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Molecular population phylogeny of the hazel grouse *Bonasa bonasia* in East Asia inferred from mitochondrial control-region sequences

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A total of 62 mitochondrial haplotypes were detected from 174 samples of hazel grouse *Bonasa bonasia* from Hokkaido, Primorskii, Sakhalin, Magadan, Siberia and Bohemia using 428 bp of the mitochondrial DNA control region. Haplotype diversity for four populations in Hokkaido was more than 0.8, suggesting that a reasonable population size had been maintained throughout their history. Haplotypes from Hokkaido and haplotypes from Primorskii were clearly separated from other Eurasian continent haplotypes, not only in the phylogenetic tree but also in the network tree. Haplotypes from Hokkaido, Sakhalin and Magadan radiated from the hypothetical root composed of a double cubic network of parallel substitutions. Most of the haplotypes were separated by three substitutions from the root, or within a maximum of five substitutions. Pairwise sequence differences for most Eurasian haplotypes had a bimodal curve consisting of the first peak at 0-1 substitution differences and the second peak at 3-4 substitution differences, whereas those for Hokkaido haplotypes had only a peak at around 4-6 substitution differences. 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 optimum over the last 10,000 years.

Key words: haplotype diversity, hazel grouse, Hokkaido, mitochondrial control region, molecular phylogeny, network tree

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Genetic characteristics of mitochondrial DNA (mtDNA), which is maternally inherited and lacks recombination, provide a useful analytical tool for estimating genetic divergence among related species (Avise 1986, Avise 1994, Avise & Hamrick 1996). The control region (or D-loop region) evolves four times faster than the remainder of the mitochondrial genome (Quinn & Wilson 1993), presumably because of a lack of coding constraints, allowing inference on sequence divergence of intraspecific populations.

Collection of feathers is a non-invasive sampling method which provides sources of DNA, and is especially well suited for endangered animals (Koike, Okayama & Baba 1998). Feathers shed by the rock ptarmigan *Lagopus mutus*, which is listed as vulnerable in the Japanese Red Data List, were used for mitochondrial DNA analysis (Baba, Koike, Okayama & Fujimaki 1997). The structure and genetic functions of the mitochondrial control region for the hazel grouse *Bonasa bonasia* have been reported elsewhere (Baba, Fujimaki & Koike 2001). In addition, its control regions have been compared with those of the domestic chicken *Gallus gallus domesticus* (Desjardins & Morais 1990), the Japanese quail *Coturnix japonica* (Desjardins & Morais 1991), the mallard *Anas platyrhynchos* (Ramirez, Savoie & Morais 1993), the snow goose *Anser caerulescens caerulescens* (Quinn & Wilson 1993), the turnstone *Arenaria interpres* and dunlin *Calidris alpina* (Wenink, Baker & Tilanus 1994).

In this paper we discuss molecular phylogeny of the hazel grouse using the mitochondrial control region sequences extracted from both fresh tissue and feather samples. Furthermore, we compare these to published sequences of the domestic chicken and the quail which belong to the same order, i.e. Galliformes. The family of grouse, Tetraonidae, extends northward from the temperate zones of the Northern Hemisphere (Short 1967). The hazel grouse is a small tetraonid, which is found in deciduous broad-leaved and coniferous forests of the Palaearctic region. The hazel grouse in Hokkaido belongs to the subspecies *B. b. vicinitas* which is distributed in forest areas of the Far-East, Sakhalin and Korea (Han & Fujimaki 1996). Hunting statistics indicate that 10,000-12,000 individuals are killed annually during the hunting season (Fujimaki & Konishi 1996).

