Limited gene flow and partial isolation phylogeography of Himalayan snowcock *Tetraogallus himalayensis* based on part mitochondrial D-loop sequences

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**Abstract**

Himalayan snowcock *Tetraogallus himalayensis* are distributed in alpine and subalpine areas in China. We used mitochondrial DNA control-region data to investigate the origin and past demographic change in sixty-seven Himalayan snowcock *T. himalayensis*. The fragments of 1155 nucleotides from the control region of mitochondrial DNA were sequenced, and 57 polymorphic positions defined 37 haplotypes. A high level of genetic diversity was detected in all populations sampled and may be associated isolation of the mountains and habitat fragmentation and deterioration from Quaternary glaciations. In the phylogenetic tree, all haplotypes grouped into four groups: clade A (Kunlun Mountains clade), clade B (Northern Qinghai-Tibetan Plateau clade), clade C (Tianshan Mountains clade) and clade D (Kalakunlun Mountains clade). We found a low level of gene flow and significant genetic differentiation among all populations. Based on divergence time we suggest that the divergence of Himalayan snowcock occurred in the middle Pleistocene inter-glaciation, and expansion occurred in the glaciation. Analysis of mtDNA D-loop sequences confirmed demographic population expansion, as did our non-significant mismatch distribution analysis. In conclusion, limited gene flow and a pattern of partial isolation phylogeographic was found in geographic populations of *T. himalayensis* based on the analysis on mtDNA D-loop sequences [Current Zoology 57 (6): 758–767, 2011].

**Keywords**

*Tetraogallus himalayensis*, Mitochondrial DNA control-region, Phylogeography

Glaciation, interglaciation and cyclical Pleistocene climatic oscillations have shaped the geographical distribution, demographic history and ultimately patterns of genetic diversification of many plant and animal species across the Palaearctic ecozone (Avise, 2000; Hewitt, 2000). With the analysis of genealogical and geographic data, phylogeography has been successfully used to infer historical biogeographic events (Avise, 2009; Mi- amiya et al., 2009; Knowles, 2009). Potentially, molecular markers can be used to analyse phylogeography and population demographic change (Avise, 2000; Rogers and Harpending, 1992). The most common markers for animal phylogeographic research are segments of mitochondrial DNA (mtDNA) (Arbogast and Kenagy, 2001). The part of the mitochondrial genome known as the noncoding control region (CR) is the most frequently targeted, as it generally provides sufficient variation for studies at the intraspecific level (Brown et al., 1979; Moritz et al., 1987). The use of these markers are particularly useful when clarifying contemporary geographical patterns of evolutionary subdivision within species (Arbogast and Kenagy, 2001) and allow for an assessment of the impact of historical events on the current genealogical relationships (e.g. Aurelle and Berrebi, 2001; Huang et al., 2010; Qu and Lei, 2009; Recueroc et al., 2006).

Himalayan snowcock *Tetraogallus himalayensis* are distributed across alpine and subalpine zones of mountains at altitudes 2,000–6,000 m (Johnsgard, 1999; Liu, 1998; Zheng, 1978). With a characteristic seasonal vertical migration, the snowcock occurs from 3,600–5,100 m during summer, and can come down to shrubby zones and spruce forests for foraging in winter (Chang and Liu, 1993; Johnsgard, 1999). Mountain meadows are its preferred habitat, and in some areas it may feed on small grass-like herbs. Grass is the major source of food, but snowcock also eat seeds and moss roots (Johnsgard, 1999). Himalayan snowcock demonstrate a high level of adaptation to the harsh alpine habitat, suggesting the area of origin of snowcock lay in the Pamirs, Tianshan, Kunlunshan, and Himalayan Mountains (Potapov, 1992; Liu, 1998). Liu (1998), Shen and Wang (1963) sug-
gested that snowcock evolution was influenced by the fact that they originated in the Qinghai-Tibetan Plateau and interglacial periods during the Tertiary and the Quaternary, based on the relationship between individual development and phylogenesis. Cyclical climatic changes and topographic variation on the Plateau in the Pleistocene are assumed to be two of the most important factors influencing the current spatial distribution of local species and their genetic diversity (Hewitt, 2000). However, the genesis of the Himalayan snowcock still remains a controversial issue. Based on analysis on mitochondrial cytochrome b partial sequences, the genetic structure and populations of phylogeographic structure were constructed in T. himalayensis (Ruan et al., 2005; 2006), but there were too many common haplotypes among different local populations for the limited sequence divergence based on mtDNA cytb.

