoaves. Predatory groups like Accipitriformes and Falconiformes appear close together, reflecting their shared ecological strategies. Similarly, Pelecaniformes and Phoenicopteriformes emerge as separate but related branches, consistent with their ties to aquatic habitats. In contrast, land bird groups such as Passeriformes, Piciformes, and Psittaciformes cluster toward the more derived parts of the tree, indicating more recent evolutionary diversification. Taken together, the tree supports the established picture of avian evolution, showing a clear split between water bird and land bird lineages, along with strong internal clustering that aligns well with ecological roles and morphological traits.

Nevertheless, we also performed phylogenetic analysis of ND1 gene among same orders of different species. The results of our analysis revealed that the avian belong to Charadriiformes orders are supported with two major clades: one comprising gulls and their relatives (Larus, Chroicocephalus, Ichthyaetus, Stercorarius, Synthliboramphus), and the other including shore/waders (Recurvirostra, Vanellus, Eurynorhynchus, Jacana). The tree highlights Jacana species as a distinct sister group, while Vanellus and Recurvirostra share a closer evolutionary relationship with 100% bootstrap support.

Likewise, the phylogenetic tree of Galliformes order shows clear genus-level clustering, with Syrmaticus, Phasianus, Polyplectron, and Coturnix forming distinct clades, while Arborophila represents the most basal lineage, indicating early divergence. Overall, the relationships are strongly supported, highlighting well-defined evolutionary separation among the major groups.

In parallel, the phylogenetic tree of Anseriformes display high nodal confidence for the grouping of the avian. The tree further reveals species *Cygnuscygnus* and *Cygnuscolumbianus* as close relatives clustering with *Anserindicus*. Also, a tight cluster formation is observed between species *Aythyaamericana* and *Tadornaferruginea* which highlights their evolutionary split between the lineages of swan-goose and duck-shelduck.

The Passeriformes avian phylogenetic tree shows well-resolved relationships with 99–100% bootstrap support, confirming reliable branching. Clustering of species into distinct clades like Garrulax and Turdus compared to higher groups like Corvidae, Paridae, and Psittaciformes,reflects strong phylogenetic differentiation and evolutionary diversification. The phylogenetic tree of Falconiformes avian indicates that *Falco cherrug*, *F. rusticolus*, and *F. peregrinus* are the most closely related species, while *F. columbarius* and *F. subbuteo* diverge later, and *F. sparverius* is the earliest branching lineage, representing the most distinct evolutionary path within this group.

The phylogenetic tree of Apodiformes avian reveals two significant clades: one comprises humming avian (*Amaziliaversicolor*, *Archilochuscolubris*, *Chrysolampismosquitus*, and *Chaeturapelagic*) and another with *Florisugafusca* forming the earliest diverging lineage. The high bootstrap value (100%) indicates strong evolutionary support for these relationships.

Earlier studies have reported phylogenetic analyses based on the complete mitochondrial genome of the Rallidae family within Gruiformes to clarify the evolutionary history and stated that avian Rallidae family diverged in the Eocene, around 40.5 million years ago. Additionally, their phylogenetic analysis resolved relationships within Gruiformes, showing Rallidae as sister to Heliornithidae, with this clade sister to Gruidae, providing a clearer understanding of avian evolutionary branching (García-Moreno et al., 2003).

Lan et al (2024) analysed the 16,723 bp mitochondrial genome of *Tarsiger indicus*, which showed conserved avian features in gene arrangement and codon usage. The analysis showed purifying selection acting across all 13 protein-coding genes. Phylogenetic analyses supported the common ancestry of Muscicapidae, Turdidae, and Paradoxornithidae. The study confirmed that *T. indicus*, along with *T. cyanurus* and *Monticolagularis*, belongs to Muscicapidae, while species like *Paradoxornisheudei* are placed in Paradoxornithidae, providing valuable insights into passerine evolution (Lan et al., 2024).

In this study, phylogenetic analyses were conducted on the mitochondrial ND1 gene, though ND1 is a well-established and informative marker in avian evolutionary research, dependence on a single mitochondrial locus cannot fully represent genome-wide evolutionary dynamics. Future studies incorporating additional mitochondrial and nuclear genes would provide a more comprehensive framework and help validate the phylogenetic and codon usage patterns identified here.

In this study, the CUB was low and ND genes were AT rich. Similarities and dissimilarities were observed in codon usage in seven ND genes among avian species. Role of mutation pressure and natural selection was not same in different ND genes among the avians.

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Availability of data and material

All the data used in the manuscript are available in the supplementary files.

Conflict of interest

The authors have declared that no competing interests exist.

Authors contribution

All the authors have equally contributed in research design, data generation, preparation of manuscript and final submission to this journal.

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