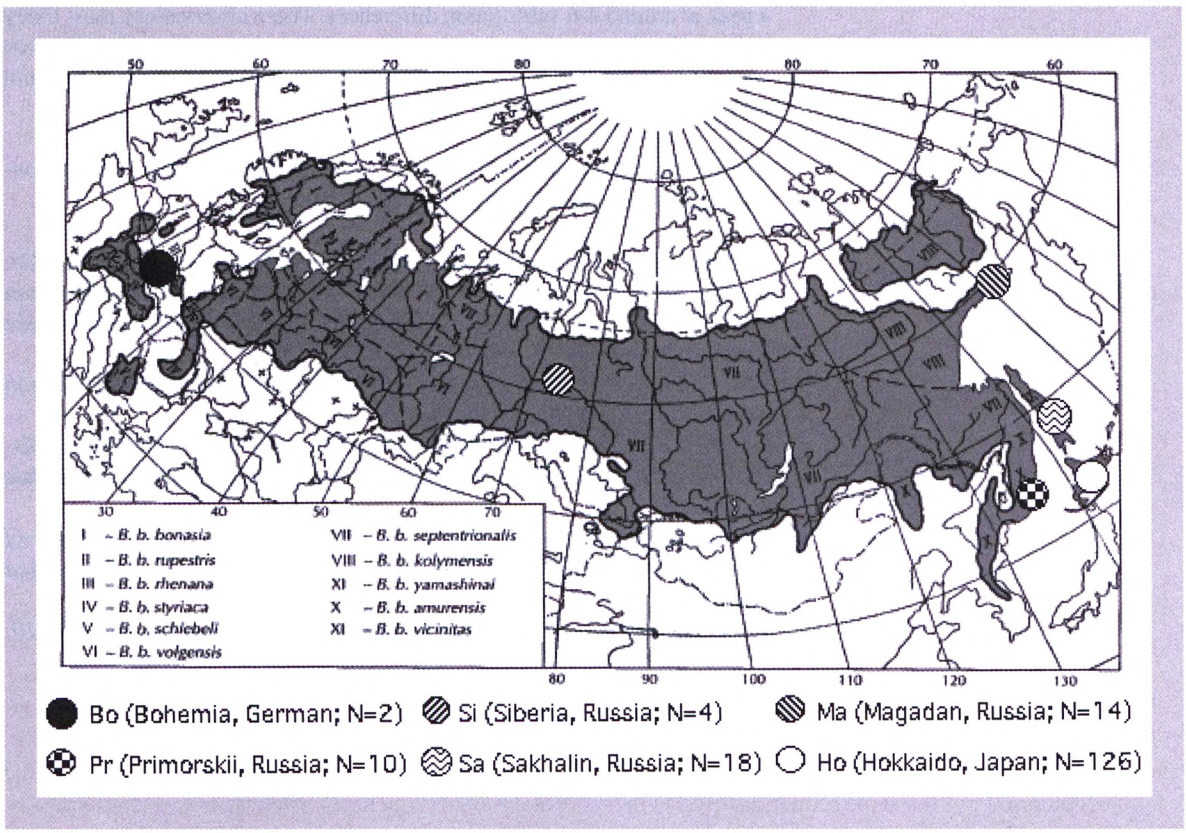

Figure 1. Distribution of the hazel grouse and sampling localities for the174 samples analysed. The map was reconstructed from Figure 3 in Bergmann, Klaus, Muller, Scherziger, Swenson & Wiesner (1996).

Material and methods

We analysed a total of 174 samples of hazel grouse from Hokkaido and other Eurasian regions (Fig. 1). Of these, 126 tissue samples collected during the hunting season in Hokkaido were provided by the Hokkaido Prefectural Government, and 37 came from northern Hokkaido (HoN), 54 from eastern Hokkaido (HoE), 16 from central Hokkaido (HoC), and 19 from southwestern Hokkaido (HoSW). The samples from Russia included 10 tissue samples from Vostretsovo in Primorskii (Pr), 18 tissue samples from Sakhalin (Sa), 14 tissue samples from Magadan (Ma), and four samples (two feather and two tissue) from Zapadano Sibirskaya in Siberia (Si). The remaining samples were two feather samples from the Bohemian forest (Bo) in eastern Europe. From China we received four feather samples of the Chinese hazel grouse *Bonasa sewerzowi*, which is the species most closely related to the hazel grouse. These data were used in our phylogenetic analysis for rooting purposes.

Approximately 2 mg of sliced tissue was placed in the 310 µl of RSB buffer, 15 µl of 10% SDS and 25 µl of 20 mg/ml Proteinase K, and incubated for two hours at 55°C on a rotator for protein digestion. Nucleic acids were extracted using an IsoQuick Nucleic Acid Extraction Kit (ORCA Research Inc., USA). In the case of feather samples, a whole feather or the root of a feather was sliced into small fragments with clean scissors, and homogenised in liquid nitrogen. The sample was placed in 450 µl of RSB buffer, and processed in the same manner as the tissue samples.

To avoid amplifications of contaminated DNA when using the Polymerase Chain Reaction (PCR) method, specific primers for the grouse were designed on the basis of comparison with the domestic chicken sequence (Desjardins & Morais 1990).

L16760.rai (5' GACTACGGCTTGAAAAGCCATTGTTGT 3') annealing within the $tRNA^{Glu}$ region and AVEH476.rai (5' GTGAAAAGTGAGAAAGTTCAGGAGTTA 3') annealing at the central conserved domain of the control region were designed to amplify the left domain of the control region (Baba et al. 2001). The PCRs were performed with 30 cycles of denaturation at 95°C for 15 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 60 seconds.

