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Complete mitochondrial genome of *Chroicocephalus brunnicephalus* from India: phylogeny with other Larids

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ABSTRACT

The complete mitogenome sequence of the brown-headed gull, *Chroicocephalus brunnicephalus* was determined in this study. The 16,771 bp genome consists of 13 protein-coding genes (PCGs), two ribosomal RNA (*rRNA*) genes, and 22 transfer RNA (*tRNA*) genes, and a control region (CR). The decoded mitogenome was AT-rich (54.77%) with nine overlapping and 17 intergenic spacer regions. Most of the PCGs were started by a typical ATG initiation codon except for *cox1* and *nad3*. Further, the usual termination codons (AGG, TAG, TAA, and AGA) were used by 11 PCGs except for *cox3* and *nad4*. The concatenated PCGs based Bayesian phylogeny clearly discriminates all the Laridae species and reflects the sister relationship of *C. brunnicephalus* with *C. ridibundus*. The present mitogenome-based phylogeny was congruent with the earlier hypothesis and confirmed the evolutionary position of the brown-headed gull as masked species. The generated mitogenome of *C. brunnicephalus* is almost identical to the previously generated mitogenome from China except for two base pairs in CR. To visualize the population structure of this migratory species, we propose more sampling from different geographical locations and the generation of additional molecular data to clarify the reality.

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1. Introduction

The family Laridae (order: Charadriiformes) comprises 105 known species under 20 genera globally (BirdLife International 2020). Among them, 38 species under 17 genera were recorded from Indian coastal or inland (Praveen et al. 2020). They are medium to large-sized shorebirds, commonly known as gulls. Typically they are white or gray in color with webbed feet and have typical harsh wailing calls. Among all extant gulls, the brown-headed gull *Chroicocephalus brunnicephalus* usually migrate from China to their wintering localities in Thailand and Cambodia; while Bangladesh, India, Myanmar, and Vietnam being their major stopover locations during such migrations. They can travel an average distance of about 2400 km from their breeding grounds and take one to two weeks to cover this distance (Ratanakorn et al. 2012). Due to habitat loss, over-fishing, and other anthropogenic pressures, the population trend of many gulls is declining throughout the world including India (Aarif et al. 2014). Furthermore, *C. brunnicephalus* is also reported to be a host of highly pathogenic avian influenza *H5N1* virus and could be a potential carrier for the outbreak within Southeast Asian countries and act as an environmental bio-indicator (Ratanakorn et al. 2012; Hasan et al. 2014).

Owing to the convergent plumage evolution, the taxonomic approach often flunks to resolve the phylogenetic relationship of Charadriiformes birds (Crochet et al. 2000). Later on, the genetic analyses evidenced that, the order Charadriiformes can be classified into three major clades (Paton and Baker 2006; Fain and Houde 2007; Livezey 2010). However, the evolutionary relationship of this group is still perplexing while examining in-depth phylogenetic analysis. Both mitochondrial and nuclear genetic information were well studied to know the systematics status, evolutionary relationships, and population structures of Charadriiformes birds (Liebers et al. 2001; Crochet et al. 2003; Ericson et al. 2003; Paton et al. 2003; Thomas et al. 2004; van Tuinen et al. 2004; Pons et al. 2005; Baker et al. 2007). The comparative genomics is also employed for estimating the genetic variability, substitution pattern, phylogenetic relationship, and evolution of avifauna (van Tuinen et al. 2001; Paton et al. 2002; Backström et al. 2008; Hackett et al. 2008; Künstner et al. 2010). As of now, 47 complete mitochondrial genomes of Charadriiformes birds are available in the GenBank database. Among them, 13 mitogenomes of 11 Laridae species were generated from different geographical regions (Slack et al. 2007; Ryu and Hwang 2012; Yang et al. 2012, 2016, 2017; Yoon et al. 2015; Dong et al. 2016; Kim and Park 2016;

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Anmarkrud and Lifjeld 2017; Skujina et al. 2019). The analysis of the complete mitogenomes was also evidenced to characterize various genomic traits within protein-coding genes (PCGs), ribosomal RNA (rRNA), transfer RNA (tRNA), and control region (CR) as well as to detect gene order evolution in birds (Bensch and Härlid 2000; Crochet and Desmarais 2000; Ruokonen and Kvist 2002; Pacheco et al. 2011). Hence, this study is aimed to generate the complete mitochondrial genome of the brown-headed gull from India and estimate the phylogeny with other Laridae species to determine the evolutionary relationships.