Glaciations have shaped patterns of genetic diversity among Asian species that inhabit mid-latitude temperate zones and formerly glaciated mountains. Snowcock provide are an ideal species to investigate the consequences of the Pleistocene climate and habitat changes on the geographical distribution of intraspecific genetic variability (An et al., 2009; Ruan et al., 2005, 2006). Here, a part of mtDNA control-region sequence was analyzed in T. himalayensis sampled across its distribution area. The aims of this study were: (1) to assess whether glaciations influenced the present distribution of Himalayan snowcock in northwestern China; (2) to reconstruct phylogeographical relationships of Himalayan snowcock on the background of Pleistocene habitat changes; (3) to infer the evolutionary and historical demographic processes that may have influenced current population structure.

1 Materials and Methods

1.1 Sample collection, DNA extraction, amplification and sequencing

Sixty-seven individual Himalayan snowcock, representing 11 populations from across their range, were collected during the breeding seasons (Fig. 1 and Table 1). Two samples of T. Tibetanus were collected from Tianzhu and Delingha and were used as the outgroup. Individual tissue or feather samples were stored in 95% ethanol at −20 °C.

![Fig. 1 Sample sites and distribution of Himalayan snowcock](https://example.com/image)

### Table 1 Number of total and unique haplotypes found within each population, pairwise difference (k), nucleotide diversity (π) and haplotype diversity (h) of the 11 populations of T. himalayensis

| Population | Latitude | Longitude | Sample size | Total Haplotype | Unique haplotype | k (SD) | π (SD) | h (SD) |
|------------|----------|-----------|-------------|-----------------|-----------------|-------|-------|-------|
| DL 37° 21.87’ | 97° 21.77’ | 5 | 3 | 2 | 12.200 (6.663) | 0.0106 (0.0068) | 0.8000 (0.1640) |
| GEM 36° 24.94’ | 94° 53.48’ | 2 | 1 | 1 | 0.000 (0.000) | 0.0000 (0.0000) | 0.0000 (0.0000) |
| SB 39° 36.00’ | 58° 58.00’ | 9 | 5 | 4 | 7.500 (3.869) | 0.0065 (0.0038) | 0.8333 (0.0980) |
| TSK 37° 49.17’ | 75° 12.34’ | 5 | 3 | 3 | 1.400 (1.019) | 0.0012 (0.0010) | 0.8000 (0.1640) |
| PS 37° 37.05’ | 16° 16.16’ | 6 | 2 | 1 | 1.200 (0.883) | 0.0010 (0.0009) | 0.6000 (0.1291) |
| HT 37° 06.69’ | 79° 54.92’ | 3 | 3 | 2 | 24.000 (14.697) | 0.0208 (0.0159) | 1.0000 (0.2722) |
| KAS 37° 49.17’ | 94° 53.48’ | 15 | 10 | 9 | 3.219 (1.759) | 0.0028 (0.0017) | 0.9333 (0.0449) |
| AKS 41° 10.32’ | 80° 15.40’ | 6 | 4 | 3 | 4.067 (2.357) | 0.0035 (0.0024) | 0.8667 (0.1291) |
| ZS 43° 09.51’ | 81° 07.19’ | 4 | 2 | 1 | 1.333 (1.025) | 0.0012 (0.0011) | 0.6667 (0.2041) |
| QT 44° 01.50’ | 89° 34.94’ | 10 | 7 | 6 | 3.622 (2.004) | 0.0031 (0.0020) | 0.9111 (0.0773) |
| HX 45° 37.09’ | 80° 05.97’ | 2 | 1 | 1 | 0.000 (0.000) | 0.0000 (0.0000) | 0.0000 (0.0000) |
Total DNA was extracted using the Guanidinium Thiocyanate and Diatomaceous Earth Protocol (Gerloff and Schlotterer, 1995). An approximately 1150-bp fragment was amplified using primers 5′-GGCTTGGAA AAGCCATTGTG-3′, 5′-CTTGGGATCCTCAGTG CCATGC-3′, which were modified versions of L16578 and H1251 (Michael and Jennifer, 1999). There was 2.5 U of Tag DNA polymerase per 50 μl of reactants. Final concentrations were 10 mmol/L Tris-HCl (PH 8.3), 50 mmol/L KCl, 1.5 mmol/L MgCl2, 150 μmol/L dNTP, 10 pmol/L primers and about 100 ng DNA template. The PCR conditions were as follows: 94°C for 4 min, 94°C for 45 s, 53°C for 45 s, 72°C for 1 min (35 cycles), and 72°C for 10 min. PCR products were purified and sequencing performed with each of the PCR primers on an ABI 377 automated sequencer. The sequences were deposited in GenBank and the Accession Nos. are from GQ343513 to GQ343549, the outgroup of T. Tibetanus deposited in GenBank and the Accession Nos. are from GQ343550 and GQ343551.

