

## Phylogenetic Relationship of *Tetraogallus* Inferred from Sequences of Cytochrome b Gene

Yifeng Gong<sup>1</sup>, Jinfu Wang<sup>1</sup>, Hongyan Li<sup>1</sup>, Li Wang<sup>2</sup>, Runlin Ma<sup>3</sup>

1. Ministry of Education Key Laboratory of Xinjiang Endemic and Ethnic Disease, Shihezi University, Shihezi, Xinjiang 832003, China
2. Department of Animal Science and Technology, Shihezi University, Shihezi, Xinjiang 832003, China
3. Institute of Genetics and Developmental Biology, Chinese Academy Sciences, Beijing 100101, China

**Abstract:** A phylogenetic tree of Neighbor-joining (NJ) for the sequences of cytochrome b (Cyto b) gene was constructed to study the phylogenetic relationship of the genus *Tetraogallus*. Numbers near the branches were bootstrap probability (BP) values coming from 1000 replications. Some bootstrap probability values were 92% (*Coturnix chinensis* / *Coturnix japonica*), 100% (*Tetraogallus altaicus* / *Tetraogallus himalayensis*), and 100% (*Tetraogallus altaicus* / *Tetraogallus himalayensis* / *Tetraogallus tibetanus*). The overall average distance was 0.112, and average genetic distance among *Tetraogallus* was 0.042. The genera *Alectoris*, *Coturnix*, *Tetraogallus* and *Pucrasia* formed a monophyletic group. The two genera, *Alectoris* and *Coturnix*, should have a common ancestor, which was the sister taxon of the ancestor of the genus *Tetraogallus*. The interspecific genetic distances of the genus *Tetraogallus* were 0.012 (*T. altaicus* vs *T. himalayensis*), 0.067 (*T. himalayensis* vs *T. tibetanus*) and 0.068 (*T. altaicus* vs *T. tibetanus*) respectively. By combining the geographical distribution pattern, morphological characteristics and the genetic distance among these species of the genus *Tetraogallus*. It can be inferred preliminarily that Tibetan snow cocks were the primitive species among the three breeds; and that 1.7 million years ago, one subspecies of Tibetan snow cocks, *tibetanus* with a deep body color, distributing in the southwest of Xinjiang and the midwest of Tibet, migrated towards the west and entered the Himalayan Mountain to become the present Himalayan snow cocks; and that later, another subspecies of Himalayan snow cocks, *koslowi*, owning a light body color and often emerging in the Altai Mountain and the east of Kunlun Mountain, evolved into Altai snow cocks. [Life Science Journal. 2005;2(1):85-89] (ISSN: 1097-8135).

**Keywords:** snow cock; cytochrome b gene; phylogeny; mitochondrial DNA

### 1 Introduction

Snow cocks, the herbivore birds with high medicinal value inhabiting in the highest sea level in the world, are the children of the *Tetraogallus* under *Galliform*, *Phasianidae*. Nowadays, five species of snow cock have been found in Eurasia only. Except the foreign species, caspian snow cock (*T. caspius*) and Caucasian snow cock, the other three species including Altai snow cock (*T. altaicus*), Himalayan snow cock (*T. himalayensis*) and Tibetan snow cock (*T. tibetanus*), can all be seen in Xinjiang Uygur autonomous region of China (*T. caucasicus*) (Zheng, 2002). All of them had been listed as the international first-grade endangered and national second-protected wildlife. Presently, foreigners have little research about them except some researchers of Soviet Union who once had a shallow study on them at the beginning of 20th century. In the 1990s, people began to re-

search their domestication and reproduction in Xinjiang, Gansu and Qinghai. In China, researches were promoted at the beginning of 1960s, and have been limited by the domains of their ecological distribution and process. Now, we know that snow cocks are an excellent group for biological studies. However, the phylogenetic relationships among the snow cock species, their phylogenetic position and evolution process are still a secret that impedes our further researches of snow cocks.

