Complete mitochondrial genomes of five raptors and implications for the phylogenetic relationships between owls and nightjars

Gang Liu [Corresp., 1], **Lizhi Zhou** [2], **Guanghong Zhao** [2]

[1] School of Life Sciences, Anhui Medical University, Hefei, Anhui, China

[2] School of Resources and Environmental Engineering, Anhui University, Hefei, Anhui, China

Corresponding Author: Gang Liu
Email address: liugang8966@163.com

The phylogenetic relationships between owls and nightjars are rather complex and controversial. To clarify these relationships, we determined the complete mitochondrial genomes of *Glaucidium cuculoides*, *Otus scops*, *Glaucidium brodiei*, *Caprimulgus indicus*, and *Strix leptogrammica*, and estimated phylogenetic trees based on the complete mitochondrial genomes and aligned sequences from closely related species that were obtained in GenBank. The complete mitochondrial genomes were 17392, 17317, 17549, 17536, and 16307 bp in length. All mitochondrial genomes contained 13 protein-coding genes, two rRNAs, 22 tRNAs, and a putative control region. All mitochondrial genomes except for that of *Strix leptogrammica* contained a pseudo-control region. ATG, GTG, and ATA are generally start codons, whereas TAA is the most frequent stop codon. All tRNAs in the new mtDNAs could be folded into canonical cloverleaf secondary structures except for $tRNA^{Ser\ (AGY)}$ and $tRNA^{Leu\ (CUN)}$, which missing the “DHU” arm. The phylogenetic relationships demonstrated that Strigiformes and Caprimulgiformes are independent orders, and Aegothelidae is a family within Caprimulgiformes. The results also revealed that Accipitriformes is an independent order, and Pandionidae and Sagittariidae are independent families. The results also supported that Apodiformes is polyphyletic, and hummingbirds (family Trochilidae) belong to Apodiformes. Piciformes was most distantly related to all other analyzed orders.

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Authors' names:

Gang Liu[1,2], Lizhi Zhou[1], Guanghong Zhao[1]

Authors' affiliations:

1. Institute of Biodiversity and Wetland Ecology, School of Resources and Environmental Engineering, Anhui University, P. R. China

2. Department of Biology, School of Life Sciences, Anhui Medical University, P. R. China

Corresponding author: Lizhi Zhou (zhoulz@ahu.edu.cn)

Address:

1. School of Resources and Environmental Engineering, Anhui University, 111 Jiulong Road, Hefei 230601, P. R. China

2. Department of Biology, School of Life Sciences, Anhui Medical University, 81 Meishan Road, Hefei 230032, P. R. China

Tel: +86-551-63861752

Fax: +86-551-63861724

ABSTRACT

The phylogenetic relationships between owls and nightjars are rather complex and controversial. To clarify these relationships, we determined the complete mitochondrial genomes of *Glaucidium cuculoides*, *Otus scops*, *Glaucidium brodiei*, *Caprimulgus indicus*, and *Strix leptogrammica*, and estimated phylogenetic trees based on the complete mitochondrial genomes and aligned sequences from closely related species that were obtained in GenBank. The complete mitochondrial genomes were 17392, 17317, 17549, 17536, and 16307 bp in length. All mitochondrial genomes contained 13 protein-coding genes, two rRNAs, 22 tRNAs, and a putative control region. All mitochondrial genomes except for that of *Strix leptogrammica* contained a pseudo-control region. ATG, GTG, and ATA are generally start codons, whereas TAA is the most frequent stop codon. All tRNAs in the new mtDNAs could be folded into canonical cloverleaf secondary structures except for $tRNA^{Ser\ (AGY)}$ and $tRNA^{Leu\ (CUN)}$, which missing the "DHU" arm. The phylogenetic relationships demonstrated that Strigiformes and Caprimulgiformes are independent orders, and Aegothelidae is a family within Caprimulgiformes. The results also revealed that Accipitriformes is an independent order, and Pandionidae and Sagittariidae are independent families. The results also supported that Apodiformes is polyphyletic, and hummingbirds (family Trochilidae) belong to Apodiformes.

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Key words

Strigiformes, Caprimulgiformes, complete mitochondrial genome, phylogenetic relationship

INTRODUCTION

Owls and nightjars arecologically important and are among the best-studied groups of predatory birds. Traditionally, owls are a group of birds that belong to order Strigiformes, and nightjars, which are sometimes known as goatsuckers, belong to order Caprimulgiformes (Konig, 1999; Lane et al, 2004). Most owls and nightjars are solitary and nocturnal birds of prey that are distributed throughout most of the world, except Antarctica and some remote islands (Konig, 1999; Lane et al, 2004). Because of similar morphological features and habitats, the phylogenetic relationships between owls and nightjars are rather complex and controversial; in particular, the phylogenetic position of nightjars has been rearranged several times throughout history (Sibley et al, 1988; Zheng, 1994; Zheng, 2005; Lerner et al, 2008). A major source of conflict is centered around nightjars, and it is debated whether nightjars represent an independent order, Caprimulgiformes, or a suborder, Caprimulgi, within Strigiformes (Sibley et al, 1988; 1990; Zheng, 1994; Zheng, 2005).

Because there are some unique characteristics of morphological features and habits, nightjar classification has long been controversial and difficult (Zheng, 1994; Zheng, 2005). Nightjars have similarities to owls because they are nocturnal predators with a highly developed sense of sight, and swifts, because they are excellent flyers with small, weak legs. Nightjars were traditionally considered morphologically intermediate between owls and swifts; at different points in time, they were thought to be most closely related to owls, swifts, kingfishers, hoopoes, mousebirds, hornbills, rollers, bee-eaters, woodpeckers, trogons, and hummingbirds (Sibley et al, 1988; 1990; Zheng, 1994; Zheng, 2005). Based on morphological, ecological, and behavioral data, nightjars were thought to belong to suborder Caprimulgi within Strigiformes (Sibley et al, 1988; 1990). However, some authors suggested that nightjars should not belong to Strigiformes, but rather represent an independent order, Caprimulgiformes (Wang et al, 1990; Zheng, 2004). However, Caprimulgiformes remains controversial. Aegotheli/Aegothelidae is a major source of conflict at the suborder/family level, and it is debated whether this group represents an independent family (Aegothelidae) or suborder of Strigiformes (Aegotheli) (Sibley et al, 1988; 1990; Zheng, 2004).

Recently, with the development of molecular biological techniques, some authors provided new insight into owl and nightjar phylogenetic

relationships (Sibley et al, 1988; 1990). Sibley et al (1990) and Haring et al (2001) estimated the overall genomic similarity by DNA–DNA hybridization and proposed a new classification of birds In their classification, the Falconiform taxa were placed within an expanded order, Ciconiiformes, which included the infraorders Falconides (including Falconidae and Accipitridae) and Ciconiides (including Ciconiidae with the subfamilies Cathartinae and Ciconiinae) (Sibley et al, 1990; Haring et al, 2001); nightjars were a suborder placed within Strigiformes, and contained Strigida and Caprimugida (Sibley et al, 1990; Haring et al, 2001). However, phylogenetic results based on all tRNA gene sequences differed from those of previous morphological and DNA–DNA hybridization studies. The results did not support that Falconiformes should be placed within order Ciconiiformes or that nightjars should be placed into Strigiformes; instead, the results indicated that Strigiformes and Caprimulgiformes were independent orders (Wang et al, 1990).

