The Complete Mitochondrial Genome of *Aix galericulata* and *Tadorna ferruginea*: Bearings on Their Phylogenetic Position in the Anseriformes

Gang Liu¹,², Lizhi Zhou¹,²*, Bo Li¹,², Lili Zhang¹,²

¹ Institute of Biodiversity and Wetland Ecology, School of Resources and Environmental Engineering, Anhui University, Hefei, Anhui, P. R. China ² Anhui Biodiversity Information Center, Hefei, Anhui, P. R. China

Abstract

*Aix galericulata* and *Tadorna ferruginea* are two Anatidae species representing different taxonomic groups of Anseriformes. We used a PCR-based method to determine the complete mtDNAs of both species, and estimated phylogenetic trees based on the complete mtDNA alignment of these and 14 other Anseriform species, to clarify Anseriform phylogenetics. Phylogenetic trees were also estimated using a multiple sequence alignment of three mitochondrial genes (Cyt b, ND2, and COI) from 68 typical species in GenBank, to further clarify the phylogenetic relationships of several groups among the Anseriformes. The new mtDNAs are circular molecules, 16,651 bp (*Aix galericulata*) and 16,639 bp (*Tadorna ferruginea*) in length, containing the 37 typical genes, with an identical gene order and arrangement as those of other Anseriformes. Comparing the protein-coding genes among the mtDNAs of 16 Anseriform species, ATG is generally the start codon, TAA is the most frequent stop codon, one of three, TAA, TAG, and T-, commonly observed. All tRNAs could be folded into typical cloverleaf secondary structures except for tRNAser (AGY) and tRNAleu (CUN), which are missing the "DHU" arm. Phylogenetic relationships demonstrate that *Aix galericulata* and *Tadorna ferruginea* are in the same group, the Tadorninae lineage, based on our analyses of complete mtDNA sequences and combined gene data. Molecular phylogenetic analysis suggests the 68 species of Anseriform birds be divided into three families: Anhimidae, Anatidae, and Anseranatidae. The results suggest Anatidae birds be divided into five subfamilies: Anatinae, Tadorninae, Anserinae, Oxyurinae, and Dendrocygninae. Oxyurinae and Dendrocygninae should not belong to Anserinae, but rather represent independent subfamilies. The Anatinae includes species from the tribes Mergini, Somaterini, Anatini, and Aythyini. The Anserinae includes species from the tribes Anserini and Cygini.

Introduction

Anseriformes is a highly differentiated order of birds with worldwide distribution, containing more than 150 species [1], [2]. Anseriformes is one of the best-studied groups of birds, largely owing to the group’s historic importance in hunting, domestication, and aviculture [3]. The phylogenetic relationships among the Anseriformes, especially the phylogenetic position of several important species and groups, are rather complex and controversial, and have been affected by rearrangements several times throughout history [1], [2], [3].

Mandarin duck (*Aix galericulata*) and Ruddy shelduck (*Tadorna ferruginea*) are two typical Anseriform waterfowl, yet their phylogenetic status has remained controversial [3]. Traditionally, *Aix galericulata* belongs in Tadornini, and *Tadorna ferruginea* is a member of Cairinini; both of them placed inside Anatinae. The two birds have a moderately large body size, contrastingly pale dorsal wing-coverts, and blunt carpal (wing) spurs, along with other shared morphological characteristics; and both also feed through a combination of wading and dabbling [3]. All of these characteristics lie between the true ducks (Anatinae) and true geese (Anserinae) in terms of anatomy and behavior [12]. Accordingly, some authors believe *Aix* and *Tadorna* form an independent lineage together, the subfamily Tadorninae [8]. However, this view has often been challenged, and relationships within Tadorninae/Tadornini remain problematic, in particular, the taxonomy and systematic relationships within the groups. Many authors support *Cairina* a so-called “perching duck”, and *Aix* clustering together as a sister group, belonging to Tadornini [1], [2], [3]. Others suggest that *Aix* and *Cairina* should be placed in their own tribe, Cairinini, within Anatinae [6]. However, still other authors consider the genera *Aix* and *Cairina* as forming the subtribe Cairinina, placed in the tribe Anatini [9]. Even other authors suggest that Tadornini (containing *Aix* and *Cairina*) should not belong to Anatinae at all, but rather represents the independent subfamily Tadorninae, placed in Anatidae [3].
| Gene          | Direction | mtDNA of Aix galericulata | mtDNA of Tadorna ferruginea |
|--------------|-----------|---------------------------|----------------------------|
|              |           | Nucleotide no | Size  | Start codon | Stop codon | Nucleotide no | Size  | Start codon | Stop codon |
| D-loop       | F         | 1–1071       | 1071  |             |            | 1–1077       | 1077  |             |            |
| tRNA^Acc     | F         | 1072–1139    | 68    |             |            | 1078–1147    | 70    |             |            |
| 12S rRNA     | F         | 1140–2136    | 997   |             |            | 1148–2129    | 982   |             |            |
| 16S rRNA     | F         | 2137–2208    | 72    |             |            | 2130–2200    | 71    |             |            |
| tRNA^Val     | F         | 2209–3812    | 1604  |             |            | 2201–3810    | 1610  |             |            |
| 12S rRNA     | F         | 3813–3886    | 74    |             |            | 3811–3884    | 74    |             |            |
| ND1          | F         | 3893–4870    | 978   | ATG         | AGG        | 3891–4868    | 978   | ATG         | AGG       |
| tRNA^Ile     | R         | 4869–4941    | 73    |             |            | 4867–4939    | 73    |             |            |
| tRNA^Gln     | F         | 4949–5019    | 71    |             |            | 4947–5017    | 71    |             |            |
| tRNA^Met     | F         | 5019–5087    | 69    |             |            | 5017–5085    | 69    |             |            |
| ND2          | F         | 5088–6128    | 1041  | ATG         | TAA        | 5086–6126    | 1041  | ATG         | TAG       |
| tRNA^Gly     | R         | 6127–6203    | 77    |             |            | 6125–6199    | 75    |             |            |
| tRNA^Trp     | R         | 6207–6275    | 69    |             |            | 6203–6271    | 69    |             |            |
| tRNA^Ala     | R         | 6276–6353    | 78    |             |            | 6274–6346    | 73    |             |            |
| tRNA^Cys     | R         | 6354–6418    | 65    |             |            | 6347–6411    | 65    |             |            |
| tRNA^Tyr     | F         | 6419–6492    | 74    |             |            | 6412–6482    | 71    |             |            |
| COI          | R         | 6494–8044    | 1551  | GTG         | AGG        | 6484–8034    | 1551  | GTG         | AGG       |
| tRNA^Ser (UCN) | F          | 8036–8108    | 73    |             |            | 8026–8098    | 73    |             |            |
| tRNA^Ser     | F         | 8111–8179    | 69    |             |            | 8101–8169    | 69    |             |            |
| COII         | F         | 8181–8867    | 687   | GTG         | TAA        | 8171–8857    | 687   | ATG         | TAA       |
| tRNA^Thr     | F         | 8869–9037    | 69    |             |            | 8859–8927    | 69    |             |            |
| ATP8         | F         | 8939–9106    | 168   | ATG         | TAA        | 8929–9096    | 168   | ATG         | TAA       |
| ATP6         | F         | 9097–9780    | 684   | ATG         | TAA        | 9087–9770    | 684   | ATG         | TAA       |
| COIII        | F         | 9780–10563   | 784   | ATG         | T-         | 9770–10541   | 772   | ATG         | T-         |
| tRNA^Phe     | F         | 10364–10632  | 69    |             |            | 10354–10610  | 69    |             |            |
| ND3          | F         | 10633–10984  | 352   | ATG         | TAG        | 10611–10962  | 352   | ATG         | TAG       |
| tRNA^Arg     | F         | 10986–11055  | 70    |             |            | 10964–11033  | 70    |             |            |
| ND4L         | F         | 11056–11352  | 297   | ATG         | TAA        | 11034–11330  | 297   | ATG         | TAA       |
| ND4          | F         | 11355–12723  | 1369  | ATT         | T-         | 11333–12701  | 1369  | ATT         | T-         |
| tRNA^Ser (AGY) | F          | 12724–12792  | 69    |             |            | 12702–12770  | 69    |             |            |
| tRNA^Ser (UCN) | F          | 12793–12859  | 67    |             |            | 12771–12836  | 66    |             |            |
| ND5          | F         | 12859–12929  | 71    |             |            | 12836–12907  | 72    |             |            |
| Cyt b        | F         | 14756–15898  | 1143  | ATG         | TAA        | 14728–15870  | 1143  | ATG         | TAA       |
Researchers have also used molecular evidence, based on combined Cyt b and ND2 gene sequences, to suggest placing *Aix* and *Tadorna* together as a tribe in the Anatinae [2]. These authors have also grouped the shelducks (*Tadorna*) together with the sheldgeese (*Cyanochen, Alopochen, Neochen* and *Chloephaga*), forming the tribe *Tadornini*, based on morphological and molecular data, but consider it a non-monophyletic grouping [2]. To add to the confusion, traditional primitive characters used to define “perching ducks” describes a polyphyletic grouping, because similar morphological, biochemical, and behavioral characteristics occur in many differing genera, including *Anseranas, Dendrocygna, Sarkidiornis, Tadorna, Cairina, Aix*, and *Chenonetta* [13].