Mitochondrial DNA can be used as a molecular marker since people have paid overwhelming attention to its traditional characteristics such as rigorous maternal inheritance (Gyllensten, 1985), rapid evolution (Vawter, 1986) and non-rearrangement (Cann, 1987). In recent years, the polymorphic analyses of mitochondrial DNA have become the effectual means for phylogeny reconstruction and taxonomy (Kirchman, 2000; Meyer, 1990). For example, cytochrome b (Cyto b) gene of mitochon-

drial DNA, is widely used for avian molecular phylogenetics at the levels of species and genus (Tuinen, 2000), which can be used to resolve the problems bequeathed by morphological taxa (Avice, 1994). In this study, snow cocks and their close related genera were selected as the experimental materials in order to study the phylogenetic position of the genus *Tetraogallus*, understand the phylogenetic relationships among the available snow cock species (the foreign species of *T. caspius* and *T. caucasicus* are very difficult to find and sample, and the related sequence data can not be found in other literatures), and infer the evolution process of the *Tetraogallus* by combining the molecular data, distribution patterns and morphological traits.

## 2 Materials and Methods

### 2.1 Materials and nucleotide sequences

Altai snow cocks and Himalayan snow cocks were captured in the Altai Mountain and the areas of Tianshan, respectively. The blood samples were collected. The other sequence data were retrieved from GenBank as shown in Table 1.

**Table 1.** The sequence data used in this study<sup>a</sup>

Code	Latin name	English name	Accession No
AG	<i>Alectoris graeca</i>	Rock Partridge	ZA8772
CA <sup>b</sup>	<i>Crossoptilon auritum</i>	Blue Eared-Pheasant	AF534552
CC	<i>Coturnix chinensis</i>	Blue-breasted Quail	NC004575
CJ	<i>Coturnix japonica</i>	Japanese Quail	NC003408
FF	<i>Francolinus francolinus</i>	Black Francolin	AF013762
PM	<i>Pucrasia macrolopha</i>	Koklass Pheasant	AF028800
TT1 <sup>c</sup>	<i>Tetraogallus tibetanus</i>	Tibetan snow cock	AY563128
TT2 <sup>c</sup>	<i>Tetraogallus tibetanus</i>	Tibetan snow cock	AY563130
TT3 <sup>c</sup>	<i>Tetraogallus tibetanus</i>	Tibetan snow cock	AY563133

Notes:<sup>a</sup> The information included the supplementary materials' Latin names, English names and sequence accession numbers in the GenBank, and, at the same time, the codes for these supplementary materials were formed by the two first letters of their Latin names in this study. <sup>b</sup> This species was selected as outgroup in this study. <sup>c</sup> The digitals behind the codes represent the individuals we selected in this study.

### 2.2 Extraction of total genomic DNA

The blood samples were digested with SDS/Protease K. Then the supernatant was extracted with phenol/chloroform. Finally, ethanol precipitation would be preformed to condense the total genomic DNA. The concentrated DNAs were dis-

solved in TE (pH 8.0) and stored at  $-20^{\circ}\text{C}$  for later use (Ausubel, 1995).

### 2.3 PCR amplification

Total genomic DNA was extracted from blood using a modified protocol, and used as the template in the polymerase chain reaction (PCR) amplifications. The primers were designed using DNAMAN software according to the published Himalayan snow cock Cyto b sequence (Accession No. AY678108). The upstream primer (5'-CTATAC-TACggCTCCTACCTg-3') and downstream primer (5'-gTTTgggATTgAgCgTA ggATg-3') were used in this study. Then, reactions were conducted in volumes of 50  $\mu\text{l}$ , typically with a amplification profile as follows: 3 min at  $95^{\circ}\text{C}$ , 35 cycles of 45 sec at  $95^{\circ}\text{C}$ , 45 sec at  $52^{\circ}\text{C}$ , and 3 min at  $72^{\circ}\text{C}$ , followed by a final extension at  $72^{\circ}\text{C}$  for 10 min using the HiFi Ready-to-use PCR kit (Sangon, Cat. #SK 2073, USA) on PTC-100<sup>TM</sup> Thermocycler.

### 2.4 Purification, cloning, and sequencing of PCR products

PCR products were purified from agarose gels using the EZ-10 spin column PCR product purification kit (BBI, Cat. #BS363, USA) and cloned directly into pGEM-T-easy vector (Promega, Cat. #A3600, USA). Sequencing was carried out with an ABI 3100 Genetic Analyzer, using the BigDye Terminator Cycle Sequencing Kit according to the manufacturer's protocols.

