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The Mitochondrial Genome Sequence and Molecular Phylogeny of the Turkey, *Meleagris gallopavo*

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Summary

The mitochondrial genome (mtGenome) has been very little studied in the turkey (*Meleagris gallopavo*), for which there is no publicly available whole genome mitochondrial sequence. Here, we used PCR-based methods with 19 pairs of primers designed from the chicken and other species to develop a complete turkey mtGenome sequence. A total length of 16, 717 bp of the whole turkey mtGenome was obtained, with 85% similarity to chicken mtGenome. There were 13 genes and 24 RNA (22 tRNA and 2 rRNA) annotated. The mtGenome-based phylogenetic analysis suggests that the turkey is most closely related to the chicken, *Gallus gallus*, and quail, *Corturnix japonica*. Given the importance of the mitochondria genome, the present work adds to the growing genomic resources needed to define the genetic mechanisms that underlie some economic traits in the turkey.

Keywords

turkey; mitochondrial genome; phylogenetics

Introduction

The turkey, *Meleagris gallopavo*, is native to North America and exists widely as both an important wild bird and the second most widely consumed poultry meat species in developed countries. It is surprising that the turkey, an ecologically and agriculturally important species, lacks a whole mtGenome sequence, which is one of the reasons why the phylogenetic relationship between the turkey and other avian species, especially gallinaceous birds, is also very little understood. The complete turkey mtGenome sequence could provide an opportunity to use non-recombining sequences with diverse rates of evolution for turkey phylogenetics. The few avian phylogenetic studies that involved the turkey include that by Helm-Bychowski and Wilson (1986), which used restriction enzyme-based maps involving 161 restriction sites to describe relationships among the turkey and six other phasianoids that were different from those by traditional and protein-based comparisons. Using allozymes, Randi et al (1991) grouped phasianoids into two main categories, one including the turkey and the other the quails, with the guinea fowl distantly related to both groups. Using DNA-DNA hybridization, Sibley and Ahlquist (1990)

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reported, in broad terms, that based on DNA-DNA hybridization data and in contrast to some earlier reports involving morphology as well as mtDNA, galliform birds form two clades one of which include chicken, guinea fowl, Old World quail, and turkey. That work subsequently called for more data to permit resolution of the Phasianidae.

Because of the lack of consensus of the relationships among galliform birds, especially among the chicken, quail, and turkey, phylogenetics studies of these poultry species continue. In a recent study, Kaiser et al. (2007) reported that based on insertion events of *CR1* retrotransposable elements, the turkey was more closely related to quail and chicken but distant to the guinea fowl. Despite the use of these diverse genetic markers, our understanding of galliform relationships continues to be marginal and the phylogenies continue to be without congruence. The mtGenome sequence of the turkey will provide access to genes with different evolutionary rates than those that have been used to assess galliform phylogenies.

In addition to its utility for phylogeny of galliform birds, the sequence of the turkey mtGenome could be a useful tool for establishing the influence of the mitochondria and its genes on economically important phenotypes. Here, the primary objective was to sequence and annotate the turkey mtGenome. Further, the validity of the sequence and the utility of this genomic resource were evaluated by inter-species phylogenetic analysis.

Materials and methods

Mitochondrial genome sequencing

Two Blue Slate turkeys were used to develop two whole mtGenome sequences from PCR products or amplicons obtained using heterologous primers. The primers included universal oligos previously described by Sorenson et al. (1999) and those developed for the present work (VT Primers, Table 1). All but four primer-pairs used to generate amplicons that were sequenced are new. In addition to the conventional criteria for selecting primers, oligos were designed and chosen by Primer 3 (Rozen and Skaletsky, 1997) for their ability to produce overlapping amplicons of 2 to 4 kb. Primers were optimized at an annealing temperature of 56°C using the FailSafe™ PCR PreMixes kit according to the manufacturer's recommendation (Epicentre Technology, Madison, WI). Following optimization, the successful premix that produced a single amplicon was used to carry out PCR for the specific primer pair. For each primer-pair, at least two independent PCR products were purified and sequenced using both reverse and forward primers as previously described (Lin et al., 2006). Internal primers were also developed to complete the sequencing of some long-range PCR products. The internal primers also ensured that some regions of the turkey mtGenome were sequenced at least three times including in those in the regions were two or more primers produced overlapping amplicons. The sequences were assembled using a combination of bioinformatics tools including of Phred, Phrap and Consed (Gordon et al., 1998).