2. Materials and methods

A naturally dead adult specimen of *C. brunnicephalus* was collected on 27 February 2019 from the seashore of Coringa Wildlife Sanctuary (16.58 N 82.30 E) in Andhra Pradesh, India (Figure 1(A)). As the sample was naturally dead, no prior permission was acquired for this biological sampling. The live photograph of the same species was captured from the same locality. The country-level map has been downloaded from the spatial data platform and overlaying by DIVA-GIS 7.5 software (http://www.diva-gis.org). The muscle tissue was collected from the carcass specimen in a sterile condition and stored in 70% ethanol at −80 °C in the National Zoological Collections of Bird Section, Zoological Survey of India, Kolkata under voucher No. 41301/AVES.

The collected tissue sample was thoroughly chopped by the surgical blade and re-suspended in 200 µl of buffer (50 mM Tris–HCl, 25 mM of EDTA, and 150 mM NaCl), with the addition of 20 µl of proteinase K (20 mg/ml) followed by incubation at 56 °C for overnight. The sample was further religiously vortex with 10 ml buffer (0.32 M Sucrose, 1 mM EDTA, 10 mM Tris–HCl) and centrifuged at 700 × *g* for 5 min at 4 °C to remove nuclei and cell debris. The supernatant was collected in 1.5 ml Eppendorf tubes and centrifuged at 12,000 × *g* for 10 min at 4 °C to precipitate the mitochondrial pellet. The mitochondrial DNA was extracted by Qiagen DNeasy Blood & Tissue Kit (QIAGEN Inc., Germantown, MD)..

The complete mitochondrial genome was obtained commercially and the high-quality paired-end data was assembled through NOVOPlasty version 2.6.7 with default parameters (Dierckxsens et al. 2017). The genome library was sequenced using the Illumina platform (2 × 150 bp PE chemistry), to generate ~6 GB data (Illumina, Inc, San Diego, CA). The raw reads were screened using cutadapt tool (http://code.google.com/p/cutadapt/) and low-quality bases were trimmed with a cutoff of Phred quality score (Q score =20). The *cox1* sequence of the same species (accession no. NC_018548/JX155863) was used as a reference seed sequence for the present assembly. To confirm the assembly, a similarity search was carried out in the GenBank database using BLASTn version 2.2.28 search (https://blast.ncbi.nlm.nih.gov) algorithm.

The spherical representation of the generated mitogenome of *C. brunnicephalus* was plotted by CGView Server (http://stoth ard.afns.ualbe rta.ca/cgview_server/) with default parameters (Grant and Stothard 2008). The strand direction and arrangements of each PCG, tRNA, rRNA, and CR were checked through MITOS version 806 online web server (http://mitos.bioinf.uni-leipzig.de) (Bernt et al. 2013). The overlapping regions and intergenic spacers between the neighbor genes were counted manually through Microsoft Excel. The sequences of PCGs were translated into the putative amino acid sequences on the basis of the vertebrate mitochondrial genetic code. The initiation and termination codons were identified in ClustalX using other publicly available reference sequences of Laridae (Thompson et al. 2003). The mitogenome sequence was submitted to the GenBank database through the Bankit submission tool. The mitogenome size and nucleotide composition were calculated using MEGA version 6.0 (Tamura et al. 2013).

On the basis of homology search in the Refseq database (https://www.ncbi.nlm.nih.gov/refse q/), 11 Laridae species mitogenomes were downloaded from GenBank and incorporated in the dataset for their phylogenetic relationships. The PCGs were aligned individually by codons using MAFFT algorithm in TranslatorX with L-INS-i strategy with GBlocks parameters (Abascal et al. 2010). The dataset of all PCGs was concatenated using SequenceMatrix version 1.7.84537 (Vaidya et al. 2011). The best suitable model (GTR + G + I) for phylogenetic analysis was calculated by PartitionFinder 2 (Lanfear et al. 2016) at CIPRES Science Gateway version 3.3 (Miller et al. 2015). The Bayesian analysis (BA) was performed through Mr. Bayes version 3.1.2 and the metropolis-coupled Markov Chain Monte Carlo (MCMC) was run for 100,000,000 generations with sampling at every 100th generation and 25% of samples were discarded as burn-in (Ronquist and Huelsenbeck 2003). The BA phylogeny was further illustrated in iTOL version 4 (https://itol.embl.de/login.cgi) and edited with Adobe Photoshop CS version 8.0. The database sequence of *Gallus gallus* (order Galliformes) was as outgroup taxa in the phylogenetic analysis.