Nonetheless, important groups in the Anseriformes remain controversial. Traditionally, the order Anseriformes has been considered to be composed of the families Anhimidae (two genera and three species) and Anatidae (approximately 41 genera and 147 species, including *Anseranas semipalmata*) [1]. However, some authors suggest that it should be divided into three families: Anhimidae, Anatidae, and Anseranatidae, the latter only containing one species, *Anseranas semipalmata* [1], [2]. The family Anhimidae is supported by both morphological and molecular data, usually without controversy [1], [3]. A major source of conflict at the family level is centered around *Anseranas semipalmata*, that is, whether it should be considered a member of an independent family by itself, or whether it is contained within a subfamily of Anatidae [1], [2], [4], [5], [6]. The Anatidae comprises the largest number of species in Anseriformes, and traditionally was divided into two subfamilies: Anatinae and Anserinae [1], [2], [3], [7], [8]. However, this view has been challenged by several authors, who recognize five subfamilies within Anatidae: Anatinae, Anserinae, Oxyurinae, Dendrocygninae, and Anseranatinae [2], [9], [10]. Anatinae, Anserinae, and Dendrocygninae are supported by previously published mtDNA data [10]. The stiff-tailed ducks (Oxyurinae/Oxyurini) include some of the most distinctive waterfowl species, showing the greatest sexual size dimorphism [11]. Most of its members have long stiff-tail feathers, which are erected at rest, and relatively large, swollen bills [3]. According to morphological and behavioral characteristics, stiff-tailed ducks (Oxyurinae/Oxyurini) appear to be closer to swans and true geese than they are to typical ducks [3]. Previously the group was considered to be a comparatively primitive member of the tribe Oxyurini; however, some authors do not support this view and considered it to be a subfamily, Oxyurinae [3]. Its relationships are still enigmatic, and are subject to considerable debate regarding its validity and circumscription [3], [11]. Systematic controversies concerning the stiff-tailed ducks (Oxyurinae/Oxyurini) have focused on whether they constitute a tribe or a subfamily, and often consider it to be close to Anserinae, not within Anatinae at all, and agree with morphology-based studies [8], [9], [11].

Mitochondrial DNA is a powerful, increasingly popular, and widely used molecular marker for the estimation of the animal phylogenetic relationships. It has become a major tool of comparative genomics and plays an important role in phylogenetic studies, comparative and evolutionary genomics, and molecular evolutionary analyses, owing to its maternal inheritance, lack of recombination, and accelerated nucleotide substitution rates compared with those of the nuclear DNA [14], [15]. Here, we attempt to resolve the controversial Aves species using mtDNA analyses. Our newly completed mitochondrial genomes should provide new insights into the phylogenetic position of important species, and yield insight into the higher-level systematics of Anseriform birds. Early molecular work disentangling the phylog-
eny of the Anseriformes was mostly based on one or a few mitochondrial loci, almost always including Cyt b, ND2, and/or control region (CR) sequences [1], [2]. However, complete mtDNA sequences have become increasingly important for comprehensive evolutionary and phylogenetic studies [10], [15], [16]. Several analyses have demonstrated that complete mtDNA provides higher levels of phylogenetic support than those based on individual or partial mitogenomes [10], [15], [16], [17], [18], [19]. Complete mtDNA sequences are not only more informative than shorter sequences of individual genes, but also provide reliable information toward the inference of phylogenetic relationships among controversial animals [17], [19], [20], [21]. Consequently, complete mtDNA genomes are becoming a preferred marker for resolving controversial species relationships, and are increasingly important for comprehensive evolutionary studies [10], [14], [15], [16], [22]. However, very few Anseriform birds are currently represented with complete mtDNAs, consequently, a number of Anseriform species and their phylogenetic relationships remain unresolved. We sequenced the complete mtDNA of two important Anseriform birds, from the genera Aix and Tadorna, in this study. We also analyzed the nucleotide composition, codon usage, and compositional biases of the mitogenomes. Our phylogenomic analysis should shed increased light on the phylogenetic status of Aix galericulata and Tadorna ferruginea, and on the phylogenetic relationships of other important groups of Anseriformes.

Results

Genome organization and arrangement

The complete mtDNAs of Aix galericulata and Tadorna ferruginea are 16,651 and 16,639 bp in length, respectively. Both contain the typical set of 37 genes, including 13 protein-coding genes (PCGs) (ATP6, ATP8, COI-III, ND1-6, ND4L, and Cyt b), two rRNAs (12S rRNA and 16S rRNA), 22 tRNAs and a putative CR (D-loop) (Table 1). The heavy (H-) DNA strand carries most of the genes, 12 PCGs, two rRNAs, and 14 tRNAs. ND6 and eight tRNAs are located on the light (L-) strand (Table 1).

Protein-coding genes

Through the 13 protein-coding genes, ATG is the start codon in nine of the 13 PCGs in Aix galericulata, but ND4 starts with ATT, while COI, COII, and ND5 begin with the nonstandard start codon GTG. In Tadorna ferruginea ten PCGs start with ATG, and ATT is the start codon only in ND4, while COI and ND5 begin with GTG. The standard stop termination codon TAA occurs in most of the same genes in the two birds’ mtDNAs, except ND2 stops with TAA in Aix galericulata and Tadorna ferruginea. Furthermore, AGG terminates the ND1 and COI genes, TAG terminates the ND3 and ND6 gene, and the incomplete termination codon T- occurs in the COII and ND4 genes in both birds.

