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Genetic Relationships among Different Breeds of Chinese Gamecocks Revealed by mtDNA Variation*

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ABSTRACT : There are currently five primary breeds of Chinese gamecock, the Henan, Luxi, Tulufan, Xishuangbanna and Zhangzhou. Though there is historical evidence of cockfighting in China dating as far back as 2,800 years, the origin and genetic relationships of these breeds are not well understood. We used sequence variation from the mtDNA cyt*b* gene and control region (1,697 bp) to examine the domestication history and genetic relationship of the Chinese gamecock. From 75 samples (14-16 per breed) we found 34 haplotypes, and 45 variable nucleotides. Phylogenetic reconstruction indicated multiple origins of the gamecock breeds. The breeds in the north and center of China, Tulufan, Luxi and Henan, clustered together in a haplogroup and may have the same ancestor. However the southern breeds, Zhangzhou and Xishuangbanna clustered into two isolated haplogroups, suggesting another two origins of Chinese gamecock. Meanwhile, extensive admixture was also found because samples from different breeds, more or less, were always grouped together in the same clades. Based on these results, we discuss the possibilities of multiple origins of gamecock breeds, from both ancestral gamecocks as well as other domestic chickens and red jungle fowl. (**Key Words** : Gamecock, mtDNA, Variation, Phylogenetic Tree, Domestication)

INTRODUCTION

Domestic chickens have been dated archaeologically in China as far back as 6,000 B.C. (West and Zhou, 1988). At present, there are over 80 local breeds of chickens distributed extensively throughout China. The gamecock (douji in Chinese), a colorful and distinct group, was developed for entertainment rather than food. According to documents in "Lie Zi", "Shi Ji", "Zuo Zhuan" as well as archaeological evidence, gamecocks can be traced back about 2,800 years to an area around the Wei and Yellow Rivers in central China (Wu, 1993a). The number of gamecocks in China has increased during the past 20 years and currently is estimated at over 50,000, since the cockfight is becoming more popular recently in China. Gamecock breeds are characterized by high levels of aggression, muscular and robust bodies, almost vertically long and strong legs, a short tail and a large head. The face is fiery red and the shanks are thick and long. At present these gamecocks are generally classified into five main breeds distributed in distinct geographical areas. The Tulufan gamecock is found in Xinjiang, an area in north-west China and the Xishuangbanna gamecock is found in Yunnan, a south-western province. The Zhangzhou gamecock is located in south-eastern Fujian province while Luxi and Henan gamecocks are found in central China.

Many studies have suggested that the Southeast Asian red jungle fowl is the common ancestor of all chickens (Crawford 1990; Akishononomiya et al., 1994, 1996; Dundes, 1994). That the red jungle fowl remains at several sites had been interpreted as evidence that it had a much wider distribution in ancient China than at present, with the assumption that domestication had multiple origins (Ho, 1977). The controversy has been maintained for a long time between supporters for monophyletic and multiple origins of domestic chicken (Crawford, 1990, 1995; Akishononomiya et al., 1994, 1996; Liu et al., 2006), and the multiple origin hypothesis was well documented recently (Liu et al., 2006). It was also reported recently that the domesticated chicken was not only originated from the red jungle fowl but also the grey jungle fowl (Eriksson et al.,

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2008). However, the origins and ancestors of gamecock are still unclear even after some research on gamecock origins in Japan (Komiyama et al., 2003, 2004).

Mitochondrial DNA (mtDNA) has been widely used in biodiversity studies of birds (Baker and Marshall, 1997; Mindell et al., 1997; Moore and Defilippis, 1997; Wayne et al., 2002) including chickens (Komiyama et al., 2003, 2004) as well as other domestic animals (Odahara et al., 2006; Sasazaki et al., 2006; Lei et al., 2007; Liu et al., 2007; Wang et al., 2007; Li et al., 2008). The advantages of mtDNA as molecular markers include maternal inheritance (Lansman et al., 1983), a higher rate of evolution than in single-copy nuclear DNA (Brown et al., 1979), the lack of recombination, and variable evolutionary rates of genes within the genome itself (Aquadro and Greenberg, 1983; Cann et al., 1984). Traditionally, coding genes of the mitochondrial genome have been reserved for phylogenetic studies above the species level (Moore and Defilippis, 1997), whereas the control region has been considered more suitable for inter-specific population studies (Baker and Marshall, 1997). The control region evolves three to five times more rapidly than the remainder of the mitochondrial genome (Aquadro and Greenberg, 1983; Cann et al., 1984).