Mitochondrial DNA is a powerful, increasingly popular, and widely used molecular marker to estimate animal phylogenetic relationships. It has become a major tool of comparative genomics and plays an important role in phylogenetic, comparative and evolutionary genomics, and molecular evolutionary analyses, because it is maternally inherited, lacks recombination, and has accelerated nucleotide substitution rates

compared with nuclear DNA (Liu et al, 2013; 2014). Analyses of complete mitochondrial genomes provide not only sequence information about structural genomic characteristic analysis, but also data for phylogenetic studies. Consequently, complete mitochondrial genomes are becoming a preferred marker for resolving controversial species relationships, and have become increasingly important for comprehensive evolutionary studies (Lin et al, 2004; Gissi et al, 2008). However, there are only 16 complete mitochondrial genome sequences of owls and nightjars in GenBank; consequently, their phylogenetic relationships remain unresolved.

In this study, we clarified the phylogenetic relationships between owls and nightjars using mtDNA analyses. Our newly completed mitochondrial genomes should provide new insights into the phylogenetic position of some important species, and yield insight into the higher-level systematics of owls and nightjars. We sequenced the complete mitochondrial genomes of five ecologically important owls and nightjars: *Glaucidium cuculoides*, *Otus scops*, *G. brodiei*, *Caprimulgus indicus*, and *Strix leptogrammica*. We also analyzed the nucleotide composition, codon usage, and compositional biases of the mitogenomes. Our phylogenomic analysis elucidates the phylogenetic relationships between owls, nightjars, and other important groups of birds.

MATERIALS AND METHODS

Ethics Statement and Sample Collection

The *G. cuculoides*, *O. scops*, and *G. brodiei* tissue samples were collected from dead birds that were killed by bird repellent at Hefei Xinqiao International Airport, Anhui Province, China. The *C. indicus* sample was collected from a dead bird that was illegally hunted and transported, and was confiscated by Huangpu Mountain Forest Police Station on 7 May 2013, Chuzhou City, Anhui Province, China. The *S. leptogrammica* feather samples were collected from two young rescued birds at the South Anhui National Wild Animal Rescue Centre, Anhui Province, China on June 1, 2012. All samples were deposited at the Institute of Biodiversity and Wetland Ecology, School of Resources and Environmental Engineering, Anhui University (sample codes AHU-RP20160501–20160505). All experimental procedures complied with the current laws on animal welfare and research in China, and were specifically approved by the Animal Research Ethics Committee of Anhui Zoological Society. In addition, *C. indicus* and *S. leptogrammica* were sequenced by our lab and we only published about the sequence structure, but did not use these sequences for phylogenetic analysis.

DNA Extraction, PCR Amplification, and Sequencing

Whole genomic DNA was isolated from samples using the

phenol/chloroform method. Extracted DNAs were examined on a 1.0% agarose/TBE gel and stored at −20°C as templates for PCR. The mtDNA sequences of *Tyto longimembris* (KP893332), *Asio flammeus* (NC_027606), and *Aegotheles cristatus* (NC_011718) were aligned using Clustal X 1.8 (Thompson et al., 1997). Conserved primers were then designed using Primer 5.0, and each pair of primers generated a product with more than 100 bp of overlap. PCR amplifications were referenced to the manual of Trans Taq-T DNA Polymerase (Beijing, China). PCR amplification conditions were refered to Liu (Liu et al. 2017). PCR products were purified and bidirectionally sequenced by Sangon Biotech Co., Ltd. (Shanghai, China).

Genome Annotation and Sequence Analysis

DNA sequences were analyzed using Seqman (DNASTAR 2001), BioEdit, and Chromas 2.22, and then manually adjusted. The boundaries of protein-coding genes (PCGs) and rRNA genes were initially identified via the MITOS and DOGMA webservers, and refined by alignment with mitochondrial genomes of other species of Strigiformes. The 22 tRNA genes were identified using tRNA Scan-SE 1.21 (http://lowelab.ucsc.edu/tRNAscan-SE), with their cloverleaf secondary structures and anticodon sequences determined using DNASIS 2.5 (Hitachi Software Engineering, San Bruno, CA, USA). The complete

mtgenome maps were visualized using the software CGView Comparison Tool (CCT). The complete mitochondrial genome sequences of *G. cuculoides*, *O. scops*, *G. brodiei*, *C. indicus*, and *S. leptogrammica* were deposited in GenBank under accession numbers KY092431, KY471456, MF155890, NC_025773, and KC953095, respectively.

Phylogenetic Analyses

To more extensively and accurately explore phylogenetic relationships between owls and nightjars, we collected complete mtDNA sequences from GenBank of birds that are closely related to owls and nightjars, which included members of Accipitriformes (19 species), Falconiformes (eight species), Apodiformes (four species), Trochiliformes/Trochilidae (five species), Coraciiformes (six species), and Piciformes (six species). All of these species are predatory birds, and have similar morphological features and habits. Phylogenetic trees were estimated using ML and BI methods to study the phylogeny of the 16 owls and nightjars, for which we collected all complete mtDNA sequences that were available in GenBank. The corresponding *Gallus gallus* sequence (NC_001323) was used as the outgroup. Phylogenetic trees were estimated for two cases: one based on the complete mitochondrial genomes of 63 species (Table 1), and the other based on multiple sequence alignment of two rRNA

sequences (12S rRNA and 16S rRNA) from GenBank. Avian species generally exhibit moderate levels of sequence divergence in mitochondrial gene regions, such as 12S rRNA and 16S rRNA; a combination of these two genes is suitable for resolving phylogenetic relationships at different taxonomic levels, which ranges from species to genera and families in raptors. The 107 species contains all the 12S rRNA and 16S rRNA sequences of Falconiformes and Accipitriformes, and all the 19 owls and nightjars in GenBank to this day. It also contains some typical species from Apodiformes (four species), Trochiliformes/Trochilidae (five species), Coraciiformes (six species), and Piciformes (six species).

Phylogenetic analyses were first performed on the individual genes to assess congruence of the phylogenetic signal among genes. Then, all of the sequences were aligned using Clustal X 2.1 as implemented in Mega 5.0 with manual adjustment. The mtDNA sequence files were saved in .meg format to under the each species bird name, and then turned it into .nex format. The optimal substitution model of each partition was determined by Modeltest 3.7, using the corrected Akaike information criterion (AICc). ML and BI phylogenetic trees were reconstructed using PAUP* 4.0b and MrBayes 3.1.2, respectively. The ML and BI trees were constructed refered to Liu (Liu et al. 2017)

RESULTS

Genome Organization and Gene Arrangement in the Five Raptors

The complete mitochondrial genomes of *G. cuculoides*, *O. scops*, *G. brodiei*, *C. indicus*, and *S. leptogrammica* were determined to be 17392, 17317, 17549, 17536, and 16307 bp in length, respectively. All mitochondrial genomes contained 13 PCGs, 22 tRNAs, and a putative control region (CR). All of the mitochondrial genomes except for that of *S. leptogrammica* contained a pseudo-CR (Figure 1). The heavy DNA strand (H-strand) carries most of the genes: 12 PCGs, two rRNAs, and 14 tRNAs. ND6 and eight tRNAs are located on the L-strand.