Ribosomal RNA, transfer RNA, and non-coding regions

In Aix galericulata and Tadorna ferruginea 12S rRNA (997 bp and 982 bp, respectively) and 16S rRNA (1,604 bp and 1,610 bp, respectively) genes are located between the tRNAPhe and tRNALeu genes, separated by the tRNA Val gene. The two complete mtDNAs contain 22 tRNAs genes, and except for tRNASer (AGY) and tRNALeu (CUN), which lack dihydrouridine (DHU) arms, all other tRNAs could be folded into the typical cloverleaf structure. The longest tRNAs are tRNAAsn (78 bp) and tRNAAsp (87 bp) in...
mtDNA of *Aix galericula* and *Tadorna ferruginea*
Aix galericulata and Tadorna ferruginea, respectively, and the shortest is tRNA^{Cys} (65 bp in both).

Non-coding regions in the mtDNAs include the CRs and a few intergenic spacers. The CRs are located between the tRNA^{Cys} and tRNA^{Phe} genes, which are 1,071 bp and 1,077 bp, respectively, in Aix galericulata and Tadorna ferruginea. Additionally, 11 gene junction regions spacer by a total of 31 bp, with the longest one being 10 bp between ND6 and tRNA^{Glu} in Aix galericulata (Table 1). There are a total of 53 bp spacer region at 12 gene junctions in Tadorna ferruginea (Table 1).

Phylogenetic reconstructions

Our chosen 16 Anseriform species represent two major branches of the Anseriformes phylogeny with highly similar topologies and only slight differences in bootstrap support and posterior probability values (Figure 1). The first branch is Anatidae and the second is Anseranatidae. Anatidae contains Anatinae, Tadorninae, Anserinae, and Dendrocygninae; and Anseranatidae only contains Anseranas semipalmata. Anatinae and Tadorninae are sister groups, grouped together nestled within the clades Anserinae and Dendrocygninae. Mergini, Anatini, and Aythyini form Anatinae; Anserinae contains Anserini and Aythyini. Aix galericulata and Tadorna ferruginea are in the Tadorninae group.

Phylogenetic analysis was also performed on a concatenated Cyt b, ND2, and COI genes among 68 Anseriform species. The trees from the maximum likelihood (ML) and Bayesian inference (BI) analyses share identical topologies and high node support values (Figure 2). The results indicate that Anseriformes could be divided into three branches: Anatidae, Anseranatidae, and Anhilmidae. Anatidae and Anseranatidae are sister branches, then grouped with Anhilmidae. Anatidae contains five clades: Anatinae, Tadorninae, Anserinae, Oxyurinae, and Dendrocygninae. The Anatinae, includes species from the tribes Mergini, Somaterini, Anatini, and Aythyini. This subfamily is a sister group to Tadorninae, comprising Aix, Cairina, Tadorna, and Chlorophaga. These two subfamilies form a clade that is in a sister group relationship with Anserinae, comprising the tribes Anserini and Cygnini. In turn, this clade is sister to the remaining Oxyurinae and Dendrocygninae. All of them are grouped with families Anseranatidae and Anhilmidae.

Discussion

Mitochondrial genome annotation and features

The gene order and arrangement of the two new mtDNA sequences, including such features as gene length, base composition, and RNA structure, are extremely conservative, and similar to that of other Anseriform birds [10], [16], [23], [24]. The overall base composition is similar to other Anseriforme species, for example, A+T content is higher than C+G content, conforming to other Anseriforme species [51.6–55.7%] [10], [16], [23], [24]. The relative abundance of nucleotides is C>A>T>G, reflecting the strong AT bias [10], [16]. Guanine is the rarest nucleotide.

Results indicate that all Anseriformes mtDNAs so far sequenced have the same gene order and arrangement, no introns, no long intergenic spacers, and only a few overlapping sequences [10], [16]. All genes are encoded on the same arrangement, and there are no missing or duplicated genes [10], [16]. Among the 16 mtDNAs the longest is from Anseranas semipalmata (16,870 bp), and the shortest is Anas formosa (16,594 bp). Homologous regions comprise 12,748 bp, representing 79.68% of the complete genome (Table 2). The 16 genomes generally have the highest transition/transversion ratio in closely-related species [25]. The A, T, and A+T compositions are similar, and shared with a strong AT bias and rare guanines (Table 2). Metazoan mtDNA usually present a clear strand bias in nucleotide composition; this strand bias can be measured as AT-skew and GC-skew [26]. All of the Anseriformes mtDNAs exhibit a slight AT-skew (average value: 0.137), ranging from 0.125 in Dendrocygna javanica to 0.147 in Tadorna ferruginea (Table 2). The GC-skew ranges from −0.577 (Anseranas semipalmata) to −0.927 (Dendrocygna javanica), with an average value of −0.353 (Table 2).

Comparison of protein-coding genes

We compared the total length of the 13 PCGs in Aix galericulata and Tadorna ferruginea with other Anseriform birds. Lengths among them are quite similar and very conservative; the longest one is Aix galericulata (11,403 bp) and the shortest is Anser fabalis (11,328 bp). The 13 PCGs have a total length of 11,351 bp in Aix galericulata and 11,385 bp in Tadorna ferruginea, which is 68.05% and 68.42% of each entire mtDNA genome, respectively. In both species, the longest gene is ND5, located between the tRNA^{Lys} and Cyt b genes, and the shortest is ATP8, which is between the tRNA^{Ly} and ATP6 genes. Most PCGs used ATG as start codons, only a few start with GTG, GTC, or ATA. Stop codons are also similar across species, with TAA, TAG, and T- occurring most frequently. In Tadorna ferruginea and Tadorna ferruginea, the start codons are ATG, GTG and ATT, and TAA, AGG, TAG and T- as stop termination codons occur in most of the same genes in the two birds’ mtDNAs. Among the 13 PCGs, specific examples include the following: the COI initiation codon is GTG and the termination codon is AGG in all 16 species; Cyt b starts with ATG and ends with TAA; COI starts with ATG and ends with TAG, except in Branta canadensis, where it starts with GTG; ND6 starts with ATG and ends with TAG, except in Anser fabalis it ends with TAA; ND1 starts with ATG and ends with AGG, except in Anser albinus where the stop codon is TAA; and ND2 starts with ATG and ends with TAG, except in Anseranas semipalmata, Cygnus atratus, and Tadorna ferruginea, where the stop codon is TAA (Table 3).

Some mtDNA PCGs are particularly worthy of note. Avian species generally exhibit moderate levels of sequence divergence in some mitochondrial genes, including Cyt b, ND2, and COI. These genes are of special interest, because they have been widely used to resolve the taxonomy of controversial groups in Anseriformes [2]. A combination of these three genes is often adequate and has been used for resolving phylogenetic problems at many different taxonomic levels, ranging from related species to genera and families [25]. They have been valuable for clarifying phylogenetic relationships within many controversial animal groups, especially that of Anseriform birds [16], [23], [27].