### 2.5 Sequence analysis and phylogenetic tree construction

Mitochondrial Cyto b gene sequences from Altai and Himalayan snow cocks were generated in this study. The other data was obtained from GenBank or from the literatures (Table 1). All nucleotide sequences were aligned with the CLUSTAL X (version 1.8) multiple alignment program and refined manually. Phylogenetic analysis was performed with MEGA version 2.1 (Kumar, 2001) and TREE PUZZLE version 5.0. We applied two different methods of phylogenetic analysis, Maximum parsimony (MP) and Neighbor joining (NJ), to ensure that our analyses were fit enough for the reality. Phylogenetic trees were inferred from the ML distances calculated with Kimura 2-parameter with *Crossoptilon auritum* as outgroup.

### 3 Results and Discussion

Snow cocks are an excellent kind of birds for biological studies. There are five species of this genus living in Eurasia only, and *T. altaicus*, *T. himalayensis* and *T. tibetanus* can be seen in China except *T. caspius* and *T. caucasicus*. Traditionally, *Tetraogallus* were classified into different species according to their morphological characteristics and geographical distributions. Tibetan snow cocks, one of these species, can be divided into six subspecies whose distribution areas are composed of Himalayan Mountain, Pamir Plateau, Mishmi Mountain and Tibetan Plateau. Nowadays, it is said that there are 0.2 million Tibetan snow cocks in Tibet. Altai snow cocks are composed of two subspecies, which can be discovered from Altai Mountain to the Northwest Territories of Mongolia. Himalayan snow cocks include four subspecies, which have a wide distribution including Afghanistan, Turkey to Nepal, and the northwest territories of China (Zheng, 2002). Nowadays, only shallow researches have been done. However, the evolutionary relationship among the Snow cocks still remain unclear, and even the evolution process of the whole genus is sometimes in doubt.

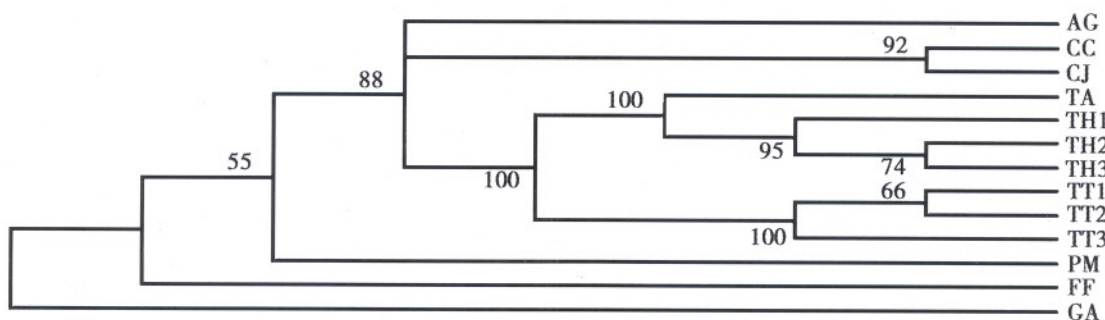
The overall average distance was 0.112, and average genetic distance among *Tetraogallus* was 0.042. The genetic distances between *C. auritum*

and all the other species was the biggest one of all the genetic distances in the same lines. The interspecific genetic distances of the genus *Tetraogallus* were 0.012 (*T. altaicus* vs *T. himalayensis*), 0.067 (*T. himalayensis* vs *T. tibetanus*) and 0.068 (*T. altaicus* vs *T. tibetanus*), respectively (Table 2). It is well known that phylogenetic reconstruction benefited from increased character inclusion, which provided all characters sharing a common evolutionary history. In recent avian genetic evolutionary researches, people propose that the genetic distance of Cyto b gene is bigger than 0.01 (>0.01) between species and smaller than 0.01 (<0.01) between subspecies (Krajewski, 1996). So, the result suggested that Altai snow cocks and Himalayan snow cocks had an intimate relationship, and Altai snow cocks and Tibetan snow cocks had a distant relationship among the three species. The length of mtDNA Cyto b gene sequence in our sampled species was 535 base pairs, and the average base composition of *Tetraogallus* was 25% thymine (T), 35% cystine (C), 27.5% adenosine (A), and 13% guanine (G), and the T and A contents (52%) was higher than that (47.7%) of C and G. This composition was very similar to that of other Snow cocks. At the same time, we found that no gaps were introduced into the Cyto b gene alignment, and there were 227 sites that were variable ones, and conservative sequence composing of 113 base pairs was discovered.