PCGs

All five raptor mitochondrial genomes in this study contained 13 PCGs (ATP6, ATP8, COI-III, ND1-6, ND4L, and Cyt b) and two rRNAs (12S rRNA and 16S rRNA). The 13 PCGs were similar in length and very conservative in all owls and nightjars. The 13 PCGs represented 68.05% of the mitochondrial genomes. The longest PCG was ND5 (1799, 1827, 1825, 1827, and 1825 bp), which is located between $tRNA^{Leu(CUN)}$ and Cyt b. The shortest was ATP8 (684, 684, 684, 684, and 686 bp), which is between $tRNA^{Lys}$ and ATP6 in the new five mitochondrial genomes. Five start codons (ATG, GTG, ATT, ATC, and ATA) were detected in the 13 PCGs; TAA was the most frequent stop codon, but AGG, TAA, TAG, and

T- were also commonly observed.

Ribosomal RNA, Transfer RNA, and Non-coding Regions

The new mtDNA sequences contained 12S rRNA and 16S rRNA, which are located between $tRNA^{Phe}$ and $tRNA^{Leu}$, and separated by $tRNA^{Val}$. All mitochondrial genomes contained 22 tRNAs, and all tRNAs could be folded into the typical cloverleaf structure, except for $tRNA^{Ser(AGY)}$ and $tRNA^{Leu(CUN)}$ which lack a dihydroxyuridine (DHU) arm. The non-coding regions included some intergenic spacers and a CR. All the first CR located between $tRNA^{Thr}$ and $tRNA^{Pro}$, except *Strix leptogrammica* only contains an extra CR.

Phylogenetic Reconstructions

The ML and BI phylogenetic trees for 63 species shared identical topologies and high node support values for complete mtDNA sequences (Figure 2). The results indicated that the species could be divided into seven branches: Falconiformes, Accipitriformes, Strigiformes, Caprimulgiformes, Apodiformes, Coraciiformes, and Piciformes. Falconiformes and Accipitriformes formed a clade that was sister to a clade that included Strigiformes, Caprimulgiformes, Apodiformes, and Coraciiformes. Strigiformes was the sister clade to Caprimulgiformes, and Apodiformes and Coraciiformes were sister clades. Piciformes was

present at the base of the tree Accipitriformes contained Accipitridae, Pandionidae, and Sagittariidae. The results showed that the 16 owls and nightjars could be divided into two branches: Strigiformes and Caprimulgiformes (Figure 2). Strigiformes and Caprimulgiformes are sister branches. Strigiformes included Tytonidae and Strigidae species, whereas Caprimulgiformes included Aegothelidae and Caprimulgidae species.

The ML and BI trees shared identical topologies and high node support values for the 107 analyzed species based on 12S rRNA and 16S rRNA genes (Figure 3). The results revealed that the species could be divided into seven branches: Falconiformes, Accipitriformes, Strigiformes, Caprimulgiformes, Apodiformes, Coraciiformes and Piciformes. The results also revealed that the 16 owls and nightjars could be divided into two branches: Strigiformes and Caprimulgiformes. Strigiformes included Tytonidae and Strigidae species, whereas Caprimulgiformes included Aegothelidae and Caprimulgidae species. Tytonidae contained *Tyto* and *Phodilus*. Strigidae contained *Otus*, *Ninox*, *Athene*, *Glaucidium*, *Asio*, *Strix*, and *Bubo*. Aegothelidae contained *Aegotheles* and *Chordeiles*, and Caprimulgidae contained *Caprimulgus*.

DISCUSSION

Mitochondrial Genome Annotation and Features

All genes identified in the five mitochondrial genomes are typical raptor mitochondrial genes (Xiao et al, 2006; Song et al, 2015; Jiang et al, 2015). All genomes contained 37 genes (13 PCGs, 22 tRNAs, and two rRNAs) and a CR, and the gene order was identical to that of the other raptor mitogenomes sequenced to date, but different from the gene order in other birds (Song et al, 2015; Jiang et al, 2015). The arrangement of the five new owl and nightjar mitochondrial genomes in this study differed from those all other birds studied (Song et al, 2015; Jiang et al, 2015), and the mitochondrial genomes were also longer than typical mitochondrial genomes (Xiao et al, 2006; Song et al, 2015; Jiang et al, 2015).

The gene length, base composition, and RNA structure were similar to those of other raptor birds (Jiang et al, 2015; Zhao et al, 2016). Among the mitochondrial genomes of the 16 owls and nightjars, the longest was *Asio flammeus* (18966 bp) and the shortest was *Tyto alba* (16148 bp) (Table 2). All results showed that the five new mitogenomes are consistently AT-biased, with values that ranged from 51.5% in *Ninox strenua* to 57.7% in *Accipiter nisus* (average, 54.2%); this indicates that A+T content was higher than C+G content, which is consistent with those of other bird species (51.6–55.7%) (Liu et al, 2013; 2014). The overall base composition was also similar to those of other raptor species (Liu et al, 2014; Zhao et al, 2016). The relative abundance of nucleotides

was C>A>T>G, which reflected a strong AT bias; guanine (13.9%) was the rarest nucleotide (Ryu et al, 2012; Liu et al, 2013; 2014). Metazoan mtDNA usually present a clear strand bias in nucleotide composition; this strand bias can be measured as AT-skew and GC-skew (Guan et al, 2009). Across all 16 mitogenomes, there were 5856 conserved sites and 8215 variable sites, 6455 of which were parsimony-informative. All 16 mitochondrial genomes exhibited a slight AT-skew, which ranged from 0.327 in *N. novaeseelandiae* to 0.405 in *Asio flammeus* (average, 0.386; Table 2, Figure 4). The GC-skew ranged from −0.423 (*N. scutulata*) to −0.368 (*T. alba*, *T. longimembris*), with an average value of −0.398 (Table 2, Figure 4).

Comparison of PCGs

Comparison of PCGs among the 16 owls and nightjars revealed that the start codon ATG accounted for 82.69% of the start codons in all 13 PCGs, followed by GTG (9.13%), which is similar to the start codons in mitochondrial sequences of other birds (Table 3, Figure 5) (Guan et al, 2009; Liu et al, 2013; 2014). All PCGs were initiated by the typical ATN or GTG, as was observed in owls and nightjars, and terminated with TAA, AGG, and TAG, or T- is presumed to be completed via post-transcriptional polyadenylation (Harrison et al, 2004; Gibb et al, 2009; Jiang et al, 2015). Among the 13 PCGs, all of the ATP8, COIII,

ND4, ND4L, and ND6 start codons were ATG. GTG was common in ND1, COI COII, and ND5, whereas ATC and ATA were only found in ND1, ND2, and ND3 (Table 3, Figure 5), and these patterns are frequently observed in other avian orders (Guan et al, 2009). In *Athene brama* and *S. leptogrammica*, the start codons of all 13 PCGs were ATG (Liu et al, 2014). TAA, AGG, TAG, and T- occurred as stop termination codons in most of the same genes in the bird mitochondrial genomes (Gissi et al, 2008; Jiang et al, 2015). The most common stop codon, TAA, was only observed in ND5 and Cyt b, T- was observed in COIII, and AGG was found in ND1, ND2, COI, COII, ATP6, ATP8, and ND4L (Table 3, Figure 5). Among the 13 PCGs in all 16 species, specific examples included ATG as the COIII initiation codon and TAA as the termination codon; ND6 had ATG as the initiation codon and T- as the termination codon (Table 3, Figure 5).