3. Results and discussion

The complete mitochondrial genome of *C. brunnicephalus* was determined by using next-generation sequencing approach. The total length of the decoded mitogenome was 16,771 bp and deposited with the accession number (MT876573/NC_050864) in the GenBank database. The complete mitogenome contains 13 PCGs, 22 tRNAs, two rRNAs, and a CR as depicted in other avian mitogenomes (Figure 1(B), Table S1). A total of nine genes (*nad6* and eight tRNAs) were located on the light strand, while the other genes were encoded on the heavy strand. The overall base-composition of this mitogenome was 30.72% A, 14.13% G, 31.10% C, and 24.04% T. The AT and GC content of the complete mitogenome was 54.77 and 45.23%, respectively. A total of nine overlapping regions with a total length of 34 bp were identified. These sequences varied in length (1–10 bp) with the longest overlapping region present between ATP synthase F0 subunit 8 (*atp8*) and ATP synthase F0 subunit 6 (*atp6*). Further, the intergenic spacers spread over 17 regions ranging from 1 to 19 bp with a total length of 68 bp. The longest spacer (19 bp) occurred between NADH dehydrogenase

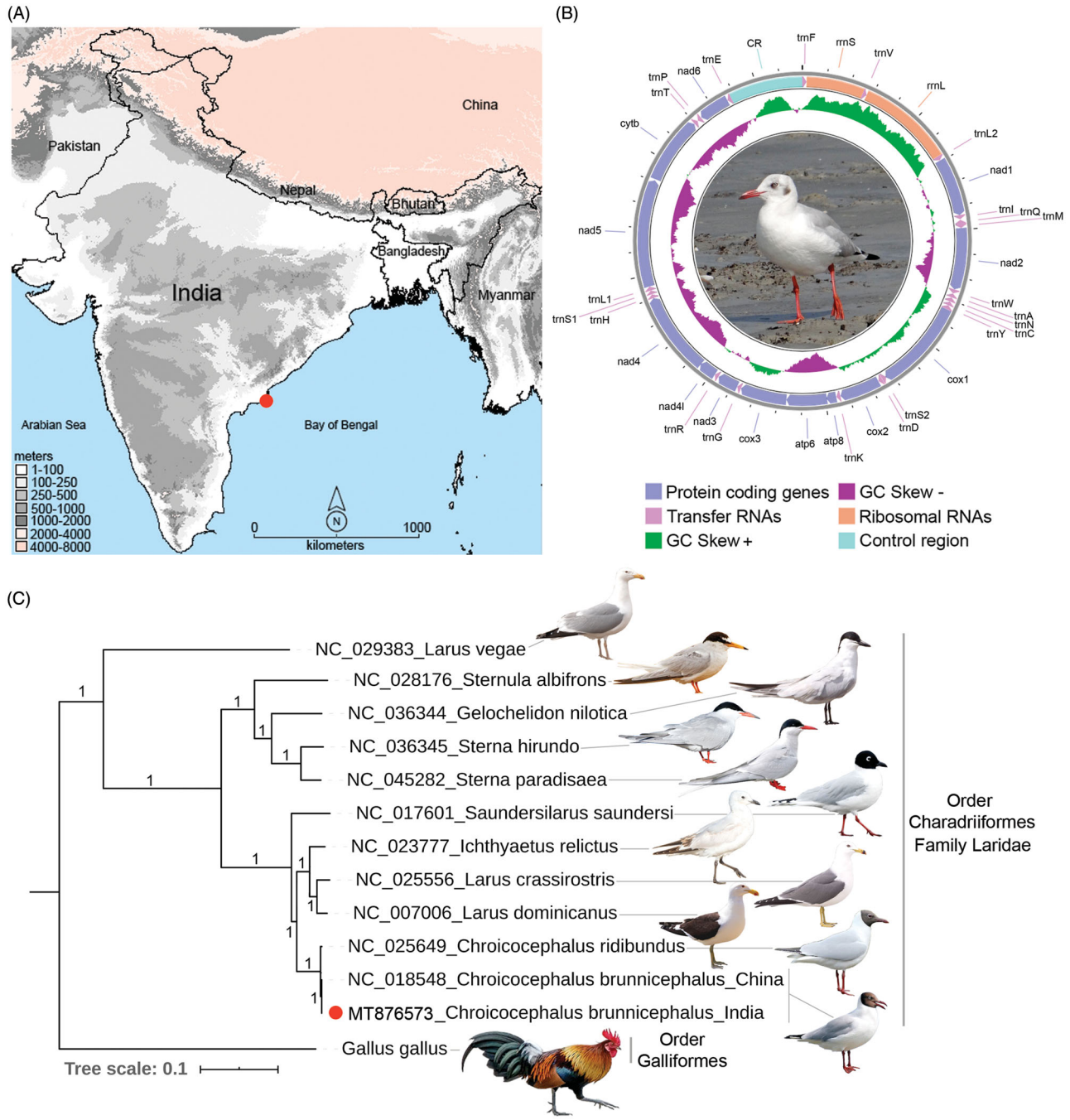

Figure 1. (A) Map showing the collection locality of *C. brunnicephalus* from the southern coast of India. (B) The spherical representation of mitochondrial genome of *C. brunnicephalus*. Direction of gene transcription is indicated by arrows. PCGs are shown as blue arrows, *rRNA* genes as coral arrows, *tRNA* genes as orchid arrows, and non-coding control region as mint rectangle. The GC-skew is plotted using green and violet color sliding window as the deviation from the average in the complete mitogenome. The species photograph was taken by the third author (G.M.) (C) The BA phylogeny based on the concatenated nucleotide sequences of 13 PCGs showing the evolutionary relationship of the studied taxa with other Laridae species. The posterior probability support values were superimposed with each node. The figure was edited with the representative species photograph acquired from the internet using Adobe Photoshop CS version 8.0.

subunit 5 (*nad5*) and Cytochrome b (*Cytb*) (Table S1). Most of the PCGs were started by the typical ATG initiation codon with an exception in Cytochrome oxidase subunit I (*cox1*) with GTG and NADH dehydrogenase subunit 3 (*nad3*) with ATT. The 11 PCGs used usual termination codons (AGG, TAG, TAA, and AGA), except for Cytochrome oxidase subunit III (*cox3*) and NADH dehydrogenase subunit 4 (*nad4*) with incomplete termination codon (T) (Table S1).