Control region comparisons

The CR is the only major non-coding segment of mtDNA, and has higher variability, evolving three to five times more rapidly, than other vertebrate mtDNAs [28]. Its primary function is thought to be the regulation of replication and transcription [29]. In Aves the CR is located between the tRNA^{Cys} and tRNA^{Phe} genes. Sequence variation in the CR results in length variability in
| Species                | T (%)  | C (%)  | A (%)  | G (%)  | A+T (%) | G+C (%) | AT-skew | GC-skew | Total nucleotide |
|------------------------|--------|--------|--------|--------|---------|---------|---------|---------|-----------------|
| Anser albirostris      | 22.63  | 32.05  | 30.15  | 15.18  | 52.78   | 47.23   | 0.142   | −0.357  | 16 737          |
| Anser anser            | 22.58  | 32.14  | 30.19  | 15.09  | 52.77   | 47.23   | 0.144   | −0.361  | 16 738          |
| Anser fabalis          | 22.74  | 31.84  | 30.06  | 15.36  | 52.80   | 47.20   | 0.138   | −0.349  | 16 688          |
| Anas formosa           | 22.51  | 32.44  | 29.52  | 15.53  | 52.03   | 47.97   | 0.134   | −0.353  | 16 594          |
| Anas platyrhynchos     | 22.19  | 32.52  | 29.21  | 16.08  | 51.40   | 48.60   | 0.136   | −0.338  | 16 606          |
| Aythya americana       | 22.24  | 32.75  | 29.39  | 15.62  | 51.60   | 48.40   | 0.138   | −0.354  | 16 616          |
| Aythya semipalmata     | 23.49  | 31.38  | 30.92  | 14.21  | 54.41   | 45.59   | 0.136   | −0.377  | 16 870          |
| Branta canadensis      | 22.60  | 32.07  | 30.18  | 15.15  | 52.78   | 47.22   | 0.143   | −0.358  | 16 760          |
| Cygnus columbianus     | 22.79  | 31.89  | 30.10  | 15.22  | 52.89   | 47.11   | 0.138   | −0.354  | 16 728          |
| Cygnus atatus          | 22.20  | 32.55  | 29.52  | 15.73  | 51.72   | 48.28   | 0.142   | −0.348  | 16 748          |
| Cairina moschata       | 21.93  | 32.95  | 29.00  | 16.12  | 54.88   | 45.12   | 0.129   | −0.373  | 16 610          |
| Dendrocygna javanica   | 23.67  | 30.44  | 30.44  | 15.45  | 54.11   | 45.89   | 0.125   | −0.327  | 16 753          |
| Mergus squamatus       | 22.26  | 32.76  | 29.04  | 15.94  | 51.30   | 48.70   | 0.132   | −0.345  | 16 595          |
| Anas falcata           | 22.35  | 32.65  | 28.89  | 16.11  | 51.24   | 48.76   | 0.128   | −0.339  | 16 601          |
| Aix galericulata       | 22.36  | 32.65  | 29.24  | 15.75  | 51.60   | 48.40   | 0.133   | −0.349  | 16 651          |
| Tadorna ferruginea     | 22.00  | 32.96  | 29.57  | 15.47  | 51.57   | 48.43   | 0.147   | −0.361  | 16 639          |

doi:10.1371/journal.pone.0109701.t002
### Table 3. Predicted initiation and termination codons for 13 mitochondrial PCGs in the 16 Anseriforme species.

| Gene   | Predicted initiation and termination |
|--------|--------------------------------------|
|        | A | B | C | D | E | F | G | H | I | J | K | L | M | N | O | P |
| ND1    | ATG/TAA | ATG/AGG | ATG/AGG | ATG/AGG | ATG/AGG | ATG/AGG | ATG/AGG | ATG/AGG | ATG/AGG | ATG/AGG | ATG/AGG | ATG/AGG | ATG/TAA | ATG/TAA | ATG/TAA |
| ND2    | ATG/TAG | ATG/TAG | ATG/TAA | ATG/TAG | ATG/TAG | ATG/TAG | ATG/AGG | ATG/TAG | ATG/TAA | ATG/TAA | ATG/AGG | ATG/TAA | ATG/TAA | ATG/TAA | ATG/TAA |
| COI    | ATG/AGG | ATG/AGG | ATG/AGG | ATG/AGG | ATG/AGG | ATG/AGG | ATG/AGG | ATG/AGG | ATG/AGG | ATG/AGG | ATG/AGG | ATG/AGG | ATG/AGG | ATG/AGG | ATG/AGG |
| COII   | ATG/AGG | ATG/AGG | ATG/AGG | ATG/AGG | ATG/AGG | ATG/AGG | ATG/AGG | ATG/AGG | ATG/AGG | ATG/AGG | ATG/AGG | ATG/AGG | ATG/AGG | ATG/AGG | ATG/AGG |
| COIII  | ATG/AGG | ATG/AGG | ATG/AGG | ATG/AGG | ATG/AGG | ATG/AGG | ATG/AGG | ATG/AGG | ATG/AGG | ATG/AGG | ATG/AGG | ATG/AGG | ATG/AGG | ATG/AGG | ATG/AGG |
| ATP6   | ATG/TAA | ATG/TAA | ATG/TAA | ATG/TAA | ATG/TAA | ATG/TAA | ATG/TAA | ATG/TAA | ATG/TAA | ATG/TAA | ATG/TAA | ATG/TAA | ATG/TAA | ATG/TAA | ATG/TAA |
| ND4L   | ATG/TAA | ATG/TAA | ATG/TAA | ATG/TAA | ATG/TAA | ATG/TAA | ATG/TAA | ATG/TAA | ATG/TAA | ATG/TAA | ATG/TAA | ATG/TAA | ATG/TAA | ATG/TAA | ATG/TAA |
| ND5    | ATG/AGG | ATG/AGG | ATG/AGG | ATG/AGG | ATG/AGG | ATG/AGG | ATG/AGG | ATG/AGG | ATG/AGG | ATG/AGG | ATG/AGG | ATG/AGG | ATG/AGG | ATG/AGG | ATG/AGG |
| Cytb   | ATG/AGG | ATG/AGG | ATG/AGG | ATG/AGG | ATG/AGG | ATG/AGG | ATG/AGG | ATG/AGG | ATG/AGG | ATG/AGG | ATG/AGG | ATG/AGG | ATG/AGG | ATG/AGG | ATG/AGG |
| ND6    | ATG/AGG | ATG/AGG | ATG/AGG | ATG/AGG | ATG/AGG | ATG/AGG | ATG/AGG | ATG/AGG | ATG/AGG | ATG/AGG | ATG/AGG | ATG/AGG | ATG/AGG | ATG/AGG | ATG/AGG |

Notes: A: *Anser albirostris* (NC_004539), B: *Anser anser* (NC_011196), C: *Branta canadensis* (NC_0071011), D: *Cygnus atratus* (NC_012843), E: *Cygnus columbianus* (NC_007691), F: *Mergus merganser* (NC_016723), G: *Cairina moschata* (NC_010965), H: *Anas platyrhynchos* (NC_009684), I: *Aythya americana* (NC_000877), J: *Anseranas semipalmata* (NC_005933), K: *Dendrocygna javanica* (NC_012844), L: *Anas formosa* (NC_015482), M: *Anas fabalis* (NC_016922), N: *Anas falcata*, O: *Aix galericulata* (this study), P: *Tadorna ferruginea* (this study).

doi:10.1371/journal.pone.0109701.t003
Table 4. The genetic distances among the 16 Anseriforme species of mtDNA CR based on kimura-2-parameter model in this study.

