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Abstreact

The hazel grouse is distributed throughout much of northern Asia and Europe, whereas the closely related Chinese grouse is endemic to central China. The molecular phylogeny of these two species was analyzed using a 420 bp segment of mitochondrial DNA control region domain I. We analyzed a total of 201 samples of hazel grouse collected from Hokkaido to Europe and 11 samples of the Chinese grouse collected in China. In total, 79 and 7 haplotypes were found for the hazel grouse and the Chinese grouse, respectively The phylogenetic tree showed a clear separation of the hazel grouse cluster from the Chinese grouse cluster. Two haplotypes from Italy (n = 1) and the Southern Alps (n = 1) in Switzerland (Tessin) were clearly clustered and distinctly separated from the other hazel grouse haplotypes cluster. Two haplotypes from northern part of the population were separated from the southern part of the population. These observations indicate that the Chinese grouse and hazel grouse were separated at least 200,000 years ago and the southern Alps population separated from the other hazel grouse at least 100,000 years ago. These three grouse populations reached their present distribution during the last several ten thousand years.

Introduction

The family of grouse, Tetraonidae, extends northward from the temperate zones of the Northern Hemisphere (Short 1967). The hazel grouse *Bonasa. bonasia* is a small member of Tetraonidae, and is found in deciduous broad-leaved tree and coniferous tree forests of the palearctic region. The hazel grouse is distributed from forest areas of Europe (Bergman and Klaus 1994) to East Asia, Sakhalin, Hokkaido Japan (Fujimaki and Konishi 1996)and Korea (Han and Fujimaki 1996, Rhim and Lee. 2001). Some populations are decreasing (Bergman and Klaus 1994, Fujimaki and Konishi 1996).

The Chinese grouse *B. swerzowi* is the smallest Tetraoidae and endemic in China (Sun et al. 2005). The habitat consists of isolated remains of the mountain coniferous forests of Gansu, Qinghai, Sichuan, Yunnan and Tibet. The area of the most closely related species, the hazel grouse, is separated byabout 1000 km by the dessert Gobi (Klaus et al. 1996).

The purpose of this work is to study the phylogenetic relationships in hazel grouse and in Chinese grouse. The genetic characteristics of mitochondrial DNA (mtDNA), which has an almost strictly maternal mode of inheritance and lack of recombination provides us with a useful analytical tool for estimating molecular phylogeny among related species. Feathers provide a non-invasive source of DNA, especially for endangered animals (Koike et al. 1998). Moulted feathers from rock ptarmigan *Lagopus mutus* were successfully used before for mitochondrial DNA analysis (Baba et al. 2001). DNAanalysis of Hazel grouse was carried out using material of *B. b. vicinitas* (Baba et al. 1999) and of birds from different parts of East Asia (Baba et al. 2002). These observations most likely indicate that the populations analysed were differentiated about 40,000 years ago, and have expanded to the present distribution during the climatic optium within the last 10,000 years. In this paper we describe the population history of the western part of the hazel grouse population and of the Chinese grouse using the mitochondrial control region sequences extracted from both fresh tissue and feather samples.

Material and Methods

Sampling

Chinese grouse

A total of 11 samples of Chinese grouse were analyzed from China. Among them, 9 feather and tissue samples of Chinese grouse were analyzed from south part of distribution, 2 samples from north part of distribution.

Hazel grouse

Among them, 133 tissue samples collected during the hunting season in Hokkaido were provided by the Hokkaido Prefectural Gorverment,which consisted of 36 samples from north Hokkaido (HoN), 45 samples from eastern Hokkaido (HoE), 33 samples from central Hokkaido (HoC), and 19 samples from south-western Hokkaido (HoSW) (Baba 2001). The samples from Russia were included 10 tissue samples from Primorskii (Pr), 18 tissue samples from Sakhalin (Sa), 14 tissue samples from Magadan (Ma), and 5 samples (3 feathers and 2 tissues) from Zapadano Sibirskaya in Siberia (Si). Remaining samples were 22 samples from the Europe. 22 Europe samples are split 2 samples from South part of Europe (Swaziland, Italy) (Al) and 20 from another area (Eu) at population analysis, and 2 samples (Al), 12 samples from Germany (10 samples), Poland (1 sample), Austria (1samples) (EuN) and 8 samples from Swaziland (2 samples), Fence (4 samples), Italy (2 samples) (EuS).

Amplification

We amplified the first 686 bp of the control region with L16575 (Baba 2001) and H476 (Baba et al. 2002), and 2nd amplified 469 bp of the control region L16760 (Baba et al. 2002) and H454.gr (Baba et al. 2002), use the protocols described Baba et al. (Baba et al. 2002). The amplified products were isolated single strand DNA using PCR Product Pre-Sequencing Kit (USB) according to manufacturer's directions. Direct sequence was undertaken in a DNA Processer (Beckman Coulter Co. Ltd) with DTCS kit (Beckman Coulter Co. Ltd), using L16760 or H454 primer.

