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Complete mitochondrial genome sequence and gene organization of Chinese indigenous chickens with phylogenetic considerations

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ABSTRACT. In this study, we sequenced the complete mitochondrial DNA of Chinese indigenous Jinhu Black-bone and Rugao chickens. The two chicken mitochondrial genomes were deposited in GenBank under accession Nos. KP742951 and KR347464, respectively. The complete mitochondrial genomes of Jinhu Black-bone and Rugao chickens were sequenced and found to span 16,785 and 16,786 bp, respectively, and consisted of 22 tRNA genes, two rRNA genes (12S rRNA and 16S rRNA), 13 protein-coding genes, and one control region (D-loop). The majority of genes were positioned on the H-strand,

and the ND6 and eight tRNA genes were found to be encoded on the L-strand. The mitogenomes showed a similar gene order to that of the published *Gallus gallus* genome, as neither included a control region. The overall base composition of the genome of the two chickens was A = 30.22/30.28%, G = 13.57/13.49%, T = 23.74/23.76%, and C = 32.48/32.48%. Nucleotide skewness of the coding strands of the two chicken genomes (AT-skew = 0.12, GC-skew = -0.41) was biased towards T and G. Phylogenetic analysis revealed 29 subspecies, and the molecular genetic relationship among the 29 subspecies was identical to that of traditional taxonomy.

Key words: Chicken; Mitochondrial genome; Phylogenetic relationship

INTRODUCTION

Mitochondrial (mt) genomes are short in length, as they include short intergenic regions and lack introns. mtDNA has been used extensively as a genetic marker for phylogenetic analyses and is an ideal model of gene rearrangement and genome evolution. Complete mtDNA genomes have been reported in many studies on chicken, relying on the (partial) control region sequence (CR; displacement loop [D-loop]: nucleotide positions 1-1232; NC_007235). However, the relatively small size of the CR limits the resolution of mtDNA phylogeny. Because the mutation rate in this region is higher than in coding regions, high levels of recurrent mutations can blur the structure of the matrilineal genealogy. The D-loop is found in the main non-coding region of the mtDNA molecule in the CR or D-loop region (Pereira et al., 2004). mtDNA is a major control site for mtDNA expression and is important in maternal inheritance. This region varies in different populations and contains essential transcription and replication elements that can be used to investigate maternal inheritance and human evolution.

Jinhu Black-bone and Rugao chickens are indigenous Chinese breeds distributed in southeast China, and are bred for different purposes. The Jinhu Black-bone chicken is a dual-purpose chicken bred for ornamental value, whereas the Rugao Yellow chicken is bred for its meat and eggs. Therefore, it is important to obtain the complete mitogenome sequences to resolve their phylogenetic positions and interrelationships within Gallus. In the present study, we determined the complete mtDNA sequence of these two chicken breeds and described the organization of the genome, nucleotide composition, gene order, and codon use. Furthermore, we analyzed the molecular phylogenetic relationships between these and a further 27 chicken breeds. Our results will be useful for studies on population genetic structure, stock identification, evolution and phylogeny, and conservation genetics.

MATERIAL AND METHODS

Ethics statement

All animals used in the study were housed and handled following the guidelines for experimental animals established by the Council of China. Experimental procedures were approved and conducted in accordance with the guidelines of the China Agricultural University Animal Care and Use Committee.

Sample collection and DNA extraction

Two Jinhu Black-bone chickens and two Rugao yellow chickens were obtained from local conservation farms in Jiangsu, China. All chickens were young, healthy females. Blood samples (~10 mL) were drawn from the jugular vein by a licensed veterinarian and placed in K3 EDTA vacuum tubes. Total genomic DNA was extracted from all samples using the Qiagen DNeasy Blood & Tissue Kit (Valencia, CA, USA) and stored at -20°C until use.

Polymerase chain reaction (PCR) amplification and sequencing

The full mitogenomes of Rugao and Jinhu Black-bone chickens were amplified by PCR in 23 overlapping fragments, using previously published conserved primers (Bao et al., 2008). PCR amplifications were performed in a total volume of 25 µL containing 12.5 µL premixed enzyme, 2.5 µL DNA template, 1.0 µL of each primer (10 ppm), and 8.0 µL sterile deionized water. The PCR protocol was as follows: initial denaturation at 94°C for 3 min, followed by 35 cycles in an ABI 9700 Cycler (Applied Biosystems, Foster City, CA, USA) at 94°C for 30 s (denaturation), 60°C for 30 s (annealing), 72°C for 1 min (extension), and a final extension at 72°C for 10 min. The PCR products (5 µL) were detected on 1% agarose gel electrophoresis to confirm the length of the amplified fragment. Each amplicon was purified using the QIAquick PCR Purification Kit (Qiagen) and subjected to automated sequencing using an ABI 3730 sequencer (Applied Biosystems).