1.2 Sequence analysis and phylogenetic structure

Sequences were aligned using CLUSTAL_X (Thompson et al., 1997) and rechecked by eye. Then, DnaSP4.0 (Rozas et al., 2003) was used to define haplotypes and estimate average and population haplotype diversity (h), mean number of pairwise differences (k), nucleotide diversity (π). Population differentiation index (Fst) and gene flow (Nm) were determined using the Arlequin2.0 analyses of molecular variance (AMOVA) (Schneider et al., 2002).

Mismatch distributions were used to test for demographic signatures of population expansions (Rogers, 1995) by Arlequin2.0 (Schneider et al., 2002). To compare observed distributions with those expected under the expansion model, we calculated the raggedness index and goodness of fit. These statistics are expected to have insignificant positive values under demographic expansion. Arlequin2.0 (Schneider et al., 2002) was also used to compute time since population expansion (τ), relative population size before (θi) and after (θf) expansion and mismatch distributions.

Mutation rate and expansion time of the mtDNA control-region haplotypes in Himalayan snowcock were computed following Rooney et al. (2001). We estimated the number of nucleotide substitutions per site (d) using the formula: \( d = (tv + tvR)/m \) (tv = the number of transversions between T. himalayensis and T. Tibetanus; R = transition/transversion ratio in T. himalayensis; m = length of the sequence). The rate of nucleotide substitution per site per lineage per year is \( \lambda = d/2T \), where T is the divergence time between the ingroup and the outgroup species. Based on Ruan et al. (2005), divergence between T. himalayensis and T. Tibetanus occurred in 4.06 Myr. The mutation rate per nucleotide site per generation is \( \mu = \lambda g \), where g is the generation time (g = 3.00 years in the snowcock, Huang et al., 1990). The mutation rate per haplotype is \( u = m\mu \), and the expansion time in generations is \( t = \tau/2u \) (Rogers and Harpending, 1992).

Bayesian phylogenetic analyses were performed using MRBAYES version 3.1.2 (Huelsenbeck and Ronquist, 2001). Models of sequence evolution were assessed using MRMODELTEST (Nylander, 2004). For Bayesian analyses, four simultaneous Monte Carlo Markov chains (MCMCs) were run for \( 5 \times 10^6 \) generations, sampling a tree every 100 generations. After summarizing the substitution model parameter values, we made sure that the Potential Scale Reduction Factor (PSRF, a convergence diagnostic) was reasonably close to 1.0 for the parameters. The posterior probabilities (PP) were calculated with the last 37,500 sampled trees after the loglikelihood values had stabilized. Maximum-parsimony (MP) analyses were performed in PAUP*4b10 (Swofford, 1998) with 1000 bootstrap replicates. Gaps were treated as a fifth character, and searches were heuristic with random addition of taxa (five replicates). The shortest trees were retained and zero length branches were collapsed. A 50% strict consensus tree was computed for the 1000 bootstrap trees. The phylogenetic tree was rooted using a mtDNA control-region sequence of T. Tibetanus. Networks were processed first by the median-joining method (Bandelt et al., 1999) and then by the maximum-parsimony Steiner method (Polzin and Daneschmand, 2003). Networks were constructed using Network 4.5.1.6 (www.fluxus-engineering.com).