CR Comparisons

In bird mitochondrial genomes, the CR is usually located between $tRNA^{Glu}$ and $tRNA^{Phe}$, but all CRs of the five new mitochondrial genomes were located between $tRNA^{Thr}$ and $tRNA^{Pro}$; this is similar to the findings of previous studies on other raptors (Jiang et al, 2015; Liu et al, 2013; 2014). In this study, most of the new mitochondrial genomes (excluding that of *S. leptogrammica*) also contained a pseudo-CR that

was approximately 1 kbp in length, which was located between $tRNA^{Glu}$ and $tRNA^{Phe}$; this is similar to what was observed for other raptors, such as *Asio flammeus*, *Tyto longimembris*, and *Phodilus badius* (Ryu et al, 2012; Song et al, 2015; Jiang et al, 2015). CR is believed to be the regulation of replication and transcription, sequence variation may result in length differences among bird mitochondrial genomes (Boore et al, 1999; Bensch et al, 2000). Additionally, some raptor mitochondrial genomes contained the pseudo-CR, which made these genomes longer than those of other birds (Song et al, 2015). Many studies also show that the extensive size variation of CR in bird mtDNA is attributed to the insertion-deletion of some segments and/or the variation of the copy number-length of tandem within its 5′ and 3′ ends; however, there were no repeat sequences in the five new mitochondrial genomes that were sequenced in this study (Bensch et al, 2000; Xiao et al, 2006).

Phylogenetic Analyses

The phylogenetic positions of owls and nightjars have changed several times throughout history. Traditionally, nightjars were thought to belong to suborder Caprimulgi within Strigiformes (Sibley et al, 1988; 1990). The ML and BI trees based on the complete mitochondrial genomes of 16 owls and nightjars shared similar topologies and high node support values with trees based on the two concatenated mitochondrial gene

sequences of 19 species (Figures 3, 4). All trees in this study showed that Strigiformes and Caprimulgiformes were independent orders. Our results do not support Caprimulgiformes being a suborder within Strigiformes, but instead that Caprimulgiformes is an independent order; this differs from what was suggested by Sibley et al (1988).

The taxonomy and systematic relationships within Strigiformes and Caprimulgiformes have been considerably debated (Zheng, 1991; 1994; Sibley et al, 1998). Traditionally, some authors considered Aegothelidae to be a suborder (Aegotheli) of Strigiformes; however, some authors suggested that Aegothelidae should be an independent family within Caprimulgiformes, or should to with owlet-nightjars formed Aegotheli (Sibley et al, 1988; 1990; Zheng, 2004). In this study, the results support that Aegothelidae is a family that belongs to Caprimulgiformes and is not a suborder with Strigiformes. Our molecular results show that Aegotheles grouped with Chordeiles, which formed an independent family (Aegothelidae) within Caprimulgiformes; this finding is slightly different from that reported by Sibley et al (1998). Molecular phylogenetic results also indicated that the studied birds could be divided into two families: Tytonidae and Strigidae. According to our study, Tytonidae diverged first, followed by Strigidae; this finding is similar to those of previous molecular phylogenetic and morphological studies (Zheng, 1991; 1994; Sibley et al, 1998).

Accipitridae is a family of raptors that has been proposed to include most diurnal birds of prey, such as hawks, eagles, and vultures; traditionally, these birds have been included with falcons in Falconiformes, but some authorities have recognized a separate order, Accipitriformes (Chesser et al, 2010; 2012). Based on our findings, we suggest that Accipitriformes does not belong to Falconiformes, but rather represents an independent order, Accipitriformes. Molecular phylogenetic analysis revealed that the Accipitriformes species should be divided into three families: Accipitridae, Sagittariidae, and Pandionidae. Our results showed that Accipitridae and Pandionidae clustered together, which was previously claimed based on a morphological and molecular study (Jiang et al, 2015). In our study, the results revealed that Pandionidae (*Pandion haliaetus*) and Sagittariidae (*Sagittarius serpentarius*) are independent families; this result is consistent with those of an increasing number of recent studies (Lerner et al, 2005; Mahmood et al, 2014; Jiang et al, 2015).

In traditional taxonomy, hummingbirds are placed in family Trochilidae within order Apodiformes, and Apodiformes also contains swifts (Mayr, 2003; 2005). However, some taxonomists have separated hummingbirds into their own order, Trochiliformes (Bleiweiss et al, 1999). In this study, we suggest that hummingbirds are not an independent order; instead, they belong to family Trochilidae within

Apodiformes. According to our study, Trochilidae diverged earlier than other lineages within Apodiformes, which shows that Apodiformes is polyphyletic (Mayr, 2003). Based on these results, Piciformes was the basal-most lineage, which indicates that it diverged earlier than the other predatory birds; this is similar to the findings of a previous report (Lanyon et al, 1994).

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Competing Interests

The authors declare that they have no conflict of interest.

Author contributions

LG and ZLZ conceived and designed the experiments; LG and ZGH performed the experiments; LG analyzed the data; LG and ZLZ wrote the paper.

Ethics

All samples were provided by at the Institute of Biodiversity and Wetland Ecology, School of Resources and Environmental Engineering, Anhui University (sample codes, AHU-RP20160501–20160505). All experimental procedures complied with the current laws on animal welfare and research in China, and were specifically approved by the

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Figure legends

Figure 1 Mitochondrial genomes of the five raptors reported in this study. A contains a pseudo-control region (*Glaucidium cuculoides*, *Otus scops*, *Glaucidium brodiei*, *Caprimulgus indicus*) and B is a normal mitochondrial genome (*Strix leptogrammica*). Genes encoded in the H-strand (clockwise orientation) are colored red or orange. Genes encoded in the L-strand (anticlockwise orientation) are colored dark or light blue. Abbreviations for genes are as follows: COI-III = cytochrome oxidase subunits, Cyt b = cytochrome b, ND1–6 = NADH dehydrogenase components, rrnL = 16S rRNA, and rrns = 12S rRNA. tRNAs are denoted as one-letter symbols based on IUPAC–IUB single-letter amino acid codons.

Figure 2 Phylogenetic relationships among 63 species based on complete mtDNA sequences.

Numbers at each node are Bayesian posterior probabilities (left) and maximum likelihood bootstrap proportions (right).

Figure 3 Phylogenetic relationships among 107 species based on 12S rRNA and 16S rRNA genes. Numbers at each node indicate Bayesian posterior probabilities (left) and maximum likelihood bootstrap proportions (right).

Figure 4 AT-skew and GC-skew in the 16 owls and nightjar mitogenomes. Each point represents a species.