Nuclear homologous of mitochondrial markers (Numt) and duplications of the mitochondrial control region have been identified in other bird species (e g Quinn 1997; Eberhard et al. 2001). To verify that we are sequence mitochondrial DNA control region, we amplified about 2000bp of cytochrome-b to 12s rRNA at 1st PCR and amplified control region from few tissue samples (Data not shown). We found no difference in the sequence from the long 1st PCR and short 1st PCR products.

Phylogenetic Analysis

Sequence was aligned by eye using GENETYX-MAC Ver. 11.0 (Software development Co., Ltd 2001). Gamma alpha-parameter was estimated from the sequence matrix by Hidden Markov Model without correction (Felsenstein and Churchill 1996) using PUZZLE Ver.5.0 (Strimmer and von Haeseler 1996). Number of haplotypes (HT) and polymorphic sites (S), nucleotide diversity (π) with variance V(π), haplotypic diversity (h) with variance V(h) (Nei and Tajima 1981), mean number of pairwise differences (d) (Nei and Jin 1989) and Tajima's test (Tajima 1989) were derived for all area using the program ARLEQUIN Ver.2.0 (Schneider et al. 2000). Mitochondrial genetic differentiation between population and taxa was assessed by pairwise ϕST (FST) value and sequence divergence in the program ARLEQUIN. The transition/transversion parameter was estimated from distance matrix. We calculated genetic distance between control region haplotypes using Kimura 2-parameter model (Kimura 1980), and constructed a neighbor-joining tree (Saitou and Nei 1987) and minimum evolution tree at between populations were constructed with a MEGA Ver.2.1 (Kumar et al. 2001).

Results and Discussion

Genetic variation

A total 215 mitochondrial control region sequence were obtained. Sequence Aliment required the insertion of gaps at 5 positions between hazel grouse to Chinese grouse and total 72 sites were variable. Transition/transversion ratio was estimated to be 4.95 (Using PUZZLE Ver.5.0).

From 201 samples of the hazel grouse, we observed 51 variable sites defining 79 haplotypes in the 428 bp fragments in the domain I, or left domain, of the control

Table 1 Genetic diversity of the Hazel grouse and Chinese grousebased on domein I sequence of the control region.

Species	Area	n	HT	π	±	V(π)	h	±	V(h)	S	d	S/d	Tajima's test
Hazel grouse	Hokkaido (HoSW)	16	11	0.00860	±	0.00518	0.933	±	0.048	11	3.48	3.2	0.192
	Hokkaido (HoN)	36	20	0.00914	±	0.00525	0.943	±	0.023	15	3.70	4.1	0.074
	Hokkaido (HoC)	33	16	0.00669	±	0.00405	0.921	±	0.031	13	2.71	4.8	-0.500
	Hokkaido (HoE)	45	20	0.00901	±	0.00516	0.924	±	0.024	16	3.65	4.4	-0.007
	Magadan (Ma)	12	4	0.00460	±	0.00319	0.561	±	0.154	7	1.86	3.8	-0.767
	Primorskii (Pr)	10	5	0.00538	±	0.00367	0.756	±	0.130	6	2.18	2.8	0.111
	Sakhalin (Sa)	18	7	0.00528	±	0.00344	0.797	±	0.066	5	2.14	2.3	1.488
	Siberia (Si)	5	3	0.00395	±	0.00326	0.700	±	0.218	4	1.60	2.5	-1.094
	Europe (Eu)	20	15	0.00957	±	0.00560	0.974	±	0.022	17	3.87	4.4	-0.717
	South Alps (Al)	2	2	0.00494	±	0.00605	1.000	±	0.500	2	2.00	1.0	0.000
Chinese Grouse	China (Bs)	15	10	0.00969	±	0.00577	0.924	±	0.053	14	3.92	3.6	-0.352

Number of individuals (n) ; Number of haplotypes (HT) ; nucleotide diversity (π) with variance V(π) ; number of haplotypic diversity (h) with variance V(h) ; number of segreating sites (S) ; number of segregating sites (S) ; mean number of pairwise nucleotide difference (d) ; expan coefficient (S/d) ; Tajima's test (Tajima's test).

Table 2 Pairwise sequence divergence and differentation between area.