Divergence times between clades were estimated using the program MDIV (Nielsen and Wakeley, 2001). MDIV applies an isolation-with-migration model to the data and uses a Bayesian approach to simultaneously approximate the posterior distribution of three parameters: divergence time between populations (\( T = l_{3456}/2N_e \), where \( N_e \) is the effective population size), the migration rate between populations since divergence (\( M = 2N_em \)), and the population parameter theta (\( \theta = 4N_em \), where \( \mu \) is the mutation rate per site per generation). The program was first run using default search settings and default priors (for the parameters of interest, \( \theta \) and \( T \)). Then, we set our prior value for \( T \) to equal 10 and M to equal 1, because it produced consistent and well-behaved posterior distributions (Spellman and
Klicka, 2007). MDIV analyses were run for 5 million generations following a burn-in period of 500,000 generations, and repeated three times to ensure convergence upon the same posterior distributions for each of the parameter estimates.

2 Results

2.1 mtDNA control-region sequence description and variability

The mtDNA control region sequence (1,155 nucleotides) alignment showed 37 different haplotypes (55.22% of all samples), defined by 57 polymorphic sites including 59 substitutions (41 transitions and 18 transversions), and two insertion/deletion (Table 2). The number of observed haplotypes within populations ranged from one in GEM and HX to 10 in KAS (Table 1). The percentages of unique haplotypes per population was calculated by dividing the number of unique haplotypes by the same size. Within each population, this percentage varied from 16.7% in PS to 100% in GEM and HX. A total of 34 haplotypes (91.89% of all the haplotypes) were unique to the 11 populations. The most common haplotype was H9, which was found in six samples from populations of AKS, PS and ZS, and the haplotype H8 was shared by populations of DLH and TSK, haplotype H35 by populations of HT and SB, respectively (Table 2).

Mean haplotype diversity and nucleotide diversity in all populations was 0.977 ± 0.0069 and 0.0103 ± 0.00523 respectively. Nucleotide diversity among the populations varied from 0.0000 (HX and GEM) to 0.0208 ± 0.0159 (HT) (Table 1). Haplotype diversity ranged from 0.000 (GEM and HX) to 1.00 ± 0.0272 (HT) (Table 1). Intrapopulation pairwise divergence was the lowest (k = 0.000) in the GEM and HX populations and the highest (k = 24.000 ± 14.697) in the HT population (Table 1). Tajima’s D was –0.0205 in the total sample (P = 0.59), and suggests that the null hypothesis of neutral evolution of these mtDNA CR sequences was not rejected in the total samples.

2.2 Phylogenetic structure of Himalayan snowcock mtDNA haplotypes

The Bayesian tree, computed with the best-fit model (HKY + I + G), was rooted using CR sequence from T. tibetanus. Convergence was obtained by a convergence diagnostic (PSRFparameters closed to 1.0). In the phylogenetic tree, all haplotypes were grouped into four clades: clade A (Kunlun Mountains clade), clade B (Northern Qinghai-Tibetan Plateau clade), clade C (Tianshan Mountains clade) and clade D (Kalakunlun Mountains clade) (Fig. 2). Clade A included two haplotypes sampled from HT (two samples) and SB (one sample). Clade B encompassed seven haplotypes: H1 and H3 from DLH (three samples), H2 from GEM (two samples), H4, H5, H6 and H7 from SB (eight samples). Clade C contained twenty-five haplotypes present in QT, HX, AKS, ZS, PS, KAS and HT. Clade D (Kalakunlun Mountains clade, located in the east of Pamirs and in the north of Kalakunlun Mountains), which could be monophyletic, included haplotypes that were only present TSK (Fig. 2). The MP procedure produced a similar topology as shown by the bayesian tree. The result of the haplotype network (Fig. 3) showed that the distribution of haplotypes was consistent with the phylogenetic tree.