| Genetic distances | 1 | 2  | 3  | 4  | 5  | 6  | 7  | 8  | 9  | 10 | 11 | 12 | 13 | 14 | 15 | 16 |
|------------------|---|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|
| 1                |   | 0.191 |    | 0.203 | 0.198 | 0.329 | 0.346 | 0.331 | 0.550 | 0.219 | 0.305 | 0.171 | 0.334 | 0.318 | 0.184 | 0.314 |
| 2                |   | 0.191 |    | 0.203 | 0.198 | 0.329 | 0.346 | 0.331 | 0.550 | 0.219 | 0.305 | 0.171 | 0.334 | 0.318 | 0.184 | 0.314 |
| 3                |   | 0.203 |    | 0.206 | 0.198 | 0.329 | 0.346 | 0.331 | 0.550 | 0.219 | 0.305 | 0.171 | 0.334 | 0.318 | 0.184 | 0.314 |
| 4                |   | 0.198 |    | 0.206 | 0.198 | 0.329 | 0.346 | 0.331 | 0.550 | 0.219 | 0.305 | 0.171 | 0.334 | 0.318 | 0.184 | 0.314 |
| 5                |   | 0.329 |    | 0.329 | 0.329 | 0.550 | 0.550 | 0.550 | 0.550 | 0.219 | 0.305 | 0.171 | 0.334 | 0.318 | 0.184 | 0.314 |
| 6                |   | 0.346 |    | 0.346 | 0.346 | 0.550 | 0.550 | 0.550 | 0.550 | 0.219 | 0.305 | 0.171 | 0.334 | 0.318 | 0.184 | 0.314 |
| 7                |   | 0.331 |    | 0.331 | 0.331 | 0.550 | 0.550 | 0.550 | 0.550 | 0.219 | 0.305 | 0.171 | 0.334 | 0.318 | 0.184 | 0.314 |
| 8                |   | 0.550 |    | 0.550 | 0.550 | 0.550 | 0.550 | 0.550 | 0.550 | 0.219 | 0.305 | 0.171 | 0.334 | 0.318 | 0.184 | 0.314 |
| 9                |   | 0.219 |    | 0.219 | 0.219 | 0.219 | 0.219 | 0.219 | 0.219 | 0.219 | 0.219 | 0.219 | 0.219 | 0.219 | 0.219 | 0.219 |
| 10               |   | 0.305 |    | 0.305 | 0.305 | 0.305 | 0.305 | 0.305 | 0.305 | 0.305 | 0.305 | 0.305 | 0.305 | 0.305 | 0.305 | 0.305 |
| 11               |   | 0.171 |    | 0.171 | 0.171 | 0.171 | 0.171 | 0.171 | 0.171 | 0.171 | 0.171 | 0.171 | 0.171 | 0.171 | 0.171 | 0.171 |
| 12               |   | 0.334 |    | 0.334 | 0.334 | 0.334 | 0.334 | 0.334 | 0.334 | 0.334 | 0.334 | 0.334 | 0.334 | 0.334 | 0.334 | 0.334 |
| 13               |   | 0.318 |    | 0.318 | 0.318 | 0.318 | 0.318 | 0.318 | 0.318 | 0.318 | 0.318 | 0.318 | 0.318 | 0.318 | 0.318 | 0.318 |
| 14               |   | 0.411 |    | 0.411 | 0.411 | 0.411 | 0.411 | 0.411 | 0.411 | 0.411 | 0.411 | 0.411 | 0.411 | 0.411 | 0.411 | 0.411 |
| 15               |   | 0.184 |    | 0.184 | 0.184 | 0.184 | 0.184 | 0.184 | 0.184 | 0.184 | 0.184 | 0.184 | 0.184 | 0.184 | 0.184 | 0.184 |
| 16               |   | 0.314 |    | 0.314 | 0.314 | 0.314 | 0.314 | 0.314 | 0.314 | 0.314 | 0.314 | 0.314 | 0.314 | 0.314 | 0.314 | 0.314 |

Notes: 1: *Aix galericulata*, 2: *Anas falcata*, 3: *Anas formosa*, 4: *Anas platyrhynchos*, 5: *Anser albifrons*, 6: *Anser anser*, 7: *Anseranas semipalmata*, 8: *Aythya americana*, 9: *Branta canadensis*, 10: *Cairina moschata*, 12: *Cygnus atratus*, 13: *Cygnus columbianus*, 14: *Dendrocygna javanica*, 15: *Mergus squamatus*, 16: *Tadorna ferruginea*.

doi:10.1371/journal.pone.0109701.t004
different birds [28], [29]. Similar to other Aueriform birds, there is only one CR in *Aix galericulata* and *Tadorna ferruginea*, 1,071 bp and 1,077 bp in length, respectively. Comparing the length of the CR in the 16 Aueriform species that we researched shows lengths ranging from 1,045 bp (*Anas formosa*) to 1,335 bp (*Anseranas semipalmata*) with an average length of 1,127 bp. The high AT content (53.55%) is similar to other organisms [10], [16], [30]. Regions of recognizable homology comprise only 406 bp, representing 27.92% of the entire mtDNA genome. An alignment of the 16 CR sequences contains 406 variable sites and 537 parsimony informative sites. Its nucleotide frequencies are not significantly different from other birds. Some conspicuous macro-repeat units are found in the CR of some Aueriform species, including microsatellite-like (C)_{11} repeats in *Anser albifrons* and *Anser anser*, and (ATCAAACG)_{15} elements in *Anseranas semipalmata*. The average genetic distance (0.376) between the CR of *Anseranas semipalmata* and the other species in our analysis is higher than average (0.288) (Table 4).

**Phylogenetic analyses**

The trees from the ML and BI analyses based on the complete mtDNA of 16 Aueriform species share similar topologies and high node support values with those of the concatenated three mitochondrial gene sequences from 68 species (Figures 2 and 3). The results support the grouping of *Aix galericulata* and *Tadorna ferruginea* together in the same lineage, Tadorninae. Livezey included *Aix* and *Cairina* in the Anatini but proposed a subtribe, Cairinina, clustering these species together on the basis of a single osteological synapomorphy [9]. Dickinson considered *Aix* and *Cairina* to be members of the Anatinae [6]. Our molecular results show *Aix* and *Cairina* grouped with *Tadorna* and *Chloephaga*, supported by high bootstrap values, forming the Tadorninae, which is located between Anatinae and Anserinae. Therefore, we agree that Tadorninae should be an independent subfamily in the Anatidae, and that *Aix* and *Cairina* don't belong to Anatinae, but are members of Tadorninae, which is a little different from Dickinson's view [6]. The taxonomy and systematic relationships within the Tadorninae have been considerably debated. Our results show that the relationships between *Aix* and *Cairina*, and *Tadorna* and *Chloephaga* are much closer. *Cairina* and *Aix* cluster
Table 5. The 5 Primer sequences used in this study.