Notes: A: *Tyto alba*, B: *Tyto longimembris*, C: *Glaucidium cuculoides*, D: *Bubo bubo*, E: P*hodilus badius*, F: *Asio flammeus*, G: *Aegotheles cristatus*, H: *Glaucidium brodiei*, I: *Otus bakkamoena*, J: *Otus scops*, K: *Ninox novaeseelandiae*, L: *Ninox scutulata*, M: *Strix leptogrammica*, N: *Athene brama*, O: *Caprimulgus indicus*, P: *Ninox strenua*

Figure 5 Start (A) and stop codon (B) usage in the 13 mitochondrial PCGs of the 16 owls and nightjars. All genes are shown in the order of occurrence in the mitochondrial genome, starting from ND1.

Tables

Table 1 GenBank accession numbers for the complete mitochondrial genomes in this study.

Species	Accession number	Species	Accession number
Nisaetus nipalensis	NC_007598	*Ninox novaeseelandiae*	NC_005932
Nisaetus bartelsi	NC_007599	*Ninox scutulata*	NC_029384
Spizaetus alboniger	NC_007599	*Glaucidium cuculoides*	KY092431
Hieraaetus fasciatus	NC_029188	*Glaucidium brodiei*	MF155890
Aquila chrysaetos	NC_024087	*Athene brama*	KF961185
Aegypius monachus	NC_022957	*Bubo bubo*	AB918148

Spilornis cheela	NC_015887	*Tyto alba*	EU410491
Accipiter soloensis	KJ680303	*Tyto longimembris*	KP893332
Accipiter gentilis	NC_011818	*Phodilus badius*	NC_023787
Accipiter gularis	KX585864	*Aegotheles cristatus*	NC_011718
Accipiter virgatus	NC_026082	*Caprimulgus indicus*	NC_025773
Accipiter nisus	KJ680300	*Glaucis hirsutus*	NC_033413
Buteo lagopus	NC_029189	*Phaethornis malaris*	NC_030288
Buteo hemilasius	NC_029377	*Florisuga fusca*	NC_030287
Buteo buteo	KM364882	*Lophornis magnificus*	KT265276
Butastur indicus	NC_032362	*Calliphlox amethystina*	NC_030286
Butastur liventer	NC_032363	*Chaetura pelagica*	NC_028545
Pandion haliaetus	NC_008550	*Apus apus*	NC_008540
Sagittarius serpentarius	NC_023788	*Cypseloides fumigatus*	NC_034933
Falco tinnunculus	NC_011307	*Heliodoxa aurescens*	NC_030285
Falco naumanni	NC_029846	*Halcyon coromanda*	NC_028177
Falco columbarius	NC_025579	*Halcyon pileata*	NC_024198
Falco cherrug	NC_026715	*Todiramphus sanctus*	EU410489
Falco rusticolus	NC_029359	*Eurystomus orientalis*	NC_011716
Falco sparverius	NC_008547	*Ceryle rudis*	NC_024280
Falco peregrines	JQ282801	*Campephilus imperialis*	KU158198
Micrastur gilvicollis	NC_008548	*Campephilus guatemalensis*	KT443920
Otus scops	KY471456	*Dendrocopos leucotos*	NC_029862
Otus bakkamoena	NC_028163	*Picoides pubescens*	NC_027936
Strix leptogrammica	KC953095	*Pteroglossus azara*	DQ780882
Asio flammeus	NC_027606	*Sasia ochracea*	NC_028019
Ninox strenua	NC_033967		

Table 2 Nucleotide compositions (%) of owl and nightjar mitochondrial genomes in this study.

Species	T (%)	C (%)	A (%)	G (%)	A + T (%)	G+C (%)	CG-skew	AT-skew	Total nucleotide
Otus scops	23.0	32.8	30.8	13.4	53.8	46.2	-0.420	0.394	17413
Otus bakkamoena	24.5	31.0	30.9	13.6	55.4	44.6	-0.390	0.389	17389
Strix leptogrammica	22.6	32.7	29.8	14.9	52.4	47.6	-0.374	0.333	16307
Asio flammeus	24.1	32.1	30.7	13.0	54.8	45.2	-0.424	0.405	18966
Ninox scutulata	21.7	33.8	30.9	13.7	52.6	47.4	-0.423	0.386	16208
Ninox strenua	21.6	34.2	29.9	14.4	51.5	50.5	-0.407	0.350	16206
Ninox novaeseelandiae	22.0	33.4	30.8	13.8	52.8	47.2	-0.415	0.381	16220
Glaucidium cuculoides	25.1	30.9	30.7	13.3	55.8	54.8	-0.398	0.395	17392
Glaucidium brodiei	23.9	32.2	29.9	14.1	53.8	46.2	-0.391	0.359	17318
Athene brama	22.1	33.2	29.9	14.8	52.0	48.0	-0.383	0.338	16194
Bubo bubo	25.9	31.4	29.8	12.9	55.7	44.7	-0.418	0.396	16250
Tyto longimembris	23.5	31.8	30.1	14.7	53.6	46.4	-0.368	0.344	18366
Tyto alba	22.9	32.5	29.6	15.0	52.5	47.5	-0.368	0.327	16148
Phodilus badius	21.4	33.8	30.5	14.3	51.9	48.1	-0.405	0.362	17036
Aegotheles cristatus	24.2	31.2	30.7	13.9	54.9	45.1	-0.383	0.377	16914
Caprimulgus indicus	23.1	32.4	30.7	13.8	53.8	46.2	-0.403	0.380	17536
Average	23.2	32.5	30.4	13.9	54.6	47.4	-0.402	0.386	17601

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Table 3 Predicted initiation and termination codons for 13 PCGs in the 16 owl and nightjar mitochondrial genomes in this study.