2.3 Gene flow and population subdivision

The AMOVA analysis showed a low level of gene flow (Nm = 0.62) and significant genetic differentiation (Fst = 0.15, P < 0.001) among all populations. Moreover, most pairwise Fst-values were large and significant which indicates restricted gene flow among these populations, and significant pairwise Fst-values between geographic localities ranged from 0.152 (DLH-SB) to 0.912 (PS-TSK) (Table 3). The remaining pairwise Fst-values were not significant, suggesting high levels of gene flow.

2.4 Estimation of mutation rate and divergence time

Based on the equation $d = (tv+tvR)/m$ (Rooney et al. 2001), we computed the average number of nucleotide substitutions per site between T. himalayensis and T. tibetanus haplotypes and obtained $d = 0.105$ (tv = 32, R = 2.79, m = 1155). The rate of nucleotide substitution per site per lineage per year ($\lambda$) = 1.29×10^{-8} (T = 4.06 million years, Ruan et al. 2005), and the mutation rate per generation ($\mu$) = 3.87×10^{-8}.

MDIV estimates of divergence time (using our estimates of the mutation rate) suggest that the basal split between clade A and the other three clades (clade B, clade C and clade D) occurred in 0.291×10^{6} years ago ($\theta$ = 24.388, t = 1.067). This was followed by a divergence time between clade B and clade C and clade D of 0.152×10^{6} years ago ($\theta$ = 16.420, t = 0.827). The divergence time of clade C and clade D was 0.140×10^{6} years ago ($\theta$ = 16.052, t = 0.780). All estimates of migration rates between clades from the MDIV analyses indicated that fewer than 0.2 (range 0.00001–0.02) individuals migrated between clades as they diverged, and many of the posterior distributions for the estimate of M included zero.
Fig. 2  Bayesian tree based on HKY + I + C model of *T. himalayensis* (37 haplotypes)

The phylogenetic tree was rooted using *T. tibetanus*. Values of posterior probability (first numbers) are shown for the key node, and the bootstrap percentage values computed in maximum-parsimony (second numbers) trees. Numbers in bracket after the sample name are sample size.

Fig. 3  Haplotype network estimated from the *T. himalayensis* control-region data

Small black circles indicate missing haplotypes not observed. Distances between linked haplotypes corresponded to one mutation, except when otherwise indicated.
2.5 Mismatch distributions and expansion time

The mismatch distribution for the total samples (Fig. 4), Northern Qinghai-Tibetan Plateau clade and Tianshan Mountains clade was bell-shaped as expected under the sudden expansion model. The observed distribution was not ragged, and the goodness-of-fit statistics were not significant (Table 4). Thus, a fitting of the observed to the expected distribution under the sudden expansion model cannot be rejected. The estimated values of \( \theta \) after the expansion (\( \theta_1 \)) was higher than that before the expansion (\( \theta_0 \)), which were similar in the four groups: Kunlunshan Mountains group, Northern Qinghai-Tibetan Plateau group, Tianshan Mountains group and Kalakunlunshan Mountains group (Table 4).

We computed \( \tau = 2ut = 3.560 \) in Himalayan snowcock from Northern Qinghai-Tibetan Plateau, a value that can be used to estimate an expansion time of \( t = \tau/2u = 3.560(2\times3.87\times10^4\times1155) = 39,822 \) generations (79,644 years). The expansion time of Tianshan Mountains group was 54,879 generations (109,758 years), and the expansion time of the snowcock from Kalakunlunshan Mountains was 16,936 generations (33,872 years).

![Fig. 4 Mismatch distributions for all Himalayan snowcock samples](image)

3 Discussion

Habitat fragmentation and deterioration resulting from Quaternary glaciations and the isolation of the mountains are mostly factors leading to high genetic diversity and high population differentiation in Himalayan snowcock (Table 3).