| Primer No | Amplified region | Forward primer sequence (5'→3') | Reverse primer sequence (5'→3') |
|-----------|------------------|---------------------------------|---------------------------------|
| 1         | 840–2106         | CCACTACCGGAGACCTACG             | TAAGTCTTTTGGTCCGGAGCAT          |
| 2         | 1997–3190        | ATAGGCTTATTTAGGTAGTCTT          | TGAATTCTCTAAATACCTACCC          |
| 3         | 3017–5121        | GTCACATCTCTCATATAAGCAA          | GGCATCTGACATTGATGATTG           |
| 4         | 4944–7091        | TTACCAAAAAACATAGGCCTCACG        | GTGACTTTGGTATTCTATTGTT          |
| 5         | 6886–8340        | GATCAAAACCTCTCATACCTTCC         | TGGGTTACGGTGATGAGCTTT           |
| 6         | 8210–9346        | TGGCTATCTCCCTCTACTCACCT         | TTGTGTTGGGTGGGAGGTGC            |
| 7         | 9176–10285       | ACACCGGCTCTGTTGTGCTTCC          | CTCTCGGATTCTCTATGCT             |
| 8         | 10127–11367      | CGACTTTCCACCATCCAACT            | GAATTGTTGGGGGACAGTAG            |
| 9         | 11017–12122      | TACTAAACACAGCACAATCCCTCTC       | GAGACAGATTGAGCAGTT              |
| 10        | 11944–12925      | CTACACCTGAGCTCTCTACT            | GAGCAGATTGGATGCTGAGT            |
| 11        | 12747–13834      | TCTGACTACCAAAGCCTCCCA           | GTGACTTTGGTATTCTATTGTT          |
| 12        | 13685–14810      | GAGTTAAATCAACAAGAAGCT           | GTGCTATCTCCCTACCTTC             |
| 13        | 14702–15767      | ACTAGGCCACCAACAAACAG            | CGAAGTTTCCATCGACGAG             |
| 14        | 15663–16493      | ATGATCTTTACCAACACAGACC          | GTGCTATGATCAGAGTGAAGT           |
| 15        | 16347–1128       | CCTMCTRCTCCTCCTTAT             | CTATGCGACATATGCGAT             |

Note: M refers to A and C, R refers to A and G.
doi:10.1371/journal.pone.0109701.t005

Materials and Methods

Sample collection

Trace blood samples from *Aix galericulata* and *Tadorna ferruginea* were collected using non-invasive methods at the Hefei Wild Animal Park in May 2013. No animal was killed for the purpose of the experiment, the method will not affect the health of the animals, and it conforms to our animal ethics committee’s guideline in this study. The Hefei Wild Animal Park is authorized to administer animal rescue and medical treatment by Anhui Provincial Conservation and Management Station for Wildlife (APCMSW), a provincial government agency for wildlife conservation in the Anhui Province of China. We were authorized to study the birds by the APCSWS. The samples were stored at −20°C at the Institute of Biodiversity and Wetland Ecology, School of Resources and Environmental Engineering, Anhui University (Sample codes are AHU-WB20130522 and AHU-WB20130523, respectively).

DNA extraction, PCR amplification, and sequencing

Whole genomic DNA was isolated from blood samples using the phenol/chloroform method. Extracted DNA was examined on a 1.0% agarose/TBE gel and stored at −20°C as templates for PCR.

Based on an alignment of complete mtDNA sequences from *Cygnus atratus* (NC_012843), *Anas platyrhynchos* (NC_009684), and *Aythya americana* (NC_000877), we designed three primer pairs (primer sets 9, 11, and 14) using Primer 5.0. We also used other primers developed from *Anser fabalis* [10], [16]. These primers were used to amplify and sequence the complete mtDNAs of *Aix galericulata* and *Tadorna ferruginea* (Table 5). Generated sequences were all less than 1,200 bp each, with each segment overlapping the next by 80–150 bp.

PCR amplifications were carried out in 50 μl volumes containing 100 ng template DNA, 5 μl of 10× reaction Buffer, 2 μl of 25 mM MgCl2, 4 μl of 2 mM dNTPs, 1 μl of each 10 mM primer, 0.5 U Taq DNA polymerase (Trans Taq-T DNA

Together as a sister group adjacent to *Tadorna* and *Chloephaga*, which, based on morphological and molecular studies, has been repeatedly claimed [2]. *Aix* and *Cairina* have several similar characteristics in behavior and breeding biology, and molecular phylogenetic trees also suggest that they are sister branches, showing they have a close genetic relationship as well [8]. Our mtDNA evidence also supports *Tadorna* and *Chloephaga* having a much closer genetic relationship, congruent with morphological studies with [9].

Our results combine the data of several new mitogenomes within the growing phylogeny of the Anseriformes. Molecular phylogenetic results indicate that the 68 Anseriform birds studied could be divided into three families: Anhimiidae, Anatidae, and Anseranatidae. Anhimiidae diverged first, followed by Anseranatidae and Anatidae, similar to previous molecular phylogenetic results don't support stiff-tailed ducks (Oxyurinae) as being a tribe within Anatidae, but present them as an independent subfamily, Anseranas semipalmata does not belong in Anatidae, but represents an independent family, Anseranatidae [1], [2]. According to our study Aythyni diverged earlier than Anatini within Anatidae and is monophyletic [1], [2]. Systematic relationships concerning the stiff-tailed ducks (Oxyurinae) have also been very controversial [5], [8], [9]. According to traditional morphological studies the stiff-tailed ducks share a common ancestor with geese and swans [3], [9], are closest to the Anserinae, and are not within the Anatidae. Our results don’t support stiff-tailed ducks (Oxyurinae) as being a tribe within Anatidae, but present them as an independent subfamily, Oxyurinae, which diverged earlier than the whistling ducks (Dendrocygninae) in Anatidae [2], [10], [16].

Our mtDNA analysis suggests that the genus *Anas* is actually polyphyletic, but *Anas formosa formosa*, which is found in one of the Anatini branches, has no close relatives among living ducks, and should be placed in some distinct genera. *Anas formosa formosa* should be in a distinct genus; and *Anas discors, Anas querquedula, Anas clypeata* and *Anas platyrhynchos* should be placed in another distinct genus, closest to the multigenus duck group (*Tachyeres, Lophonetta*, and *Amazonetta*).
Polymersase, Beijing, China), and sterile doubly-distilled water to final volume. PCR amplification conditions follow: denaturation for 5 min at 94°C, followed by 30 cycles of denaturation for 30 s at 94°C, annealing for 30 s at 49–55°C (depending on primer combinations), elongation for 2 min at 72°C, and a final extension step of 10 min at 72°C. PCR products were examined using electrophoresis on a 1% agarose/TBE gel (Figure 4) and purified and bidirectionally sequenced by Sangon Biotech Co., Ltd. (Shanghai, China).

Sequence analysis

Sequences were checked and assembled using the programs Seqman (DNASTAR, 2001), BioEdit, and Chromas 2.22, and then adjusted manually. PCGs were identified by comparison with the known complete mtDNA sequences of Anseriform birds using Sequin 11.0. The 22 tRNA genes were identified using the software package tRNA Scan-SE 1.21 (http://lowelab.ucsc.edu/tRNAscan-SE), and their cloverleaf secondary structures and anticodon sequences were determined using DNASIS (Ver.2.5, Hitachi Software Engineering). Two rRNAs were identified by comparison with complete mtDNA sequences of other Anseriformes available in GenBank. The complete mtDNA sequences have been deposited in GenBank under accession numbers KF437906 and KF684946.