Species	Area	HoSW	HoN	HoC	HoE	Ma	Pr	Sa	Si	Eu	Ea	Bs
Hazel grouse	Hokkaido (HoSW)	-	4.339	3.590	4.322	6.870	3.961	4.637	5.706	7.386	20.227	58.836
	Hokkaido (HoN)	0.093	-	3.767	4.007	7.960	4.786	5.019	5.562	8.071	21.008	57.938
	Hokkaido (HoC)	0.072	0.076	-	3.959	6.400	4.028	4.326	4.691	6.848	20.861	58.690
	Hokkaido (HoE)	0.093	(-0.007)	0.122	-	8.157	4.753	5.118	5.701	8.194	20.967	57.534
	Magadan (Ma)	0.562	0.577	0.593	0.583	-	5.986	4.567	5.738	6.808	23.166	63.127
	Primorskii (Pr)	0.208	0.292	0.332	0.287	0.636	-	3.925	5.555	6.266	18.351	58.697
	Sakhalin (Sa)	0.347	0.344	0.390	0.354	0.523	0.411	-	3.463	5.356	20.276	54.612
	Siberia (Si)	0.455	0.394	0.452	0.408	0.664	0.620	0.396	-	4.985	20.040	60.160
	Europe (Eu)	0.453	0.488	0.491	0.498	0.513	0.445	0.385	0.322	-	19.166	64.287
	South Alps (Al)	0.820	0.814	0.863	0.814	0.912	0.874	0.888	0.911	0.789	-	73.862
Chinese Grouse	China (Bs)	0.931	0.928	0.943	0.929	0.947	0.939	0.941	0.938	0.933	0.943	-

Above diagonal, sequence divergence (d, base pair). Below diagonal F_{ST} values based on AMOVA (Kimura 2-parameter distance, Ganma alpha=0.14).
F_{ST} value in parentheses, not significant at P<0.05 (1000 permutation)

region. We identified total of 41 haplotypes in 133 samples from Hokkaido. Eurasian samples showed 5 haplotypes from Primorskii , 8 haplotypes from Sakhalin, 5 haplotypes from Magadan, 3 haplotypes from Siberia and 17 haplotypes from Europe (Table 1). Only 1 haplotype was shared between Sakhalin and Magadan.

From 14 samples of the Chinese grouse, we observed 14 variable sites defining 10 haplotypes in the 428 bp fragments in the domain I (Table 1).

Genetic distance and Phylogenetic relationship between populations

The haplotype composition of the population showed as π and h (Table 1) with pairwise sequence F_{ST} value ranging from 0.947 to -0.007(Table 2). These indicated significant genetic differentiation (P <0.05) between all pairs of population expect between HoN and HoE. Number of pairwise differences (d) value ranging from 3.463 to 73.862. Pairwise difference between hazel grouse and Chinese grouse ranging from 54.612 to 73.862 are high value.

The neighbor-joining tree was constructed using the genetic distances between populations (Fig. 1) showing that Chinese grouse and hazel grouse was clearly separated at the species level. The neighbor-joining tree showed that these 2 species exhibited clear separation of 23.905 (5.69%). Using a molecular clock rate of 20.8% divergence per million years for the domain I in the mitochondrial control region, (Quinn 1992, Baba 2001), we found that the two species were splitted at least 200,000 years ago.

The Hazel grouse cluster was divergent between the Southern Alps population (Al) and the other sub cluster originating from the Northern Alps. Pairwise difference between both populations are of twofold higher value than the that of the other hazel grouse populations and neighbor-joining tree branch is about two times longer than the other hazel grouse branch, suggesting that hazel grouse population of the Southern