Sequence validation and annotation

The whole genome sequence was validated at two levels: multiple sequencing of each region and sequence comparison with GenBank mtGenome sequences from other birds. An additional validation of the turkey mitochondrial DNA sequences was based on sequence similarity as revealed by a Clustal-X (Thompson et al., 1997) based multi-alignment using mitochondrial DNA sequences publicly available for *Coturnix japonica* (NC_003408), *Numida meleagris* (NC_006308), and *Gallus gallus gallus* (NC_007236). To annotate the sequence, BLAST 2 (Tatusova & Madden, 1999) and GeneDoc (Nicholas et al., 1997) were used to compare the assembled sequence to the database of mtGenome sequences.

Additionally, ORF-Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) and vsfold4 (http://www.rna.itchiba.ac.jp/~vsfold/vsfold4/), a DNA sequence viewer and annotation tool and an RNA secondary structure prediction program, respectively, were also used to further annotate and/or validate the BLAST 2 and GeneDoc annotation of the turkey mitochondrial DNA sequence.

Phylogenetic analysis

Using the rationale that different segments of the mtGenome undergo varying rates of evolution, the two phylogenetic analyses carried out to evaluate the turkey's relationship with other birds were based on the 16S rRNA and the coding region that included 12 protein-coding genes but not ND6 which is encoded on a different strand, two rRNA genes and 19 tRNA genes. A total of 57 species, including the turkey, chicken, quail, and guineafowl were used (supplementary Table S1). In the 16S rRNA-based phylogenetic analysis, the American alligator and human sequences were used as outgroups for rooting. Based on the results of 16S rRNA phylogenetic analysis, Anseriformes were selected as outgroup for the coding region-based phylogenetic analysis. The outgroups were selected based on conventional criterion and as advanced by Caspers et al. (1997) and van Tuinen et al. (2000). Following Clustal-X (Thompson et al., 1997) based multiple sequence alignment, phylogenetic analysis, tree construction, model selection and statistical tests were carried out as described above. One thousand bootstrap replicates were used to assess the confidence in the grouping in minimum evolution, neighbor-joining, and maximum parsimony methods (Felsenstein & Kishino, 1993). Again, using the Akaike information criterion as the basis for selecting models for the 16S rRNA and coding region phylogenetic analyses, the General Time Reversible + Gamma + Proportion Invariant (GTR+G+I) model of evolution were selected with gamma distribution shape parameter of 0.6002 and 0.9201, respectively.

Results

Full mtDNA sequence of the turkey

A total sequence length of 16,717 bp, representing the BS turkey mtGenome was sequenced, validated and annotated. The sequence has been submitted to GenBank and assigned accession number EF153719. The sequence showed 85, 84 and 83% average similarity with the chicken, Japanese quail and guinea fowl mtGenome sequences in GenBank, respectively. The length of turkey mtGenome was similar as that of the chicken, Japanese quail and guinea fowl mtGenomes that are 16,785, 16,697 and 16,726 bp, respectively. The variable region or the D-loop was 1,164 bp, which was homologous to the only turkey mtDNA sequences available in GenBank from previous investigations including those by Drovetski (2002), Lucchini et al. (2001), and Mock et al. (2001).

Within the turkey mtGenome sequence, the most frequent nucleotide in the H-strand was A, followed by C, T and G. In the chicken, the most frequent nucleotide is a C, followed by A, T, and G. The GC content of 43.5% observed in the turkey sequence is also consistent with that reported in other birds ranging from *Apteryx hastii* to *Aythya Americana* with 42 and 48%, respectively. Though the GC content of the turkey mtGenome appears to be consistent with the average in vertebrates, it is lower than the chicken (46%) and goose (47%).