Phylogenetic analyses

Phylogenetic trees were estimated using ML and BI methods, to study the phylogeny of the Anseriformes. Corresponding Gallus gallus (NC_001323) sequence was used as outgroup. Phylogenetic trees were estimated for two cases: one based on the complete mtDNA of 16 Anseriforme species (Table 6), and another one based on a multiple sequence alignment of three mitogenome (Cyt b, ND2, and COI) sequences from 68 typical Anseriform species from GenBank (Table 7). Our previous research has shown that the combined gene sequence from Cyt b, ND2, and COI is suitable for resolving phylogenetic relationships among Anseriform species in the absence of sufficient complete mtDNA data [16].

**Table 6.** GenBank accession numbers for the 16 complete mtDNA of Anseriforme species in this study.

| Species                        | Accession number | Species                        | Accession number |
|--------------------------------|------------------|--------------------------------|------------------|
| Anas platyrhynchos             | NC_009684        | Aythya americana               | NC_000877        |
| Anas formosa                   | NC_015482        | Branta canadensis              | NC_007011        |
| Anser anser                    | NC_011196        | Cairina moschata               | NC_010965        |
| Anser albifrons                | NC_004539        | Cygnus columbianus             | NC_007691        |
| Anseranas semipalmata          | NC_005933        | Cygnus atratus                 | NC_012843        |
| Dendrocygna javanica           | NC_012844        | Anas falcate                   | KC759527         |
| Anser fabalis                  | NC_016922        | Mergus squamatus               | NC_016723        |
| Aix galericula                in this study |                     | Tadorna ferruginea             | In this study    |

Figure 4. The typical amplification results (bands 1–10) of representative sequences from Tadorna ferruginea and Aix galericula. Notes: bands 1–6 are the PCR amplification products used primers 6–11 of Tadorna ferruginea, and bands 7–10 are the PCR amplification products used primers 6–9 of Aix galericula.

doi:10.1371/journal.pone.0109701.g004
Before phylogenetic tree estimations all 16 complete mtDNAs and the three concatenated data sets of 68 Anseriforme species were aligned using ClustalX 1.8, followed by manual adjustment. Specifics of the phylogenetic tree estimation based on the three concatenated data sets among the 68 Anseriforme species follows: the corresponding amino acid sequences were translated into their corresponding protein sequences, then aligned using ClustalX 1.8, and the three alignments were concatenated into a single alignment. The alignments were then used to build a maximum likelihood (ML) tree using MEG 4.0. The ML analyses were performed in PAUP* 4.0b10 using TBR branch swapping (10 random addition sequences) and a general time-reversible model with invariant sites and among-site variation. The best fit model of evolution was selected using Modeltest (version 3.06) based on the AIC criterion. Internal ML tree branch support was evaluated using a bootstrap test with 100 iterations. Bayesian phylogenetic inference was done using MrBayes 3.1.2, with the same best-fit substitution model as that selected for the ML analysis. MrBayes 3.1.2 simultaneously initiates two Markov Chain Monte Carlo (MCMC) runs to provide additional confirmation of convergence of posterior probability distributions. Analyses were run for one million generations until the average standard deviation of split frequencies was less than 0.01, which indicated that convergence was reached. Chains were sampled every 1,000 generations.

### Supporting Information

#### Table S1 Phylogenetic Classification of the Anseriformes.

| Species | Accession number (Cyt b, ND2, COI) | Species | Accession number (Cyt b, ND2, COI) |
|---------|-----------------------------------|---------|-----------------------------------|
| Anhima cornuta | AY140735,AY140737,AY140729 | Anas falcata | KC759527,KC759527,KC759527 |
| Anseranas semipalmata | NC_005933,NC_005933,NC_005933 | Anas poecilorhyncha | EU914150,AF059143,JN703239 |
| Dendrocygn a arcuata | AF082061,UG97735,UG97739 | Anas discors | EU914146,AF059128,DQ434285 |
| Dendrocygn a eytoni | EUS85847,EU585710,UG97733 | Anas acuta | AF059055,AF059116,JN703180 |
| Dendrocygn a viduata | EUS85849,EU585712,FJ027502 | Anas platyalea | AF059084,AF059144,FJ027099 |
| Dendrocygn a javanica | NC_012844,NC_012844,C_012844 | Anas clYPEPETA | EU914154,AF059174,DQ434274 |
| Anser albiBRONS | NC_004539,NC_004539,C_004539 | Anas gibberifrons | AF059076,AF059136,JQ174015 |
| Anser anser | NC_01196,NC_01196,C_01196 | Anas bAHAMENSIS | EU914147,AF059120,JQ174013 |
| Anser brachyHYNCHUS | EUS85614,EU585767,GU79004 | Anas querquedula | AF059086,EUS85673,GU571723 |
| Anser indicus | EUS85619,EU585682,UG79002 | Anas penelope | AF059107,AF059167,JN703206 |
| Anser fabalis | NC_016922,NC_016922,C_016922 | Anas platyrhynchos | NC_009684,C_009684,C_009684 |
| Anser rossi | EU914156,EU585683,DQ434539 | Anas sLYSAENIS | AF059078,AF059138,FJ498830 |
| Anser canagica | NC_01196,NC_01196,C_01196 | Anas superciliosa | FJ498963,AF059152,JN801396 |
| Branta bernicla | EUS85628,EU585691,DQ434344 | Anas fomossa | NC_015482,NC_015482,NC_015482 |
| Branta canadensis | NC_007001,NC_007001,NC_007011 | Netta rustina | EUS85657,EUS85720,GU751988 |
| Branta leucopsis | EUS85630,EU585693,GU79003 | Aythya ferina | NC_000877,NC_000877,NC_000877 |
| Branta sandvicensis | EUS85632,EU585695,FJ498832 | Aythya affinis | EUS85621,EUS85684,DQ434310 |
| Coscoroba coscoroba | EUS85639,EU585702,FJ027452 | Aythya americana | NC_000877,NC_000877,NC_000877 |
| Cygnus aratrus | NC_012843,NC_012843,C_012843 | Aythya marila | EUS85625,EUS85688,DQ434333 |
| Cygnus columbianus | NC_007691,NC_007691,NC_007691 | Aythya nyroca | EUS85626,EUS85689,GQ481388 |
| Cygnus cygnus | EUS85643,EU585706,GU571854 | Somateria spectabilis | EUS85662,EUS85725,DQ434103 |
| Cygnus olor | EUS85645,EU585708,GU571856 | Somateria mollissima | AF512564,AF512568,DQ434751 |
| Tachyeres ptereres | AF059112,JQ408332,JN800206 | Melanitta perspicillata | EUS85652,EUS85715,DQ434655 |
| Sarkidiornis melanotus | EUS85660,EU585723,FJ028237 | Melanitta nigra | AF512563,AF512567,AY666338 |
| Chloephaga picta | AF512562,AF512566,FJ027353 | Bucephala albola | EUS85633,EUS85696,DQ434491 |
| Chloephaga poliolechuca | EUS85637,EU585700,FJ027355 | Bucephala clangula | AF512561,AF512565,EU525336 |
| Tadorna ferruginea | In this study | Bucephala islandica | EUS85635,EU58698,DQ434502 |
| Tadorna tadorna | AF059113,AF059173,GU571650 | Mergellus albellus | EUS85653,EUS85716,EU571480 |
| Cairina moschata | NC_010965,NC_010965,NC_010965 | Lophodytes cucullatus | EUS85650,EUS85713,AY666346 |
| Aix galericula | In this study | Mergus serrator | EUS56555,EUS57188,DQ434677 |
| Aix sponsa | AF059053,AF059114,DQ434254 | Mergus squamatus | NC_016723,NC_016723,NC_016723 |
| Lophotroctes specularoides | AF059102,JQ408334,FJ027113 | Mergus merganser | EUS85654,EUS85717,DQ434672 |
| Amazonetta brasiliensis | AF059054,AF059115,FJ027065 | Nomonyx dominicus | AF119165,AY747864,FJ027905 |
| Anas strepera | EU74791,AF059169,DQ434298 | Oxyura jamaicensis | EUS85658,EUS5721,AY666448 |