**Table 2.** Genetic distance (Lower-left) and standard error (Upper-right) analyzed by MEGA version 2.1 with Kimura 2-parameter<sup>a</sup>

	AG	CC	CJ	FF	PM	TA <sup>b</sup>	TH1 <sup>c</sup>	TH2 <sup>c</sup>	TH3 <sup>c</sup>	TT1 <sup>c</sup>	TT2 <sup>c</sup>	TT3 <sup>c</sup>	CA
AG		[0.018]	[0.018]	[0.017]	[0.017]	[0.018]	[0.017]	[0.018]	[0.017]	[0.017]	[0.017]	[0.017]	[0.017]
CC	0.141		[0.016]	[0.018]	[0.018]	[0.018]	[0.018]	[0.018]	[0.018]	[0.018]	[0.018]	[0.018]	[0.019]
CJ	0.139	0.121		[0.019]	[0.018]	[0.018]	[0.018]	[0.018]	[0.018]	[0.018]	[0.017]	[0.018]	[0.019]
FF	0.136	0.150	0.161		[0.016]	[0.017]	[0.017]	[0.017]	[0.017]	[0.017]	[0.017]	[0.017]	[0.016]
PM	0.129	0.149	0.151	0.112		[0.017]	[0.017]	[0.017]	[0.017]	[0.017]	[0.017]	[0.016]	[0.016]
TA	0.135	0.143	0.138	0.127	0.133		[0.005]	[0.005]	[0.005]	[0.012]	[0.012]	[0.012]	[0.019]
TH1	0.131	0.143	0.143	0.129	0.133	0.011		[0.003]	[0.003]	[0.012]	[0.012]	[0.012]	[0.019]
TH2	0.135	0.145	0.145	0.132	0.136	0.013	0.006		[0.003]	[0.012]	[0.012]	[0.012]	[0.019]
TH3	0.133	0.143	0.143	0.127	0.133	0.011	0.004	0.006		[0.012]	[0.012]	[0.012]	[0.019]
TT1	0.126	0.139	0.138	0.134	0.129	0.068	0.067	0.070	0.068		[0.002]	[0.003]	[0.019]
TT2	0.124	0.137	0.136	0.132	0.127	0.066	0.065	0.068	0.066	0.002		[0.002]	[0.019]
TT3	0.126	0.139	0.139	0.129	0.124	0.068	0.068	0.070	0.068	0.004	0.002		[0.018]
CA	0.143	0.162	0.164	0.135	0.140	0.160	0.159	0.162	0.160	0.153	0.150	0.148	

Notes:<sup>a</sup> The genetic distances (lower-left) and standard errors (upper-right) were analyzed using MEGA version 2.1 with Kimura 2-parameter. The genetic distances between CA and all the other species were the biggest ones in the same line. The overall average distance was 0.112 and average genetic distance among *Tetraogallus* was 0.042. The interspecific genetic distances of the genus *Tetraogallus* were 0.012 (*T. altaicus* vs *T. himalayensis*), 0.068 (*T. altaicus* vs *T. tibetanus*) and 0.067 (*T. himalayensis* vs *T. tibetanus*) respectively.<sup>b</sup> The species of Altai snow cock (*T. altaicus*).<sup>c</sup> The species of Himalayan snow cock (*T. himalayensis*) and Tibetan snow cock (*T. tibetanus*), and the digitals were the code of individuals used in this study.



**Figure 1.** Phylogeny of *Tetraogallus* and its close related genera inferred from mitochondrial cytochrome b gene sequences in the analysis. The tree topology was inferred from a Neighbor-joining (NJ) distance matrix calculated with Kimura 2-parameter. Numbers near the branches were bootstrap probability values coming from 1000 replications. Some bootstrap values were 92% (*Coturnix chinensis*/*Coturnix japonica*), 100% (*Tetraogallus altaicus* / *Tetraogallus himalayensis*), and 100% [(*Tetraogallus altaicus* / *Tetraogallus himalayensis*) / *Tetraogallus tibetanus*]. *Crossoptilon auritum* was used as outgroup. The tree topology was also supported by Maximum-Parsimony (MP) analyses.