Species	ND1	ND2	COI	COII	ATP8	ATP6	COIII	ND3	ND4L	ND4	ND5	Cytb	ND6
A	ATG/TAG	ATA/TAA	GTG/AGG	ATG/TAA	ATG/TAA	ATG/TAA	ATG/T-	ATC/TAA	ATG/TAA	ATG/TAA	ATG/TAA	ATA/TAA	ATG/TAG
B	ATG/AGG	ATG/TAG	ATG/AGG	ATG/TAA	ATG/TAA	ATG/TAA	ATG/T	ATA/TAA	ATG/TAA	ATG/T-	ATG/TAA	ATG/TAA	ATG/TAG
C	ATG/TAA	ATG/T-	GTG/AGG	ATG/TAA	ATG/TAA	ATC/TAA	ATG/T-	ATA/TAA	ATG/TAA	ATG/T-	GTG/TAA	ATG/TAA	ATG/TAA
D	ATG/AGG	ATG/TAG	GTG/AGG	ATG/TAA	ATG/TAA	ATG/TAA	ATG/T-	ATA/TAA	ATG/TAA	ATG/T-	GTG/TAA	ATG/TAA	ATG/TAG
E	ATG/AGG	ATG/TAA	GTG/AGG	ATG/TAA	ATG/TAA	ATG/TAA	ATG/T-	ATA/TAA	ATG/TAA	ATG/TAA	ATG/TAA	ATG/TAA	ATG/TAG
F	ATG/AGG	ATG/T-	GTG/AGG	ATG/T-	ATG/TAA	ATG/TAA	ATG/T-	ATA/TAA	ATG/TAA	ATG/T-	GTG/TAA	ATG/TAA	ATG/TAG
G	ATG/AGG	ATG/T-	GTG/AGG	GTG/AGG	ATG/TAA	ATG/TAA	ATG/T-	ATA/TAA	ATG/TAA	ATG/T-	GTG/TAA	ATG/TAA	ATG/TAG
H	GTG/TAA	ATG/T-	ATG/TAG	ATG/TAA	ATG/TAA	ATG/TAA	ATG/T-	ATA/TAG	ATG/TAA	ATG/T-	ATG/TAA	ATG/TAA	ATG/TAG
I	ATG/TAA	ATG/T-	ATG/AGG	ATG/TAA	ATG/TAA	ATG/TAA	ATG/T-	ATG/TAA	ATG/TAA	ATG/T-	ATG/TAA	ATG/TAA	ATG/TAG
J	ATG/AGG	ATG/TAA	GTG/AGG	ATG/TAA	ATG/TAA	ATG/TAA	ATG/T-	ATC/TAA	ATG/TAA	ATG/T-	ATG/TAA	ATG/TAA	ATG/TAG
K	ATG/TAA	ATG/T-	GTG/AGG	ATG/TAA	ATG/TAA	ATG/TAA	ATG/T-	ATG/TAA	ATG/TAA	ATG/TAA	GTG/TAA	ATG/TAA	ATG/TAG
L	ATG/TAG	ATG/T-	ATG/AGG	ATG/TAA	ATG/TAA	ATG/TAA	ATG/T-	ATA/TAA	ATG/TAA	ATG/T-	GTG/TAA	ATG/TAA	ATG/TAG
M	ATC/TAA	ATG/TAG	GTG/AGG	ATG/TAA	ATG/TAA	ATG/TAA	ATG/T-	ATA/TAA	ATG/TAA	ATG/TAA	ATA/TAA	ATG/TAA	ATG/TAG
N	GTG/AGG	ATG/TAG	ATG/AGG	ATG/TAA	ATG/TAA	ATG/TAA	ATG/T	ATA/TAA	ATG/TAA	ATG/TAA	ATG/TAA	ATG/TAA	ATG/TAA
O	ATG/TAA	ATG/AGG	ATG/AGG	ATG/AGG	ATG/AGG	ATG/AGG	ATG/T-	ATG/TAG	ATG/T-	ATG/TAA	ATG/TAA	ATG/TAA	ATG/TAA
P	ATG/AGG	ATG/TAG	GTG/AGG	ATG/TAA	ATG/TAA	ATG/TAA	ATG/T-	ATG/TAA	ATG/TAA	ATG/TAA	ATG/TAA	ATG/TAA	ATG/TAG

Notes: A: *Aegotheles cristatus*, B: *Asio flammeus*, C: *Ninox strenua*, D: *Ninox novaeseelandiae*, E: *Ninox scutulata*, F: *Tyto alba*, G: *Tyto longimembris*, H: *Bubo bubo*, I: *Athene brama*, J: *Phodilus badius*, K: *Otus bakkamoena*, L: *Otus scops*, M: *Glaucidium cuculoides*, N: *Caprimulgus indicus*, O: *Strix leptogrammica*, P: *Glaucidium brodiei*

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Figure 1

Figure 1 Mitochondrial genome of five raptors in this study, A contains a pseudo-control region (*Glaucidium cuculoides*, *Otus scops*, *Glaucidium brodiei*, *Caprimulgus indicus*) and B is a normal mtDNA (*Strix leptogrammica*).

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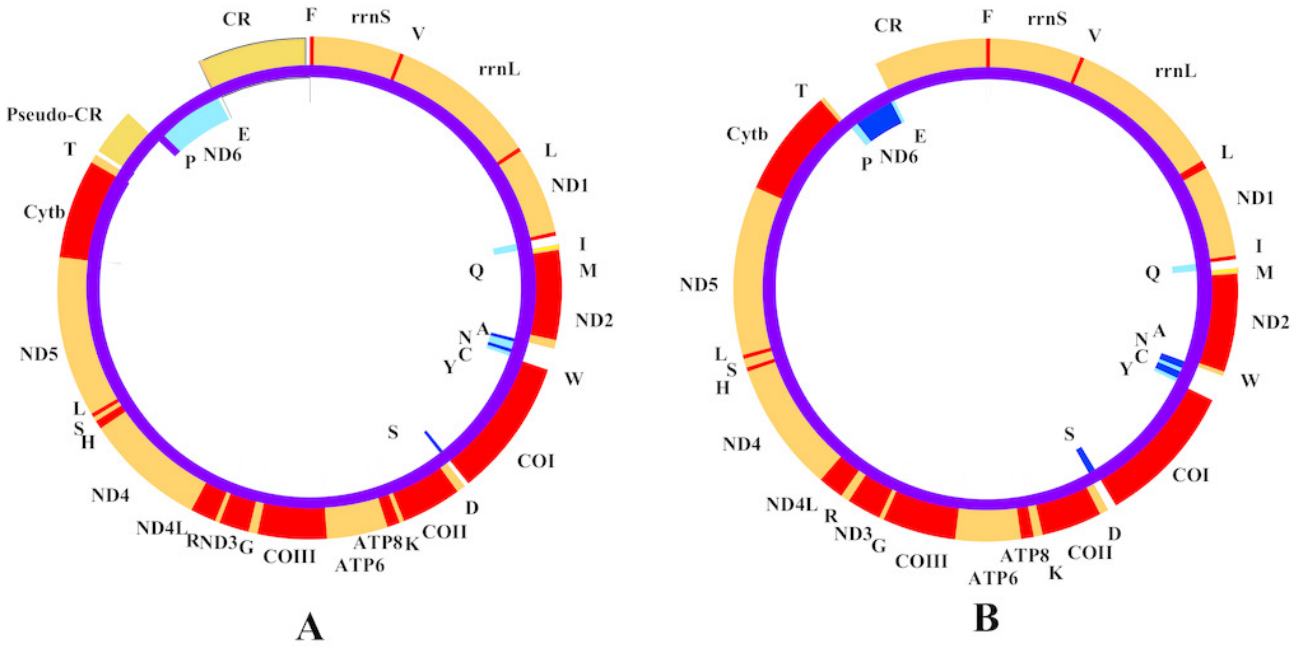

Figure 2

Figure 2 Phylogenetic relationships among the 63 species based on complete mtDNA sequences. Numbers at each node are Bayesian posterior probabilities (left) and maximum likelihood bootstrap proportions (right).