### Table 3 Population pairwise Fst-value (below the diagonal) and gene flow (above the diagonal) for comparisons between populations (n≥5)

|         | DLH | SB  | TSK | PS  | KAS | AKS | QT  |
|---------|-----|-----|-----|-----|-----|-----|-----|
| DLH     |     | 2.797 | 0.401 | 0.647 | 0.450 | 0.844 | 0.542 |
| SB      | 0.152* |       | 0.220 | 0.192 | 0.158 | 0.232 | 0.216 |
| TSK     | 0.555* | 0.695* |       | 0.0484 | 0.102 | 0.111 | 0.0996 |
| PS      | 0.436* | 0.723* | 0.912* |       | 1.964 | 1.097 | 0.394 |
| KAS     | 0.526* | 0.759* | 0.830* | 0.203* |       | 2.452 | 0.536 |
| AKS     | 0.372 | 0.683* | 0.819* | 0.313* | 0.169* |       | 0.943 |
| QT      | 0.480* | 0.698* | 0.834* | 0.559* | 0.482* | 0.346* |     |

* 0.01<P<0.05, * 0.001<δP<0.01, ** P<0.001. Inf.: the value of gene flow is infinity. Abbreviations as in Table 1.

### Table 4 Mismatch analyses

| Group                  | \( \tau \) | \( \theta_0 \) | \( \theta_1 \) | SSD   | P-value | RI  | P-value |
|------------------------|-----------|---------------|---------------|-------|---------|-----|---------|
| Kunlun Mountains       | 2.289     | 0.000         | 11.011        | 0.265 | 0.110   | 1.000 | 0.560   |
| Northern Qinghai-Tibetan| 3.560    | 0.007         | 9.902         | 0.011 | 0.660   | 0.037 | 0.870   |
| Tianshan Mountains     | 4.906     | 0.000         | 39.385        | 0.001 | 0.804   | 0.012 | 0.790   |
| Kalakunlun Mountains   | 1.514     | 0.019         | 99999.000     | 0.013 | 0.830   | 0.120 | 0.820   |

SSD: Sum of squared deviations. RI: Raggedness index.
In the phylogenetic tree of snowcock (Fig. 2), levels of geographic substructure suggest a recent population division or, more likely, historically limited gene flow (Avise et al., 1987) within each group, consistent with the findings from *T. himalayensis* by Ruan et al. (2006). Two haplotypes (H35 and H36) from populations HT (two samples) and SB (one sample) are obviously distinct from the others in group A (Kunlunshan Mountains group), which may be mostly by low levels of gene flow (Nm = 0.202) and significant genetic differentiation (Fst = 0.711, P < 0.001). Simultaneously, it was not surprising that the geographic barriers of these huge mountains (Tishan Mountains, Kalakunlunshan Mountains, Arjinshan Mountains, Qilianshan Mountains) have led to limited gene flow (Nm of Clade B, C and D = 0.221, Fst = 0.693, P < 0.001; Nm of Clade C and D = 0.166, Fst = 0.751, P < 0.001).

Based on the divergent time, the divergence of the four groups (Kunlun Mountains group, Northern Qinghai-Tibetan Plateau group, Tianshan Mountains group and Kalakunlunshan Mountains group) occurred during the period of Mindel-Riss inter-glaciation (Cao, 1996). During this inter-glaciation period, the climate became warm and the snow line moved up, forests developed, and shrub and alpine meadow shrunk. As a bird preferring low temperatures, Himalayan snowcock would migrate from the hillsides with high temperatures to peaks with low temperature and also escape from the threat of natural predators (Huang et al., 1990). This movement gave Himalayan snowcock the chance to evolve independently. According to estimated divergence time based on mtDNA control-region data, the divergence of Himalayan snowcock should occur in the inter-glaciation.

Low nucleotide diversity, high haplotype diversity and unimodal mismatch distributions in Northern Qinghai-Tibetan Plateau, Tianshan, Kalakunshan and Kunlunshan groups are consistent with recent population expansion in these four groups (Tables 1 and 3). This recent expansion hypothesis is also supported by the non-significant mismatch distribution analysis indicating a demographic population expansion (Table 4). Based on the expansion time, the expansion of Himalayan snowcock occurred in the late Pleistocene with the occurrence of the Wurm glaciation when the climate was cold and dry (Cao, 1996). Temperatures can drop very low and ice caps can form in most mountains across the bird’s current distribution area (Li et al., 1986), and individuals are known to descend to hillsides and eventually to fluvial plains for food. It is therefore unsurprising that Himalayan snowcock expanded during glaciation.

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