Another dataset used in this study was collected from the National Center for Biotechnology Information (NCBI) and included 19 local Chinese and 8 Indian chicken breeds.

Sequence annotation and genomic analysis

All sequences were inspected and assembled using DNASTAR and DNAMAN7.0. Target sequences were corrected by BLAST on the NCBI database. Protein-coding genes were determined by sequence comparisons using specialized BLAST at the NCBI database site. Next, these 13 genes were checked with the known complete mtDNA sequence of closely related species, including Daweishan Mini chickens (Yan et al., 2016), Huang Lang chickens (Yu et al., 2016), Taoyuan chickens (Liu et al., 2016b, and Xuefeng black-boned chickens (Liu, et al., 2016a). Twenty-one tRNA genes and their anticodons were annotated online using tRNAscan-SE Search Server v.1.21 (http://lowelab.ucsc.edu/tRNAscan-SE/) (Lowe and Eddy, 1997). Translation initiation and termination codons were identified by comparison with the mitochondrial genomes of known *Gallus gallus* codons. The complete mitochondrial genome was annotated with the Sequin program. Nucleotide composition was calculated for the entire genome using the EditSeq program included in the Lasergene software package, and the C, G, A, and T frequencies of the four bases were computed using the following formulas: GC-skew = (G - C) / (G + C) and AT-skew = (A - T) / (A + T).

Phylogenetic analysis

A GenBank clustering analysis was performed on all chicken breeds (Table 1). These datasets were used to construct the phylogenetic tree for the Chinese and Indian

chicken breeds. The complete mitochondrial genomes of 27 other chicken breeds were downloaded from the GenBank database in order to elucidate the phylogenetic position of the Chinese chickens. The phylogenetic tree was reconstructed using the unweighted pair group method with arithmetic means (UPGMA) in MEGA 6.0 with 1000 bootstrap replicates.

RESULTS

Complete mitochondrial genome DNA sequences

The complete mitochondrial genome sequences of Rugao and Jinhu Black-bone chickens were determined by PCR analysis and deposited in GenBank under accession Nos. KP742951 and KR347464. Twenty-three primer sets were used to determine the mtDNA sequences of Rugao and Jinhu Black-bone chickens, which were 16,786 and 16,785 bp, respectively. These lengths were similar to those reported for other chickens, including Gushi (16,785 bp), Xianju (16,784 bp), Xuefeng (16,785 bp), and Hengshan Yellow (16,785 bp) chickens (http://www.ncbi.nlm.nih.gov/).

Genome organization and structure

The overall nucleotide composition was 30.28 and 30.22% A, 23.76 and 23.74% T, 32.48 and 32.48% C, and 13.49 and 13.57% G in the order C > A > T > G for the Rugao and Jinhu Black-bone chickens, respectively. The A + T percentages (54.04 and 53.96%) were greater than the G + C percentages (45.97 and 46.05%). The mitochondrial gene arrangements of Jinhu Black-bone and Rugao yellow chicken are shown in Figures 1 and 2, respectively. The gene arrangement and transcriptional direction were similar to those of typical *G. gallus* mitogenomes (Desjardins and Morais, 1990). As shown in Figures 1 and 2, neither mtDNA genome contained a CR, but both contained a single-extra cytidine base in the ND3 gene. The total lengths of the 13 protein-coding genes in Jinhu Black-bone and Rugao yellow chickens were 11,438 and 11,437 bp, respectively, accounting for 68.14 and 68.14% of the entire mitogenome. All 13 genes of the two chickens were located on heavy chains, with the exception of ND6, which was found to be located on a light chain. Only the cytochrome c oxidase subunit I (COI) gene used GTG as a start codon, whereas the other 12 genes used ATG. The COX3 and ND4 genes were inferred to end with an incomplete stop codon (T--); the ND2 gene stopped with TAG; the COI gene terminated with AGG; and the other protein-coding genes all used a TAA termination codon. The mitochondrial genomes of the Rugao yellow and Jinhu Black-bone chickens contained 22 tRNA genes, which ranged from 66 to 76 bp in length; 14 were on a heavy chain and 8 were on a light chain (Table 1). The CRs of the Rugao yellow and Jinhu Black-bone chicken mitochondrial genomes were 1232-1233-bp long. Thirteen gene overlaps were found, of which three were on the H-strand and 10 were on the L-strand. Five intergenic spacers were detected; one spacer was on the H-strand, and four were on the L-strand. The overlaps and intergenic spacers were all 1-9 bp in size at each site (Table 1). The longest overlap (9 bp) was located between tRNA-Leu and ND1, and the largest intergenic spacer of 9 bp occurred between ATP8 and ATP6. The overlap and intergenic spacers of Rugao yellow and Jinhu Black-bone chickens were of different lengths than those of the other chicken breeds (Wang et al., 2016).