In this study, in order to elucidate the origins and domestication history of gamecock, we studied genetic variability and evolutionary relationships of the five primary Chinese gamecock breeds based on the mtDNA control region and cyt*b* sequences.

MATERIALS AND METHODS

Samples and DNA extraction

Blood samples from 13-15 adult females and 1 male were collected for each of 5 Chinese gamecock breeds, Henan, Luxi, Tulufan, Xishuangbanna and Zhangzhou. The samples (70 females and 5 males) were collected from breeders in the original location for each breed. Genomic DNA was extracted from 30 μl of fresh blood via haemolysis, proteinase K incubation, phenol, phenol/chloroform (1 v/1 v) and chloroform extraction, and ethanol precipitation. The purified DNA was then resuspended in 300 μl TE.

Primers and PCR amplification for mtDNA

Four primer pairs (Table 1) were used to amplify part of the mtDNA control region and cyt*b* gene which were located at 1-824 nt and 14,848-15,911 nt on mitochondrial DNA. PCR was carried out in a total volume of 20 μl containing 50 ng DNA, 1.5 mM $MgCl_2$, 2 μl of PCR 10×buffer (*Applied Biosystems*), 200 μM dNTP, 0.5 U *Taq* DNA polymerase (*Applied Biosystems*) and 0.5 μl of each primer (10 μM). The PCR reaction consisted of heating the mixture for 5 min at 95°C, followed by 35 cycles of 1 min at 94°C, 1 min at annealing temperature (Table 1), and 1 min at 72°C, and then a final 10 min extension at 72°C.

Direct sequencing of PCR products

For sequencing, the PCR products were cleaned by adding 1 μl ExoSAP-IT (Amersham Biosciences) to every 3 μl of PCR product and by incubating the reaction for 15 min at 37°C and for 15 min at 80°C. Five μl of cleaned PCR product was used as a template in DYEnamic cycle sequencing reactions containing 4 μl DYEnamic sequencing premix (Amersham Biosciences) and 1 μl sequencing primer (10 μM). Sequencing cycles (29) consisted of denaturation for 20 sec at 95°C, annealing for 15 sec at 50°C and primer extension for 1 min at 60°C. Excess dye terminators were removed from the sequencing reactions by gel filtration and the products were run on a MegaBACE capillary sequencing instrument (Amersham Biosciences). DNA chromatograms were edited and base calls checked using SEQUENCHERTM 4.2.2 (Gene Codes).

Statistical analysis

Gamecock sequences were aligned with other *Gallus* sequences from GenBank using BLAST on website http://www.ncbi.nlm.nih.gov/BLAST/. The nucleotide diversity (π) of each breed was calculated and the standard error was estimated with 1,000 bootstrap replicates. The migration rate (to estimate the extent of gene flow and introgression), and pair-wise Fst values with statistical test for each haplogroup and breed were assessed with Arlequin 2.0 (Schneider, et al.). Mega 2.1 (Tamura et al., 2007) was used for phylogenetic reconstruction using the NJ method

Table 1. Primers for mtDNA

Primer	Primer sequence (5'-3')	Annealing temperature for PCR condition
Cyt*b*1	L-15302: CATGGGGCCAAATATCATTC	62°C for 1 min
	H-15911: TACTGGTTGGCTTCCGATTC	
CR2	L421: TCACGAGAGATCAGCAACCC	55°C for 1 min
	H824: AACCATAACCAAATGCGATCC	
Cyt*b*2	L14848: CTTTCGCCCTCACAATCCTT	55°C for 1 min
	H15415: AATCGGGTAAGGGTTGGGTT	
CR1	L16750: AGGACTACGGCTTGAAAAGC	55°C for 1 min
	H547: TGTGCCTGACCGAGGAACCAG	

Table 2. Pairewise Fst estimates (below diagonal) and migration rates N_em (above diagonal) of breeds

Breeds	Henan	Luxi	Tulufan	Xishuangbanna	Zhangzhou
Henan	-	3.504	12.668	2.777	0.535
Luxi	0.125*	-	1.986	0.612	0.320
Tulufan	0.038	0.201*	-	3.212	0.960
Xishuangbanna	0.153**	0.450**	0.135**	-	0.680
Zhangzhou	0.483**	0.610**	0.342**	0.424**	-

* Significant ($p<0.05$). ** Significant ($p<0.01$).

with 1,000 bootstrap replicates under a Kimura 2-parameter model.