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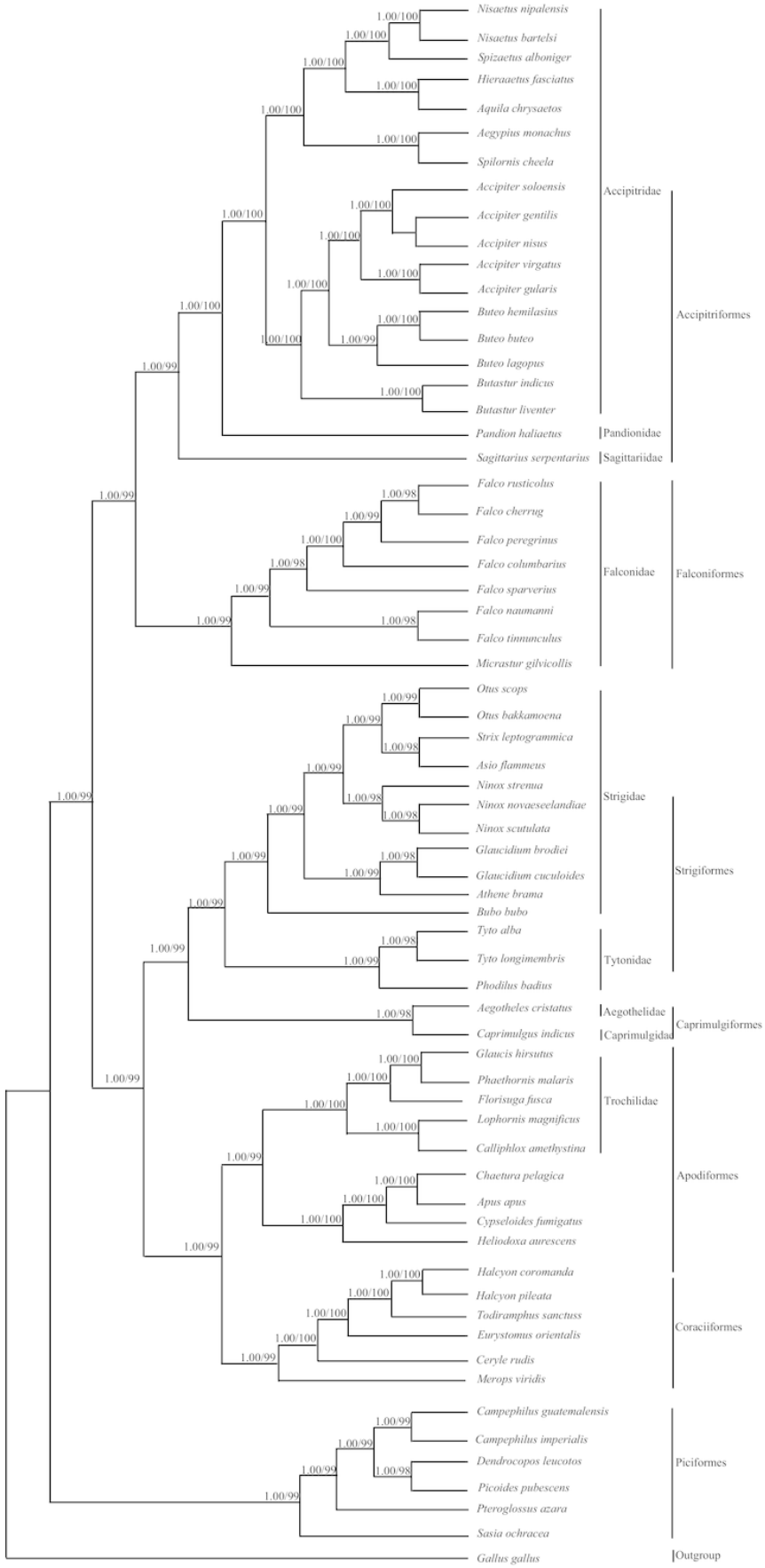

Figure 3

Figure 3 Phylogenetic relationships among the 107 species based on 12S rRNAand 16S rRNA genes. Numbers at each node indicate Bayesian posterior probabilities (left) and maximum likelihood bootstrap proportions (right).

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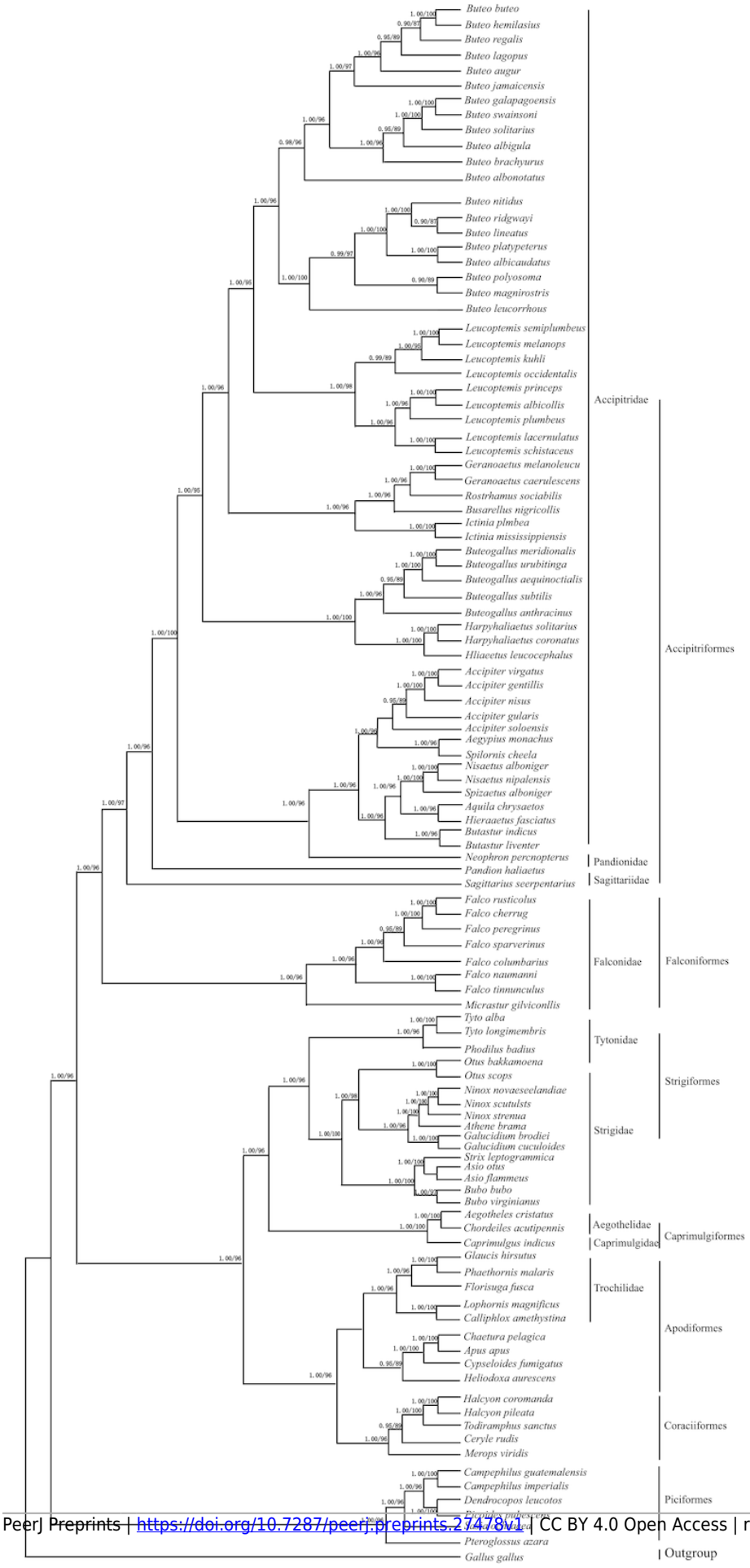
Buteo buteo
Buteo hemilasius
Buteo regalis
Buteo lagopus
Buteo augur
Buteo jamaicensis
Buteo galapagoensis
Buteo swainsoni
Buteo solitarius
Buteo albigula
Buteo brachyurus
Buteo albonotatus
Buteo nitidus
Buteo ridgwayi
Buteo lineatus
Buteo platypeterus
Buteo albicaudatus
Buteo polyosoma
Buteo magnirostris
Buteo leucorrhous
Leucoptemis semiplumbeus
Leucoptemis melanops
Leucoptemis kuhli
Leucoptemis occidentalis
Leucoptemis princeps
Leucoptemis albicollis
Leucoptemis plumbeus
Leucoptemis lacernulatus
Leucoptemis schistaceus
Geranoaetus melanoleucu
Geranoaetus caerulescens
Rostrhamus sociabilis
Busarellus nigricollis
Ictinia plmbea
Ictinia mississippiensis
Buteogallus meridionalis
Buteogallus urubitinga
Buteogallus aequinoctialis
Buteogallus subtilis
Buteogallus anthracinus
Harpyhaliaetus solitarius
Harpyhaliaetus coronatus
Hliaeetus leucocephalus
Accipiter virgatus
Accipiter gentillis
Accipiter nisus
Accipiter gularis
Accipiter soloensis
Aegypius monachus
Spilornis cheela
Nisaetus alboniger
Nisaetus nipalensis
Spizaetus alboniger
Aquila chrysaetos
Hieraaetus fasciatus
Butastur indicus
Butastur liventer
Neophron percnopterus
Pandion haliaetus
Sagittarius seerpentarius
Falco rusticolus
Falco cherrug
Falco peregrinus
Falco sparverinus
Falco columbarius
Falco naumanni
Falco tinnunculus
Micrastur gilviconllis
Tyto alba
Tyto longimembris
Phodilus badius
Otus bakkamoena
Otus scops
Ninox novaeseelandiae
Ninox scutulsts
Ninox strenua
Athene brama
Galucidium brodiei
Galucidium cuculoides
Strix leptogrammica
Asio otus
Asio flammeus
Bubo bubo
Bubo virginianus
Aegotheles cristatus
Chordeiles acutipennis
Caprimulgus indicus
Glaucis hirsutus
Phaethornis malaris
Florisuga fusca
Lophornis magnificus
Calliphlox amethystina
Chaetura pelagica
Apus apus
Cypseloides fumigatus
Heliodoxa aurescens
Halcyon coromanda
Halcyon pileata
Todiramphus sanctus
Ceryle rudis
Merops viridis
Campephilus guatemalensis
Campephilus imperialis
Dendrocopos leucotos
Pteroglossus azara
Gallus gallus
Accipitridae
Pandionidae
Sagittariidae
Falconidae
Tytonidae
Strigidae
Aegothelidae
Caprimulgidae
Trochilidae
Accipitriformes
Falconiformes
Strigiformes
Caprimulgiformes
Apodiformes
Coraciiformes
Piciformes
Outgroup