Direct sequencing was undertaken in a DNA Processor (Pharmacia L.K.B. Co. Ltd) with a Thermosequenase cycle sequencing kit (Amersham), using Cy5 fluorescent labelled primer of the same sequences used in the PCR. Twenty cycles were used with denaturation at 95°C for 30 seconds, annealing and primer extension at 65°C for 30 seconds. Sequencing was conducted using an ALFred DNA Autosequencer (Pharmacia L.K.B. Co. Ltd.).

Alignment of the sequence data was performed at BioResearch/AE (Fujitsu Ltd.), with gap penalty 2 (Kimura 1980). Neighbour-joining (NJ) tree (Saitou & Nei 1987) and genetic distances (Kimura 1983) were constructed with a BioResearch/SINCA program, version 3 (Fujitsu Ltd.). Bootstrap analysis was performed with 1,000 replications using the same program (Saitou & Imanishi 1989).

As an index for genetic diversity, haplotype diversity (h) was calculated using the formula:

$$h = (1-\sum x_i^2)\, n / (n-1)$$

where n is the number of samples, and x_i is the frequency of haplotype i (Nei & Tajima 1981). Genetic distances between populations (dxy) were calculated using the formula:

$$dxy = \sum_{ij} x_i\, y_j\, d_{ij}$$

where x_i is the frequency of haplotype i in population X, y_j is the frequency of haplotype j in population Y, and d_{ij} is the nucleotide substitution between haplotypes i and j (Nei & Jin 1989).

Results

Genetic variation in the hazel grouse

From the 174 samples of the hazel grouse, we observed 39 variable sites defining 62 haplotypes in the 428 bp fragments in the domain I, or left domain, of the control region (Table 1). Among these variable sites, we detected three deletions at sites 58, 228 and 287 from the beginning of the control region. We observed three transversions at sites 233, 328 and 358. The remaining substitution sites were transitions.

We identified a total of 41 haplotypes (Ja1-3, Jb1-15, Jc1-8, Jd1-11, Je1-4) in 126 samples from Hokkaido. Other Eurasian samples showed five haplotypes from Primorskii (haplotypes Pr1-5), eight haplotypes from Sakhalin (haplotypes Sa1-8), five haplotypes from Magadan (haplotypes Ma1-5), two haplotypes from Siberia (haplotypes Si1-2), and two haplotypes from the Bohemian forest (haplotypes Bo1-2). Haplotype Sa7 from Sakhalin and haplotype Ma 5 from Magadan had the same sequences.

Table 1. Haplotype table for the 62 haplotypes detected in the mitochondrial controlregion.

Haplo-type	Substitution site																																							Number of samples			
	56	69	128	138	157	163	170	172	191	200	204	205	206	208	218	219	221	222	226	228	230	231	233	236	240	241	249	261	256	266	287	291	300	208	328	329	358	392	406	HoC	HoE	HoN	HoSW
Ja1	G	C	T	A	G	C	G	C	C	T	G	C	T	G	T	C	C	T	G	T	C	C	T	C	T	C	T	C	A	T	A	T	C	C	A	T	A	C	C	0	0	0	1
Ja2																							A		C															0	0	0	1
Ja3																							A		C													T		0	0	0	2
Jb1																							G	T	C															3	0	2	0
Jb2																							G	T	C				G											2	2	2	4
Jb3																T							G	T	C				G											6	2	0	1
Jb4					A																		G	T	C															0	2	4	1
Jb5					A											T							G	T	C				G											1	3	0	0
Jb6					A											T							G	T	C															0	0	0	1
Jb7					A																		G	T	C				G											2	0	0	0
Jb8					A		A																G	T	C															0	1	0	0
Jb9					A		A			C													G	T	C										C					0	0	1	0
Jb10																	T						G	T	C				G											0	1	0	1
Jb11					A									C		T							G	T	C				G											1	0	0	0
Jb12																T							G	T	C				G			C								0	2	0	0
Jb13					A																		G	T	C														T	0	0	0	1
Jb14																T							G	T	C															0	0	1	0
Jb15					A			T								T							G	T	C				G											0	0	1	0
Jc1					A																		G	T	C					C										0	1	2	0
Jc2					A								C			T							G	T	C					C										0	6	1	1
Jc3					A								C										G	T	C					C										0	0	1	2
Jc4					A								C										G	T	C					C	G									0	1	1	0
Jc5					A								C								T		G	T	C					C										0	1	0	0
Jc6					A								C	C									G	T	C					C										0	3	0	0
Jc7					A							T	C			T							G	T	C					C										0	1	1	0
Jc8					A							T	C				T						G	T	C					C										0	3	2	0
Jd1					A					C											T		G	T	C															0	0	1	0
Jd2										C											T		G	T	C															0	1	1	0
Jd3										C											T		G	T	C				G											0	6	2	0
Jd4					A					C											T		G	T	C				G							T				0	1	2	3
Jd5										C							T				T		G	T	C				G											0	0	1	0
Jd6				G						C											T		G	T	C															0	1	0	0
Jd7			C		A					C											T		G	T	C				G											0	0	0	1
Jd8			C		A					C											T		G	T	C															0	1	0	0
Jd9					A					C						T					T		G	T	C															0	0	1	0
Jd10										C									A		T		G	T	C				G											0	0	1	0
Jd11					A					C											T		G	T	C				G											0	0	0	1
Je1					A		A																G	T	C								T							0	13	6	0
Je2					A		A									T							G	T	C								T							0	0	1	0
Je3					A																		G	T	C								T							0	1	0	0
Je4					A											T							G	T	C								T							0	0	1	0
Pr1																							G	T																		5	
Pr2																							G	T																		1	
Pr3																							G	T	C	T	C															1	
Pr4																							G	T	C	T	C		G													1	
Pr5																							G	T	C	T						T										2	
Sa1					A						A												G	T	C	T																6	
Sa2					A																	T	G	T	C	T												T				1	
Sa3					A																		G	T	C	T			G									T				1	
Sa4																							G	T	C	T			G									T				6	
Sa5						T																	G	T	C	T			G									T				1	
Sa6					A																		G	T	C	T																2	
Sa7					A																		G	T	C	T	C															1	
Ma1								T												C			G	T	C	T			G									T				8	
Ma2																	T			C			G	T	C	T			G													2	
Ma3																							G	T	C	T		T	G						C							1	
Ma4								T												C			G	T	C	T			G													1	
Bo1		T											C					C					G	T	C	T			G													1	
Bo2		T																C					G	T	C	T		T														1	
Si1		T			A													C					G	T	C	T			G													1	
Si2					A									C									G	T	C	T			G													3	