As the NJ tree shown that the genus *Tetraogallus* formed a sole clade, and which was well resolved with bootstrap percentage value 100%. The final results based on the molecular data supported that the genus *Tetraogallus* encompassed three (except two species, *T. caspius* and *T. caucasicus*) named groups given the rank of species: *T. altaicus*, *T. himalayensis* and *T. tibetanus*. And, our data suggested that the genera *Alectoris*, *Coturnix*, *Tetraogallus* and *Pucrasia* formed a monophyletic group. The bootstrap percentage value of the branch composed of the genera *Tetraogallus*, *Alectoris* and *Coturnix* was 88%. Therefore, the two genera, *Alectoris* and *Coturnix*, should have a common ancestor, which was the sister taxon of the ancestor of the genus *Tetraogallus* (Figure 1). Both the phylogenetic trees and the genetic distances suggested that the *Francolinus francolinus* was older than other studied species in its evolutionary history.

The NJ tree, at the same time, suggested that the genus *Tetraogallus* had evolved into two branches with bootstrap percentage (100%): one branch was composed of the Altai and Himalayan snow cock with bootstrap percentage (100%), and the other one was the Tibetan snow cock. This was also supported by the data of MP analysis. On the other hand, there are also several lines of evidence including distribution patterns and morphological traits to support our point of view for the evolutionary pattern of the three snow cock species. i) Both the Altai and Himalayan snow cocks have a similar body form, about 60 centimeters in length, while

the Tibetan snow cocks have a body form about 53 centimeters in length; ii) The two subspecies of Altai snow cocks can be discovered from Altai Mountain to the Northwest Territories of Mongolia, and have an offwhite breast and belly. *Himalayensis*, one subspecies owning a deepest body color of Himalayan snow cocks, can be detected in the areas of Tianshan and northwest of Xinjiang Uygur autonomous region, while another subspecies, *koslowi*, owning a light body color, often emerge in the Altai Mountain and the east of Kunlun Mountain. There are two subspecies holding a light body color, *Przewalskii* and *henrici*, can be discovered in the east of their distribution area, and one subspecies holding a deep body color, *tibetanus*, distributes in the southwest of Xinjiang and the midwest of Tibet.

Shields and Wilson pointed out that the divergence rate of avian mitochondrial DNA Cyto b gene is about 0.02 per million years (Shields, 1987). Therefore, according to the conclusion, we can deduce the divergence time of snow cocks was 1.7 million years ago when the terrain and environment of Hengdun mountains region had a greatly changes in the Pliocene. Considering its molecular phylogeny and geographical distribution patterns, and the evidences of the genetic distances between species are all bigger than 0.01, and the results of genetic distance (0.12) between Altai snow cocks and Himalayan snow cocks reveal that the two species have a closer relationship. It is possible that Tibetan snow cocks might be the primitive species among the three breeds and originate in the Heng-

duan mountains region according to the witness of geographical distribution pattern and morphological character combining the genetic distances among them. So we could propose that one subspecies of Tibetan snow cocks, *tibetanus*, holding a deep body color, migrated towards the West and entered the Himalayan Mountain to evolve into the present Himalayan snow cocks. Later, another subspecies of Himalayan snow cocks, *koslowi*, owning a light body color, often emerge in the Altai Mountain and the east of Kunlun Mountain to evolve into Altai snow cocks.

Although the present study provided a good start towards our understanding of the relationships of the snow cock breeds found in China, detailed interrelationships and limits of the subgroups still need further study. But in all our trees Tibetan snow cocks occupy a relatively basal position. As for Caspian snow cocks and Caucasian snow cocks, we consider that our sample of *Tetraogallus* taxa is insufficient to resolve the evolutionary processes of the foreign snow cock breeds, and that solid conclusions on the status of the total *Tetraogallus* awaited inclusion of additional related taxa, and require further investigation. But, we can give a hypothesis that, according to the geographical distribution range and morphological character, Tibetan snow cocks may be the most primitive breed among the five snow cock species, and Caucasian snow cock derived from Altai snow cocks. As to Caspian snow cocks, they might derive from Himalayan snow cocks. This hypothesis needs further investigation and study.

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#### Correspondence to:

Yifeng Gong, Jinfu Wang  
Ministry of Education Key Laboratory of Xinjiang

Endemic and Ethnic Disease  
Shihezi University  
Shihezi, Xinjiang 832003, China  
Telephone: 01186-993-205-8510  
Email: altai-2003@163.com; jfw5@163.com

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