Figure 1. Mitochondrial gene arrangement in Jinhu Black-bone chickens.

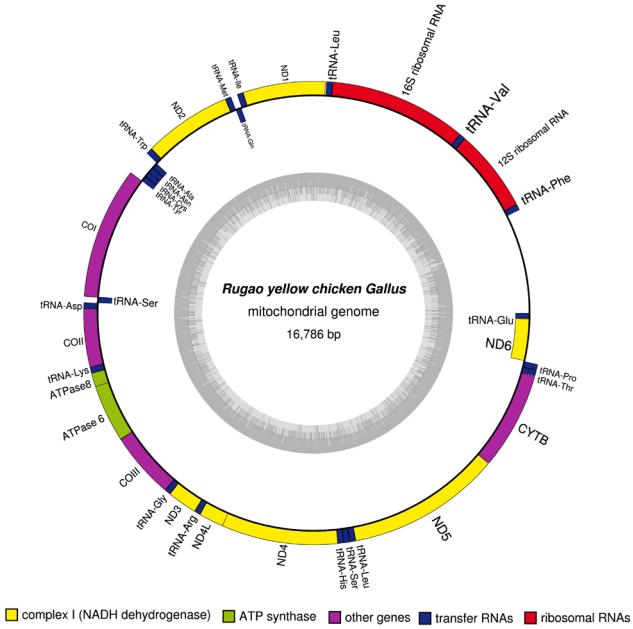

Figure 2. Mitochondrial gene arrangement in Rugao yellow chickens.

Nucleotide skewness values for the *G. gallus* coding strands (AT-skew = 0.12 and GC-skew = -0.41) were biased towards A and C. Such an A-T rich pattern reflects a typical sequence feature of the vertebrate mitochondrial genome (Mayfield and McKenna, 1978; Hiendleder, et al., 1998). The nucleotide composition of the coding strand was biased towards T and A in C, which is consistent with most *G. gallus* mitogenomes (Jiang et al., 2013).

Table 1. Complete chicken mitogenomes deposited in GenBank.

No.	Species	GenBank accession No.	Length (bp)
1	Gushi Chicken	GU261678.1	16,785
2	Chigulu Chicken	GU261719.1	16,785
3	Tengchongxue Chicken	GU261689	16,785
4	Nixi Chicken	GU261711.1	16,785
5	Dongan Black Chicken	KM886936	16,785
6	Guangxi partridge Chicken	KP681580	16,785
7	Xuefeng Chicken	GU261675.1	16,785
8	Gallus gallus Spadiceus Chicken	NC_007235.1	16,785
9	Wenshanshandi Chicken	GU261699	16,785
10	Guangxi Three-buff Chicken	KP681581	16,785
11	Jiangbian Chicken	GU261714	16,785
12	Wuding Chicken	GU261676	16,788
13	Tibetan Chicken	DQ648776	16,783
14	Taoyuan Chicken	KF981434.1	16,784
15	Xianju Chicken	GU261677	16,784
16	Tulufan Chicken	GU261683	16,784
17	Huanglang Chicken	KF954727	16,785
18	Cenxi Classical Three-buff Chicken	KM433666.1	16,786
19	Dongan Yellow Chicken	KM886937.1	16,785
20	Nicobari Brown Chicken	KP211422	16,775
21	Tellicherry Chicken	KP211424	16,775
22	Aseel Chicken	KP211418	16,775
23	Ghagus Chicken	KP211419	16,775
24	Haringhata Chicken	KP211420	16,775
25	Kadaknath Chicken	KP211425	16,775
26	Nicobari Black Chicken	KP211421.1	16,775
27	Red Jungle Fowl Chicken	KP211423.1	16,775