As expected, 13 protein coding genes and 24 RNA (22 tRNA and 2 rRNA) were identified within the turkey mtGenome sequence (Table 2). While 12 of the protein coding genes were located on the H strand, the gene for NADH dehydrogenase subunit 6 (ND6) was on the L-strand. On the other hand, while the sequences for the two rRNAs (12S and 16S) were on the H-strand, those for the 9 tRNAs were located on the L-strand. These results are consistent with the mtGenome organization in some vertebrate species (Pereira, 2000). A

characteristic that was found previously in only 46 other avian mtGenomes (Mindell et al., 1998a) was an extra base at position 174 of the gene for NADH, subunit 3 (ND3). It was suggested by Harlid et al. (1997) that the additional nucleotide causes a reading frame change which results in multiple stop codons in the ND3 gene sequence. However, since the frame shifting does not affect ND3 function, Mindell et al. (1998a) hypothesized that birds might have, as yet unknown, a mechanism such as translational frame shifting or RNA editing to correct this anomaly.

Phylogenetic analyses

The minimum evolution, maximum likelihood, maximum parsimony, and neighbor-joining trees, constructed from both 16S rRNA- and the combined sequences of 12 protein-coding genes, two rRNA genes and 19 tRNA genes were congruent and showed a closer relationship between the turkey, chicken, and quail but relatively more distant to the guinea fowl, also a gallinaceous bird (supplementary Figures 1 and Figure 2). Except for the relationship between the turkey and the guinea fowl, those defined here among the galliformes are consistent with previous reports, thus providing additional support for the vailidity of the sequence described here (Kimball et al., 1999; Sibley and Ahlquist, 1990).

Discussion

The animal mtGenome is generally considered to be under selection for both small size and a conserved gene order (Rand & Harrison, 1986; Quinn & Wilson, 1993; Boore, 1999). Animal mitochondrial genomes rarely contain either introns or intergenic spacers (Quinn and Wilson 1993, McKnight and Shaffer 1997). The turkey, an important agricultural and model avian species remains one of many birds for which there is no publicly available whole mtGenome DNA sequence. Here, we have described the turkey mtGenome sequence and showed that it is similar in length and nucleotide composition to that of most other birds. Compared to mammalian species, only a limited number of birds, which generally exceed mammals in the total number of species, have had their whole mtGenome sequenced. While the mtGenome gene content and gene order is remarkably stable across vertebrate species, the avian species are an exception to this stability. For example, the avian species have several unusual features in their mitochondrial DNA, such as the lack of the traditional origin of replication for the light strand and the as-yet unidentified splicing function to repair the one-base insertion found in the ND3 gene in most avian mtGenomes. Several gene order rearrangements have occurred in avian mtGenomes, primarily affecting the area around the ND6 gene. For instance, the genus Amazona has two duplicated control regions which are found between $tRNA^{Glu}$ and $tRNA^{Phe}$ (Eberhard et al. 2001). Other birds including the forest falcon, kestrel, and the woodpecker, have a mitochondrial genome with a different gene order that involves two control regions: one between $tRNA^{Thr}$ and $tRNA^{Pro}$, and a second region between $tRNA^{Glu}$ and $tRNA^{Phe}$ (Mindell et al. 1998b).

The new sequence was also used to evaluate the phylogenetic relationships between the turkey and other birds. To date, the most extensive comparisons of Galliformes have involved partial sequences from mitochondrial (Dimcheff et al., 2002) and nuclear genes (Smith et al., 2005; Kaiser et al., 2007) as well as the hybridization results of Sibley and Ahlquist (1990). The relationships between the turkey and other Galliformes were in general agreement with Sibley and Ahlquist (1990), Smith et al (2005) and Dimcheff et al. (2000, 2002). Briefly, these studies showed the turkey as a sister to clades containing *Gallus* and *Coturnix* species. Further, and as observed here, *Numida* was basal to the *Coturnix* and *Gallus* clades but more distant to the turkey. The high bootstrap values provide strong support for the relationships defined here between the turkey and other Galliformes.