RESULTS

Sequence variations

Totally, 1,697 bps of high quality sequences in the control region and *cytb* were bi-directionally sequenced and used in the current analyses. Gamecock and other *Gallus* sequences were nearly identical (97-100%). Thirty-four haplotypes and 45 mutation sites were present in the 75 samples. The Tulufan gamecock was the most variable breed with 12 haplogroups from 14 samples. The nucleotide diversity (π) varied between 0.0067 in Luxi and 0.0137 in Tulufan. The diversity results are shown in Figure 1.

Admixture among breeds

The breed pairwise Fst values varied from 0.038 ($p = 0.126$) for Tulufan and Henan to 0.610 ($p<0.01$) for Zhangzhou and Luxi (Table 2). The pair-wise Fst value for Henan and Tulufan was the lowest (0.038) and was not significantly different ($p = 0.126$).

Migration analysis was used to infer sample exchange and introgression among the breeds. The migration rates (N_em) ranged from 0.320 (Zhangzhou and Luxi) to 12.668 (Henan and Tulufan) (Table 2), indicating admixture among breeds is quite common.

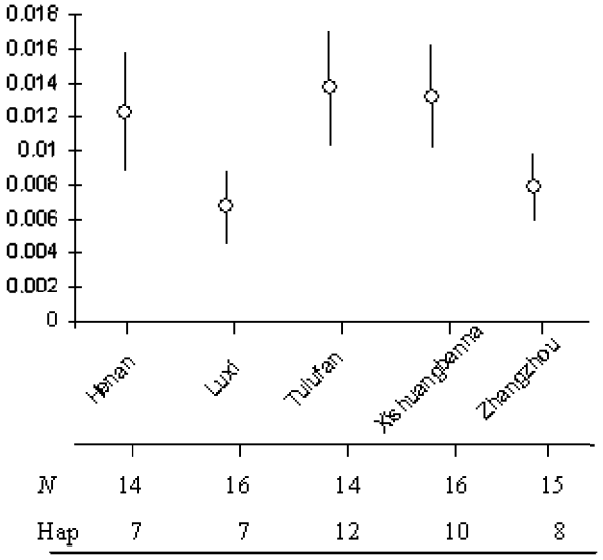

Figure 1. Genetic diversity within breeds. The graph shows estimates (and standard errors) of nucleotide diversity within breeds. Below the graph are sample size (N) and number of the haplotypes in each breed.

Phylogenetic analyses

A phylogenetic tree was constructed using the 75 sequences of Chinese gamecocks with one Ceylon jungle fowl (*Gallus lafayetii*) as an outgroup (Figure 2). The NJ phylogeny recovered 4 haplogroups (I, II, III and IV) for Chinese gamecocks, with two subgroups (I and II) in haplogroup I as well as one subgroup (III) in haplogroup IV. The Fst values among the four haplogroups ranged from 0.529 (between haplogroup III and IV) to 0.886 (between haplogroup II and III) with significant differences (all $p< 0.01$) (Table 3). Each haplogroup or subgroup was primarily comprised of individuals from the same or adjacent breeds, except for haplogroup III, which was comprised of all breeds except Luxi (Figure 1).

DISCUSSION

Domestication history of gamecock

There are two hypotheses regarding gamecock domestication. i) There was a single, monophyletic origin that gave rise to all modern gamecock breeds. ii) Gamecock breeds were developed independently in different areas from wild and domestic chicken varieties, i.e. multiple origins.

Komiyama et al. (2003) found that Japan local gamecock breeds originated directly from gamecock in China or Southeast Asia but not wild birds or other domestic chickens. That could be part of the story of gamecock domestication.