doi:10.1371/journal.pone.0109701.t007

**PLOS ONE** | www.plosone.org | 13 | November 2014 | Volume 9 | Issue 11 | e109701
Acknowledgments

We would like to thank Qianqian Sun for her kind help in sequence analysis. We also thank Guanghong Zhao and Lin Chen for their kind assistance in the laboratory work, and Meng Zheng for helping to collect the samples.

References

1. Donne-Gousse´ C, Laudet V, Hanni C (2002) A molecular phylogeny of anseriformes based on mitochondrial DNA analysis. Molecular Phylogenetics and Evolution 23, 339–356.
2. Gonzalez J, Dittmann H, Wink M (2009) Phylogenetic relationships based on two mitochondrial genes and hybridization patterns in Anatidae. Journal of Zoology 279, 310–318.
3. Livezey BC (1986) A phylogenetic analysis of recent anseriform genera using morphological characters. The Auk 103 (4), 737–754.
4. Livezey BC (1991a) A phylogenetic analysis and classification of recent dabbling ducks (tribe Anatini) based on comparative morphology. The Auk 108, 471–507.
5. Livezey BC (1991b) Phylogeny and evolutionary ecology of modern seaducks (Anatidae: Mergini). Condor 97, 233–255.
6. Dickinson EC (2003) The Havard and Moore Complete Checklist of the Birds of the World. 3rd Edition.Princeton University Press, Princeton, New Jersey.
7. Johnson P, Sorenson MD (1999) Phylogeny and biogeography of dabbling ducks (Genus: Anas): a comparison of molecular and morphological evidence. The Auk 116(3), 792–805.
8. Del Hoyo, Elliot JA, Sargatal J (1992) Handbook of the Birds of the World. Vol. 2. New World Vultures to Guineafowl. Lynx Edicions, Barcelona.
9. Livezey BC (1995b) Phylogeny and comparative ecology of modern shelducks and sheldgeese (Anatidae, Tadornini). Ibis 139, 51–66.
10. Liu G, Zhou LZ, Zhang LL, Liao ZJ, Xu WB (2013) The complete mitochondrial genome of scaly-sided merganser (Mergus squamatus) and implications for Anseriformes taxonomy,PLoS ONE 8(5): e63334:1–10.
11. Livezey BC (1995a) Phylogeny and comparative ecology of modern shelducks and sheldgeese (Anatidae, Tadornini). Ibis 139, 51–66.
12. Sraml M, Christidis L, Easteal S, Horn P, Collet C (1996) Molecular evidences for the relationship and biogeography of the Pacific black duck. A radiation within the genus Anas. The Auk 113, 985–993.
13. Ali S (1960) The Pink-headed duck Rhodonessa caryophyllacea and implications for Anseriformes taxonomy,PLoS ONE 8(5): e63334:1–10.
14. Lin CP, Danforth BN (2004) How do insect nuclear and mitochondrial gene substitution patterns differ? insights from Bayesian analyses of combined datasets. Molecular Phylogenetics and Evolution 30, 688–702.
15. Giso C, Izamelli F, Pesole G (2008) Evolution of the mitochondrial genome of Metazoa as exemplified by comparison of congeneric species. Heredity 101, 301–320.
16. Liu G, Zhou LZ, Gu CM (2012) Complete sequence and gene organization of the mitochondrial genome of scaly-sided merganser (Mergus squamatus) and phylogeny of some Anatidae species. Molecular Biology Reports 39: 2139–2145.
17. Boone JL (1999) Animal mitochondrial genomes. Nucleic Acids Research 27, 1767–1790.
18. Boone JL, Macey JR, Medina M (2005) Sequencing and comparing whole mitochondrial genomes of animals. In Molecular Evolution: Producing the Biochemical Data, Part b Volume 395. San Diego: Elsevier Academic Press Inc. 311–348.
19. Boone JL (2006) The use of genome-level characters for phylogenetic reconstruction. Trends in Ecology & Evolution 21, 439–446.
20. Dowton M, Castro LR, Austin AD (2002) Mitochondrial gene rearrangements as phylogenetic characters in the invertebrates the examination of genome ‘morphology’. Invertebrate Systematics 16, 345–356.
21. Masta SE, Boone JL (2008) Parallel evolution of truncated transfer RNA genes in arachnid mitochondrial genomes. Molecular Biology and Evolution 25, 949–953.
22. Rubinoff D, Holland BS (2005) Between two extremes: mitochondrial DNA is neither the Panacea nor the Nemesis of phylogenetic and taxonomic inference. System Biology 54, 952–961.
23. Desjardins P, Morais R (1990) Sequence and gene organization of the chicken mitochondrial genome: a novel gene order in higher vertebrates. Journal of Molecular Biology 212, 599–634.
24. Guan X, Silva P, Gynai KB, Xu J, Geng T, et al. (2009) The mitochondrial genome sequence and molecular phylogeny of the turkey, Meleagris gallopavo. Animal Genetics 40, 134–141.
25. Irwin DM, Kocher TD, Wilson AC (1991) Evolution of the cytochrome b gene of mammals. Journal of Molecular Evolution 32, 128–144.
26. Li H, Shao RF, Song F, Zhou XG, Yang QQ, et al. (2013) Mitochondrial genomes of two Barklice, Psococerus alphamasticus and Longicladus hyalophilus (Psocodea: Psocomorpha): contrasting rates in mitochondrial gene rearrangement between major lineages of psocodea, PLoS ONE 8(4), e61685.
27. Crochet PA, Desmarais E (2000) Slow rate of evolution in the mitochondrial control region of gulls (Aves: Laridae). Molecular Biology and Evolution 17(12), 1797–1806.
28. Taanman JW (1999) The mitochondrial genome: structure, transcription, translation and replication. Biochimica et Biophysica Acta (BBA) - Bioenergetics 1410, 103–123.
29. Delport W, Ferguson JW, Bloomer P (2002) Characterization and evolution of the mitochondrial DNA control region in hornbills (Bucerotiformes). Journal of Molecular Evolution 54, 794–806.
30. Slack KE, Janke A, Penny D, Arnason U (2003) Two new avian mitochondrial genomes (penguin and goose) and a summary of bird and reptile mitogenomic features. Gene 302, 43–52.
31. Ruokonen M, Kvist L (2002) Structure and evolution of the avian mitochondrial control region. Molecular Phylogenetics and Evolution 23, 422–432.

Author Contributions

Conceived and designed the experiments: LZZ GL. Performed the experiments: GL. Analyzed the data: GL. Contributed reagents/materials/analysis tools: LZZ. Wrote the paper: GL LZZ BL LLZ.