Though the phylogenetic analysis done here was to provide examples of the potential of the turkey mtGenome sequence and validation of the sequence quality, it should be noted that the placement of *G. varius* using GenBank sequences described by others appears to be inconsistent with some previous studies. Helm-Bychowski and Wilson (1986) and Fumihito et al (1996) showed that *G. varius* should not be more closely related to *G. gallus* than the Ceylon or Gray Junglefowl. It is possible that the *G. varius* (GenBank accession number: NC_007238) sequence was from a sample contaminated with domestic chicken DNA or the amplified samples were mixed up and assembled incorrectly.

In summary, the turkey mtGenome sequence was developed and used to evaluate the genetic relatedness between the turkey and other birds. Though our analyses of relationships among birds were limited in the extent of statistical parameters included, the mtGenome sequence provides a resource for extensive phylogenetic analyses. Since the evolutionary relationships among Galliformes continues to be without a general consensus, the whole mtGenome sequence described here will provide an additional tool for generating more data needed to understand the turkey's relationship with other gallinaceous birds. The sequence will also facilitate assignment of function to the mtGenome, especially the role of mitochondrial genes in variation in economically important phenotypes in the turkey. Furthermore, it will provide a foundation to begin to more widely evaluate the role of the genome of this important organelle in the turkey.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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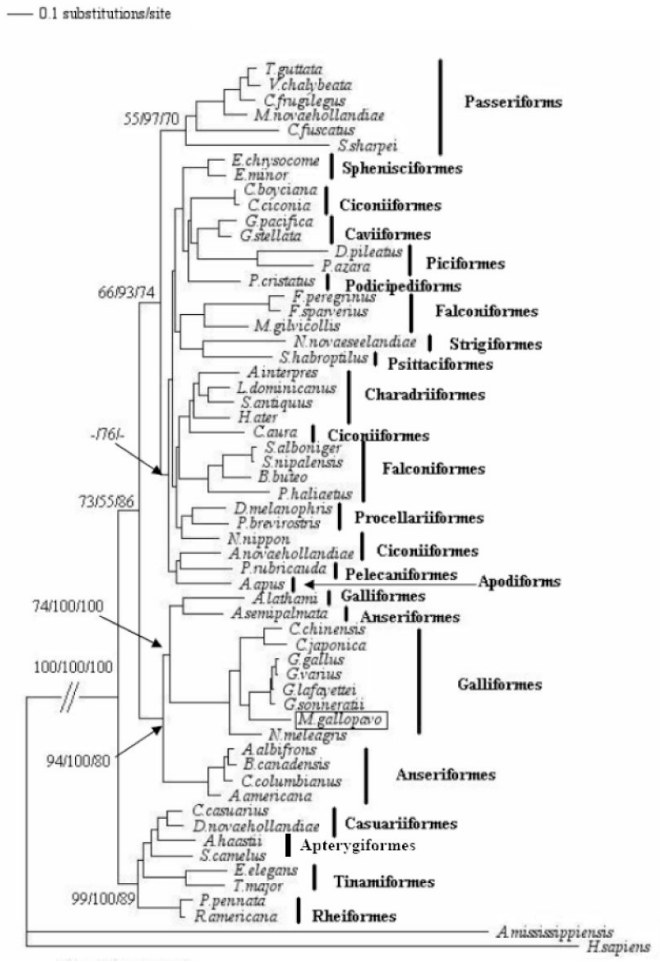

Figure 1.

A maximum likelihood-based phylogenetic tree constructed using 16S rRNA from 56 avian species described in Supplementary Table S3. The tree was rooted using *A. mississippiensis* (American alligator) and *H. sapiens* (human). The tree was congruent with those from neighbor joining, minimum evolution, and maximum parsimony methods. Confidence of the groupings was estimated using 1000 bootstrap replications. The Arabic numeral at the base of a node is the bootstrap value. The Arabic numerals at the base of a node are the bootstrap values derived from the maximum parsimony, neighbor-joining, and minimum-evolution analysis, respectively. Bootstrap values lower than 50% are not shown.