Phylogenetic analysis

A phylogenetic tree, using the complete mtDNA and CRs of Rugao yellow and Jinhu Black-bone chickens, was constructed using the UPGMA method (Tamura and Nei, 1993) and included two datasets. The first dataset was provided from our sequences, and the second was downloaded from NCBI. A bootstrap consensus tree inferred from 1000 replicates was used to represent the taxonomy of the chickens (Felsenstein, 1985). Branches corresponding to partitions reproduced with <50% bootstrap replicates collapsed. Evolutionary distances were computed using the maximum composite likelihood method (Tamura et al., 2007) and were in units of the number of base substitutions per site. The analysis involved 32 nucleotide sequences, and the first, second, third, and non-coding codon positions were included. All positions containing gaps and missing data were eliminated. Clustering analyses were conducted using MEGA6.0 (Tamura, et al., 2013).

The chicken matrilineal phylogeny was constructed using the complete mitochondrial genome (Figure 3) and CR (Figure 4). In addition to the two Chinese indigenous chicken breeds used in this study, we also downloaded the mitochondrial genome sequences of 27 other chicken breeds from NCBI, which are summarized in Table 2. The cluster tree results showed that the 29 chicken breeds could be divided into two groups using the complete

chicken mitochondrial genomes. One group included the Chinese chicken breeds and the other group included the Indian chicken breeds. Additionally, the Chinese indigenous chickens were assigned into five groups based on the geographic region and skin color. The Wuding chicken was alone in a group, which may have occurred because local farmers do not buy chickens, instead selling chickens that they have bred over a long time.

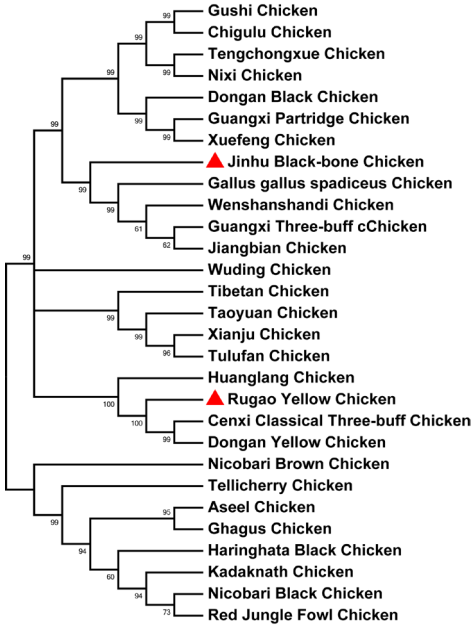

Figure 3. Evolutionary relationships between chicken breeds based on their complete mitochondrial genomes.

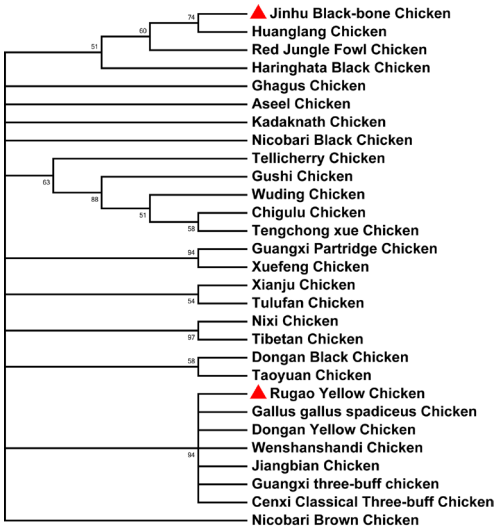

Figure 4. Evolutionary relationships between chicken breeds based on the mitogenome control region.

Table 2. Characteristics of protein coding genes and structural RNAs in the mitochondrial genome of Rugao yellow and Jinhu Black-bone chickens.