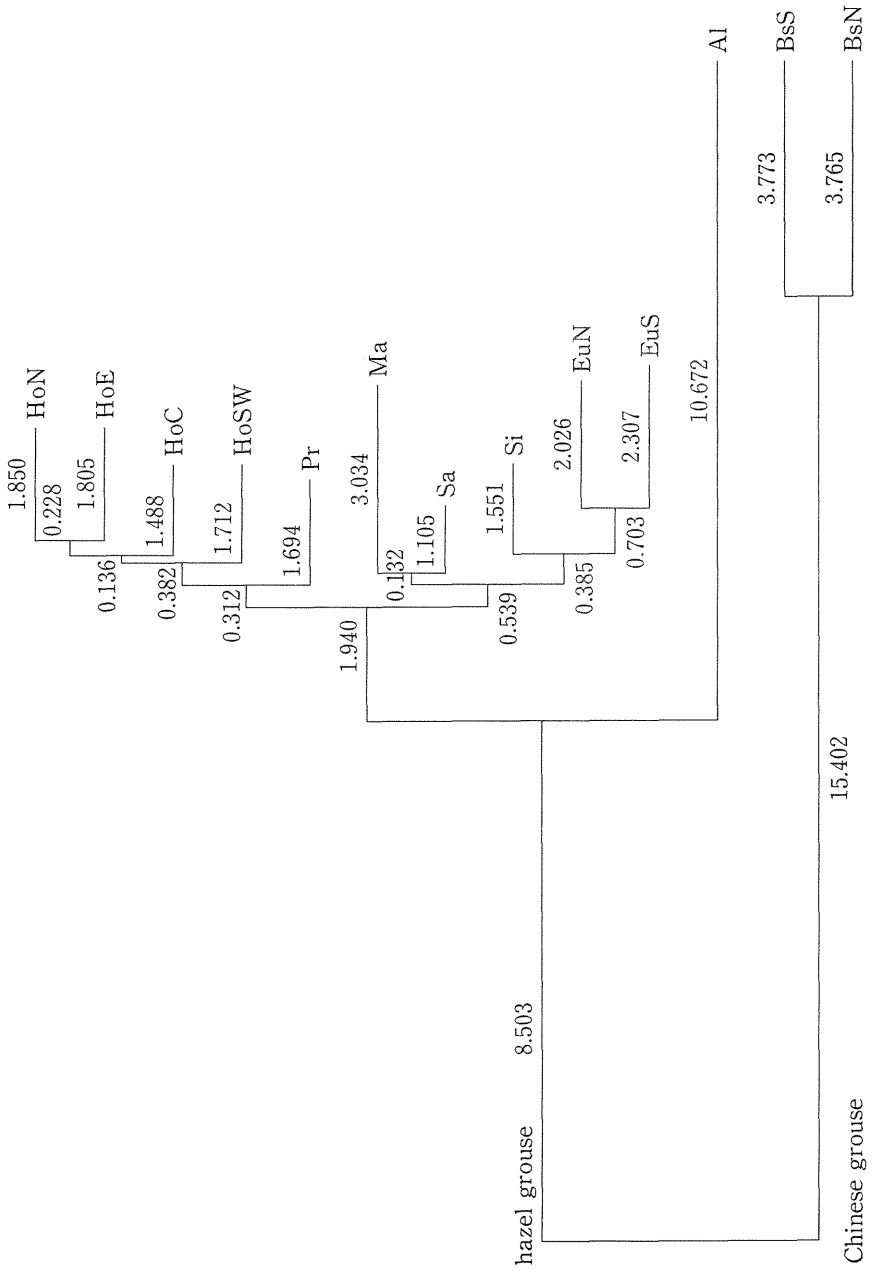

Fig.1 Neighbor joining tree using genetic distance between populations.
Number at branch in the phylogenies indicate genetic distance.

Alps was splitted earlier than the another hazel grouse populations. Because the branch length is over 10, of the hazel grouse population located in Northern Italy and Southern Switzerland (Tessin) was separated before the maximum phase of the last glaciation period and we assume that this part of the population was moving south when the ice came from north and survived in southern parts of Europe. The northen hazel grouse population diverged after 40,000 years ago together with the East Asian part of the population (Baba et al. 2002). Hazel grouse in Hokkaido were classified by classical methodas only one subspecies *B. b. visinitas*. But according to the mitochondrial DNA analysis classify three of HoN and HoE, HoC, HoSW populations by F_{ST} significant level $P < 0.05$ (Table 2).

The Chinese grouse cluster consisted south part and north part population. Each population branch length is about two times longer than that of the hazel grouse population length, expect that of population Al. It is suggested northern and southern Chinese grouse population were separated before the maximum phase of last galciation in Asia.

Population diversity

The genetic diversity of all population is summarized in table 1. Nucleotide diversity of all populations are between 0.00460 and 0.00969. Haplotypic diversity of all populations are between 0.561 and 0.974 (2sample of Al were removed), normal or high value than other bird species (Baba 2001, Baba et al. 2002). Expand coefficient value of all populations are between 2.3 and 4.8. These values suggested, that each hazel grouse and the Chinese grouse population were not bottle-necked around their population expansion.

Tajima's test of all population are between -1.094 and 1.488 but all population are not significant at $P < 0.05$. Tajima's test of Sakhalin (Sa), Magadan (Ma), Siberia (Si) and Europe (Eu) are over 0.05 or under -0.05. These populations were spared habitat from East Asia and suggested happen something weak non-neutral selection at population expanded after the maximum phase of the last glaciation. Tajima's test of Hokkaido (HoN, HoE, HoC, HoSW), Primorskii (Pr) and Chinese grouse are between 0.05 and -0.05. These hazel grouse population were keeping the same habitats during the maximum phase of the last glaciation (Baba 2002). Steppes and even desert vegetation extended to the modern coast of eastern China, but Chinese grouse habitat did not disappear during the maximum phase of the last glaciation as indicated by polynological study (Yu et al. 2000). These data suggest that the Chinese grouse population was surviving in the same area as today during the maximum phase of the last glaciation in Asia.

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