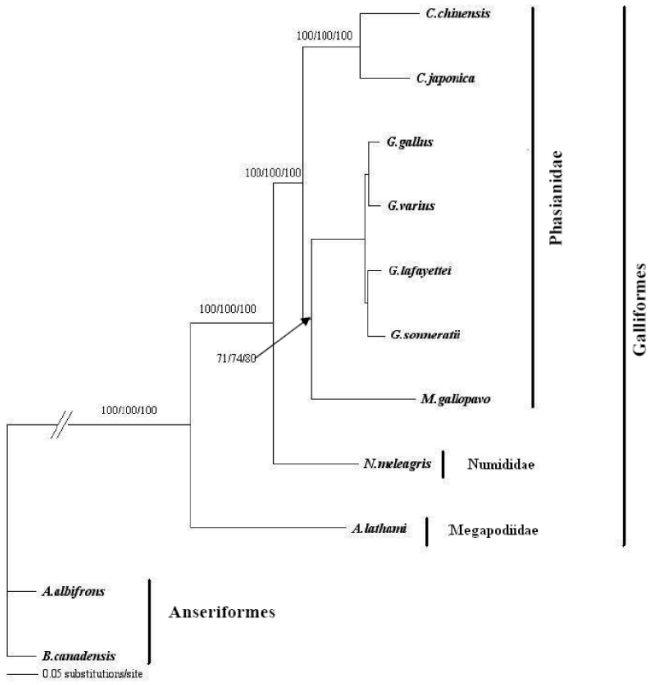

Figure 2.

A maximum likelihood phylogenetic tree based on the mitochondrial coding region that included 12 protein-coding genes, two rRNA genes, and 19 tRNA genes from Galliformes. Anserigormes were used as outgroup for rooting. The tree was congruent with those from neighbor joining, minimum evolution, and maximum parsimony methods. Confidence of the groupings was estimated using 1000 bootstrap replications. The Arabic numerals at the base of a node are the bootstrap values derived from the maximum parsimony, neighbor-joining, and minimum-evolution analysis, respectively. Bootstrap values lower than 50% are not shown. Though the tree shows a G. varius relationship that is consistent with the amino acid tree of Nishibori et al. (2005), it is not congruent with those of Helm-Bychowski and Wilson (1986) and Fumihito et al. (1996).

Table 1

Sequences of primers used in the polymerase chain reaction

Primer ID	Primer sequence
TL1F*	5′-AARCCMGAATGRTAYTTYCTWTTYGC-3′
TL1R*	5′-GTGGCTGGCACARGATTTACC-3′
TL1in1F	5′-AACCCGCGTACAAGCTCTAA-3′
TL1in1R	5′-TCTTCAGTGCCATGCTTTTG-3′
TL1in2F	5′-TCCTACCCCCAACATCCATA-3′
TL1in2R	5′-GCTTAAGGTTAATTACTGCTGAATACC-3′
TL2F*	5′-YAAAGCATGRCACTGAA-3′
TL2R*	5′-TYTCAGGYGTARGCTGARTGCTT-3′
TL3Fnew	5′-GCCCTTGGAAGGAGGATTTA-3′
TL3Rnew	5′-CAGTTCTGCACGGATTAGCA-3′
TL3in1F*	5′-CAACCGTACCGTAAGGGAAA-3′
TL3in1R	5′-CGTCTGGTTTGCACTCAGAA-3′
TL3in2F	5′-AGCCCCCTCGAAAAAGAATA-3′
TL3in2R	5′-AGGCCGGCTAGAGATAGGAG-3′
TL3in3F	5′-GTGTTCTCGTGCAAAAACGA-3′
TL3in3R	5′-GGTGGTGGGATTTTGAGATG-3′
TL3in4F	5′-CTCGGCAAATGCAAAAGACT-3′
TL3in4R	5′-TGGGAGGTTCAGGAAACTTG-3′
TL4F*	5′-CCYCTGTAAAAAGGWCTACAGCC-3′
TL4in2F	5′-CATAAAACCCCCAGCACTGT-3′
TL4Rnew2	5′-TAATTTGCTGGGTCGAAACC-3′
TL4in3F	5′-TGGAGGTCTTACGGGAATTG-3′
TL4in3R	5′-GGGTTGTTTGAGCGAGAAGA-3′
TL4in4F	5′-GAAGGAATCGAACCCTCACA-3′
TL4in4R	5′-CTGCTTTCGGTTTCCTTCTG-3′
TL4in5F	5′-GCCTGATCCTCCCTCCTATC-3′
TL4in5R	5′-ATGTCCGGCTGTAAGGTTTG-3′
TL5Fnew	5′-CAAACAACCCCAGACACAGA-3′
TL5Rnew	5′-GGCTGAGTAGGAAGGCAGTTT-3′
TL5in1F	5′-AAAACCAAACCCCATCCTTC-3′
TL5in1R	5′-GGGTTGTAGGCCTCGTGTAA-3′
TL6F*	5′-ATCCRTTGGTCTTAGGARCCA-3′
TL6R*	5′-CTTCANTYTTTGGYTTACAAGRCC-3′
TL6in1F	5′-ACAAGCAATCCAACCAGACC-3′
TL6in1R	5′-GTTTGGGATTGAGCGTAGGA-3′
TL6in2F	5′-TCCGCATGACACTGCTAGTC-3′
TL6in2R	5′-GATGAAGAAGAATGAGGCGC-3′