Genes	Position		Length (bp)	Codons		Anti-codon	Intergenic nucleotide	Strand
	Start	End		Start	Stop			
D-loop	1/1	1233/1232	1233					
tRNA-Phe	1234/1233	1303/1302	70/70			GAA	0	H
rRNA	1304/1302	2278/2277	975/976				0	H
tRNA-Val	2279/2278	2351/2350	73/73			TAC	0	H
rRNA	2352/2351	3973/3972	1622/1622				0	H
tRNA-Leu	3974/3973	4047/4046	74/74			TAA	9	H
ND1	4057/4056	5031/5030	975/975	ATG	TAA		0	H
tRNA-Ile	5032/5031	5103/5102	72/72			GAT	5	H
tRNA-Gln	5109/5108	5179/5178	71/71			TTG	-1	L
tRNA-Met	5179/5178	5247/5246	69/69			CAT	0	H
ND2	5248/5247	6288/6287	1041/1041	ATG	TAG		0	H
tRNA-Trp	6287/6286	6362/6361	76/76			TCA	0	H
tRNA-Ala	6369/6368	6441/6436	69/69			TGC	0	L
tRNA-Asn	6441/6440	6513/6512	73/73			GTT	1	L
tRNA-Cys	6515/6514	6580/6579	66/66			GCA	-1	L
tRNA-Tyr	6580/6579	6650/6649	71/71			GTA	1	L
COI*	6652/6651	8202/8201	1551/1551	GTG	AGG		-7	H
tRNA-Ser	8194/8193	8268/8267	75/75			TGA	2	L
tRNA-Asp	8271/8270	8339/8338	69/69			GTC	1	H
COX2	8341/8340	9024/9023	684/684	ATG	TAA		1	H
tRNA-Lys	9026/9025	9093/9092	68/68			TTT	1	H
ATP8	9095/9094	9259/9258	209/209	ATG	TAA		-9	H
ATP6	9250/9249	9933/99332	684/684	ATG	TAA		0	H
COX3	9932/9932	10716/10715	785/784	ATG	T--		0	H
tRNA-Gly	10717/10716	10785/10784	69/69			TCC	0	H
ND3	10786/10785	11137/11136	352/352	ATG	TAA		1	H
tRNA-Arg	11139/11138	11206//11205	68/68			TCG	0	H
ND4L	11207/11206	11503/11502	297/297	ATG	TAA		-5	H
ND4	11497/11496	12874/12873	1378/1378	ATG	T--		0	H
tRNA-His	12875/12874	12943/12492	69/69			GTG	0	H
tRNA-Ser	12944/12944	13010/13008	67/65			GCT	0	H
tRNA-Leu	13011/13010	13081/13080	71/71			ATG	0	H
ND5	13082/13081	14899/14898	1818/1817	ATG	TAA		4	H
Cyt b	14904/14903	16046/16045	1142/1142	ATG	TAA		4	H

DISCUSSION

The mtDNA sequences of the Rugao yellow and Jinhu Black-bone chickens were determined independently for each individual animal. The mtDNA sequences derived from the two local Chinese chickens matched and were registered in the NCBI database. The complete mtDNA sequences of the two chickens were 16,785 and 16,786 bp in size. Both sequences contained 13 protein-coding genes, 22 tRNA genes, two rRNA genes, and one CR. The genome organization and structure were the same as those of the three other chicken breeds (Gushi chicken, Xuefeng chicken, and Hengshan Yellow chicken). The sequence lengths differed slightly, mainly due to changes in the CR. The overall A + T contents of the Rugao yellow and Jinhu Black-bone chicken mtDNA genomes were 54.04 and 53.96%, which were similar to those of other chicken breeds, such as Daweishan Mini chickens (Yan et al., 2016), *Gallus domesticus* Brisson (Wang et al., 2016), and Huanglang chickens (Yu et al., 2016). The Rugao yellow and Jinhu Black-bone chickens possessed 13 gene overlaps and five intergenic spacers, which differs from other chicken breeds (Liu et al., 2016a,b).

Organization of the CR, 13 protein-coding genes, 22 tRNA genes, and two rRNA genes of the Rugao yellow and Jinhu Black-bone chickens was identical to the reported organization in other chickens. The protein-coding genes started with ATG or GTG. The 13 protein-coding genes possessed a variety of termination codons, including TAA, TAG,

AGG, and T--. The incomplete termination codon (T) indicates that the TAA transcription process was terminated after the PolyA tail was affixed (Clayton, 1991). This stop codon is reported commonly in many *G. gallus* breeds. In this study, we describe the organization and structure of the mitochondrial genome of two local Chinese chicken breeds and determined the molecular evolutionary relationships between these Chinese indigenous chickens and Indian chicken breeds. Neighbor-joining, maximum-likelihood, and UPGMA trees constructed using the maximum composite likelihood method showed the same results. Therefore, in this report, only the UPGMA tree is presented.

In conclusion, the complete mitochondrial genome DNA sequences of Jinhu Black-bone and Rugao yellow chickens were determined to be 16,785 and 16,786 bp in length. Our results will be of value for studies on population genetic structure, stock identification, evolution and phylogeny, and conservation genetics of *G. gallus* and related chicken breeds.

Conflicts of interest

The authors declare no conflict of interest.

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