Figure 4

Figure 4 The AT-skew and GC-skew in 16 owls and nightjar mitogenomes. Each point represents a species.

Figure 4 The AT-skew and GC-skew in 16 owls and nightjar mitogenomes. Each point represents a species. Notes: A: *Tyto alba*, B: *Tyto longimembris*, C: *Glaucidium cuculoides*, D: *Bubo bubo*, E: P*hodilus badius*, F: *Asio flammeus*, G: *Aegotheles cristatus*, H: *Glaucidium brodiei*, I: *Otus bakkamoena*, J: *Otus scops*, K: *Ninox novaeseelandiae*, L: *Ninox scutulata*, M: *Strix leptogrammica*, N: *Athene brama*, O: *Caprimulgus indicus*, P: *Ninox strenua*

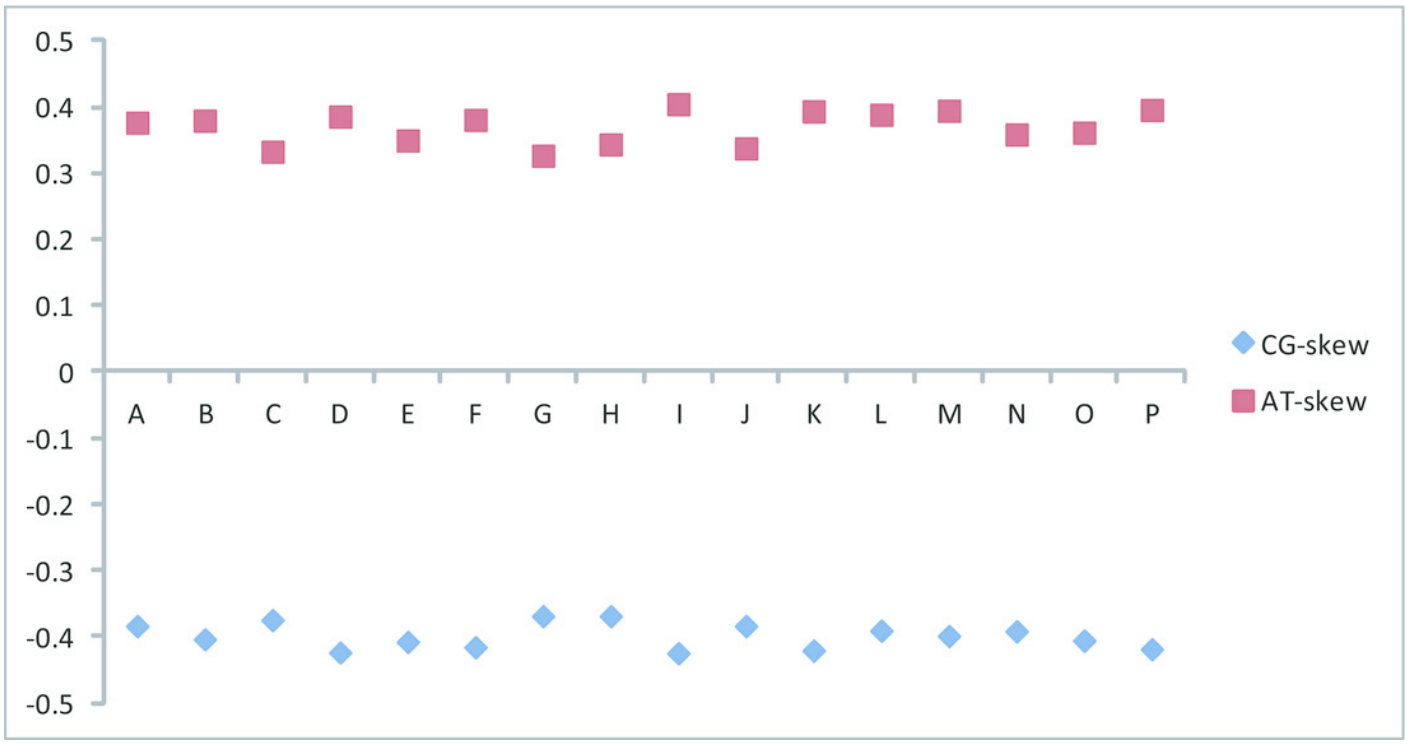

Figure 5

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