Four distinct haplogroups were defined in this study. So, similar to the results of the Liu's study (2006), which suggested multiple origins of the domestic chicken, our study indicates there are also at least four isolated genetic

Table 3. Pairwise Fst estimates of the haplogroups

Haplogroup	1	2	3	4
1	-			
2	0.850**	-		
3	0.862**	0.887**	-	
4	0.801**	0.798**	0.529**	-

** Significant ($p<0.01$).

groups of gamecocks. The breeds in the north and center of China, Tulufan, Luxi and Henan, cluster together into haplogroup I, suggesting that they may have originated from the same common ancestor. Our genetic-based data is concordant with historical locations of the three breeds (Wu, 1993a, b). The archaeological evidence and history records (West and Zhou, 1988; Wu, 1993a) showed that Central China was one of the original places for gamecock, and in the current study we found that the birds from Luxi and Henan in this region were also grouped into an isolated haplogroup. This indicated that Henan or Luxi had its own origin that could be from local domestic chicken but not from other gamecock breeds or from red jungle fowl because there was no evidence for the red jungle fowl distributing in the wild around Central China (West and Zhou, 1988). However, Zhangzhou and Xishuangbanna, both breeds from Southern China, clustered into two isolated haplogroups (II and IV respectively). In this case, geographical barriers in the form of numerous mountains and rivers may have prevented ancient trafficking of gamecocks and acted as a barrier to admixture. This suggests another two origins of Chinese local gamecocks.

Therefore, we can conclude that the gamecock could have been domesticated from both more ancient gamecock breeds and from other chicken breeds, i.e. multiple origins. Unlike domestic chickens, it seems impossible to elucidate

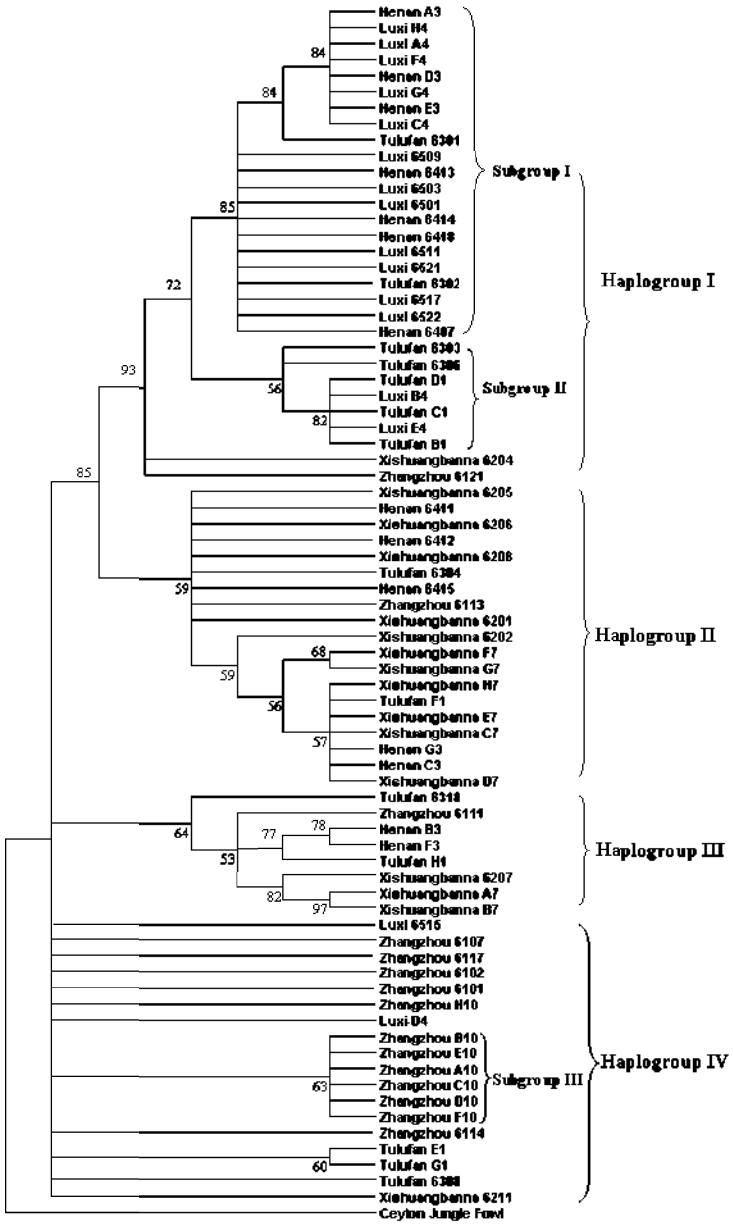

Figure 2. Majority rule neighbor-joining phylogenetic tree of the 75 Chinese gamecock individuals. Bootstrap support (based on 1,000 replicates) is shown, and nodes with less than 50% support were collapsed. The Ceylon Jungle Fowl was used to root the tree.

whether gamecock breeds originated from wild birds or domestic chicken directly.