The mitogenome based BA phylogeny clearly discriminates all the studied Laridae species with high posterior probability supports (Figure 1(C)). Both Indian and Chinese *C. brunnicephalus* sequences were cohesively clustered in the BA tree and reflected the sister relationship with the Black-headed gull, *Chroicocephalus ridibundus*. The *C. brunnicephalus* was thought to be a valid species under genus *Larus* from long back and later on shifted under *Chroicocephalus* genus based on the mitochondrial markers based phylogeny (Pons et al. 2005). This taxonomic revision was further accorded by the subsequent studies (Sternkopf 2011; Liebers-Helbig 2013). The present complete mitochondrial genome-

based phylogeny elucidates congruent results with the earlier hypothesis (Pons et al. 2005) and confirmed their evolutionary position as masked species.

The genetic applications are largely employed in the conservation of avifauna around the world (Haig et al. 2011). It is also detected that the genetic composition of birds is often altered linked with their fragmented habitats (Brown et al. 2004). Further, it is also noticed that the migratory species acquired their energy *via* mitochondrial adaptations and oxidative phosphorylation during their locomotion (Toews et al. 2014). It is also evident that locomotion plays an important role in the evolution of mtDNA and independently evolves different lineages (Pulido 2007; Shen et al. 2009). The molecular study further suggested the gene flow controlling the expression of migratory behavior of birds (Mueller et al. 2011; Pons et al. 2014). This analysis elucidates that the nucleotide composition of the Indian *C. brunnicephalus* mitogenome, sampled from the Southern coast is almost identical to the Chinese mitogenome (accession no. NC_018548/JX155863) except for two base pairs in CR. The genetic information of these two isolates is important and could be adopted for population genetics studies of this Laridae species in near future. However, we suggest more sampling from different geographical locations and subsequent generation of more molecular data to clarify the mitochondrial introgression and function of this migratory species.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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Data availability statement

The complete mitochondrial genome data that support the findings of this study are openly available in NCBI GenBank database at (https://www.ncbi.nlm.nih.gov) with the accession number (MT876573/NC_050864) which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

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