* Universal primers described by (Sorenson et al., 1999) were also used.

Table 2

Sequence annotation of the mitochondrial genome of the turkey, *Meleagris gallopavo*.

Genes	Location	Size (bp)	Initial Codon	Terminal Codon
tRNA-Phe	1-68	68		
12S ribosomal RNA (12S rRNA)	69-1040	971		
tRNA-Val	1041-1113	73		
16S ribosomal RNA (16S rRNA)	1114-2731	1618		
tRNA-Leu	2732-2805	74		
NADH dehydrogenase subunit 1 (*ND1*)	2821-3795	975	ATG	TAA
tRNA-Ile	3796-3867	72		
tRNA-Gln	3875-3945*	71		
tRNA-Met	3945-4013	69		
NADH dehydrogenase subunit 2 (*ND2*)	4014-5054	1041	ATG	TAG
tRNA-Trp	5053-5131	79		
tRNA-Ala	5138-5206*	69		
tRNA-Asn	5209-5280*	72		
tRNA-Cys	5283-5347*	65		
tRNA-Tyr	5347-5417*	71		
Cytochrome oxidase subunit 1 (*COX1*)	5419-6969	1551	GTG	AGG
tRNA-Ser	6961-7035*	75		
tRNA-Asp	7038-7106	69		
Cytochrome oxidase subunit 2 (*COX2*)	7108-7791	684	ATG	TAA
tRNA-Lys	7793-7861	69		
ATPase subunit 8 (*ATPase8*)	7863-8027	165	ATG	TAA
ATPase subunit 6 (*ATPase6*)	8018-8701	684	ATG	TAA
Cytochrome oxidase subunit 3 (*COX3*)	8701-9487	787	ATG	TGC
tRNA-Gly	9485-9553	69		
NADH dehydrogenase subunit 3 (*ND3*)	9554-9905	352	ATG	TAA
tRNA-Arg	9907-9974	68		
NADH dehydrogenase subunit 4 light-chain (*ND4L*)	9975-10271	297	ATG	TAA
NADH dehydrogenase subunit 4 (*ND4*)	10265-11645	1381	ATG	TGC
tRNA-His	11643-11711	69		
tRNA-Ser	11713-11777	65		
tRNA-Leu	11779-11849	71		
NADH dehydrogenase subunit 5 (*ND5*)	11850-13667	1818	ATG	TAA
Cytochrome b (*Cytb*)	13671-14813	1143	ATG	TAA
tRNA-Thr	14816-14884	69		
tRNA-Pro	14887-14956*	70		
NADH dehydrogenase subunit 6 (*ND6*)	14964-15484*	521	ATG	TAG
tRNA-Glu	15486-15553*	68		

*Coded on the complementary (L) strand.