Extensive admixture among Chinese local gamecock breeds

Chickens are easier to transport live than many other domesticated animals, and this is especially true of gamecock varieties. Admixture within chicken breeds is common, as is hybridization with other *Gallus* species (Nishibori et al., 2005). It is therefore not surprising to find that almost all haplogroups (with the exception of haplogroup III) are comprised of individuals from Tulufan, Luxi, Henan, Zhangzhou and Xishuangbanna gamecock breeds. Similar results were also found in Liu's study (Liu et al., 2006). However, there is some evidence of subclustering within these haplogroups, with these subgroups often consisting primarily of individuals from the same or geographically adjacent breeds.

Recently, popularity of cockfighting has increased in China, and many breeders have introgressed individuals from other Chinese, and even international, breeds in their attempts to improve fighting traits. This recent admixture could account for both the lack of monophyly in the haplogroups, as well as the high polymorphisms found in these breeds.

Origin and genetic relationship of Chinese gamecocks

Chinese gamecocks have been historically traced to the areas near the Wei and Yellow Rivers in central China, in Henan province, from roughly 2,800 years ago (Wu, 1993a). Henan province was the site of numerous ancient Chinese capitals, and the lords of these ancient population centers enjoyed cockfighting. Fighting cock breeds likely originated from Henan and spread to other places. Henan and Luxi gamecocks share numerous common characteristics such as the same feather color, similar body size, and so on, and they were grouped in one subgroup in the mtDNA phylogenetic tree. This morphological and genetic similarity is consistent with the close geographic distribution of these breeds, and likely stems from repeated admixture and common history.

Although the Tulufan samples were distributed across almost all clades in the tree, more than half of the Tulufan samples clustered in haplogroup I, suggesting that the Tulufan gamecock shares a closer genetic relationship with Henan (Fst = 0.038) or Luxi (Fst = 0.201) breeds, and indicating a possible origin in the Henan or Luxi provinces. The Silk Road, which was an important trade route in Chinese history until several hundred years ago, linked this region of central China during the Tang dynasty (618 A.D. - 907 A.D.) with western nations and, because Tulufan sits geographically in the middle of the Silk Road, it is reasonable to hypothesize that merchants and travelers brought gamecocks from central China along this route.

The Xishuangbanna region is located in Southwestern China adjacent to Thailand, and is geographically isolated from other Chinese provinces by many mountains, such as the Wuling, Hengduan, and Xuefeng mountains. These mountains presented a formidable barrier to ancient human trade, and probably prevented trafficking of gamecock individuals among people in other areas. Genetically, this barrier to trade is evident in the clustering of the Xishuangbanna individuals into an isolated breed. However, some individuals of Xishuangbanna were found in other haplogroups; the possible explanation for this is the modern sample exchanges with other gamecock breeds.

Zhangzhou region, located on the southeast coast of China, was an important port several hundreds years ago. Compared with the inland breeds, the Zhangzhou gamecock has a shorter history, and local records show that they probably appeared during the Ming dynasty around 1,500 A.D. (Wu, 1993b). The haplotypes of Zhangzhou gamecocks are significantly different from other breeds, especially for the subgroup III which only included Zhangzhou samples in haplogroup IV, indicating its unique origin. The Zhangzhou port linked the region to many areas through trade, links that continued into the Ming Dynasty. These trade links allowed for gamecock transport and admixture with many Southeast Asian breeds (Wu, 1993b), contributing to the unique genetic background of Zhangzhou gamecocks.

The possible explanation for the breed-mixed haplogroup III is the introduced gamecocks. In China, in order to improve fighting traits, breeders have commonly introduced gamecocks from Southeastern Asia, mainly from Thailand, to intercross with the Chinese local breeds during the last two decades. Even if samples in haplogroup III and Zhangzhou could have been both introduced from Southeastern Asia, they were grouped into different haplogroups. The reason for this could be the different times at which they were introduced. Zhangzhou were introduced in the Ming dynasty several hundred years ago and formed a local breed in China, whereas modern introduced samples contributed to haplogroup III.

The understanding of domestication history and basic genetic relationships of gamecock should help the modern breeder conserve and improve the breeds which have been passed down through the generations.

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