Phylogenetic tree and network tree of the hazel grouse

A phylogenetic tree using the 62 haplotypes for the hazel grouse detected in this study was generated by the NJ method. Four haplotypes for the Chinese hazel grouse (haplotypes Bse1-4; Baba, Fujimaki & Koike 1999) were used as a reference in forming the phylogenetic tree. The phylogenetic tree showed clear separation of the hazel grouse cluster from the Chinese hazel grouse cluster, with more than 98% bootstrap support.

Hokkaido haplotypes and haplotypes Pr1 and Pr2 from Primorskii were separated from other Eurasian haplotypes with only 50% bootstrap support.

Network analysis (Fig. 2) represents the evolutionary relationships between haplotypes and indicates which substitution sites distinguish each haplotype. The network tree for the hazel grouse showed that most of the haplotypes were separated by one or two substitutions, indicating that haplotypes for the hazel grouse were not much differentiated during their evolutionary history.

In this network, variable site 241 differed between Hokkaido and Eurasian haplotypes. Variable sites 157 and 256 had parallel substitutions both in Hokkaido and Eurasian haplotypes, and variable site 219 had parallel substitutions in Hokkaido haplotypes. Therefore a cubic network was formed by parallel substitutions of variable sites 157 and 256 in the first and second dimension, and variable sites 219 and 241 in the third dimension. Haplotypes in this cubic network were Jb1, Jb2, Jb3, Jb4, Jb5, Jb6, Jb7, Jb14 and Sa7. These haplotypes, especially Sa7, Jb1 and Jb4, were closest to the node for the hazel grouse in the NJ tree, indicating that the cubic network represents a hypothetical root of the hazel grouse haplotypes.

From hypothetical root of the cubic network, clade Ja consisting of haplotypes Ja1-Ja3 and Pr1-Pr2 separated from haplotype Jb1 by variable site 236, clade Je consisting of haplotypes Je1-Je4 separated from haplotypes Jb4 or Jb6 by variable site 308, clade Jc consisting of haplotypes Jc1-Jc8 separated from haplotypes Jb4 or Jb6 by variable site 266, and clade Jd consisting of haplotypes Jd1-Jd11 separated from haplotypes Jb1, Jb2, Jb4, Jb6, Jb14 by variable sites 200 and 230.

Other Eurasian haplotypes were complicated, but separated by one to three substitutions from the root. From the root, clade Sa consisting of haplotypes Sa2-Sa6 differed by variable site 392, and clade Ma consisting of haplotypes Ma1, Ma2 and Ma4 differed by variable site 228. Bohemian haplotypes differed by three substitutions from the root. It is interesting that Bohemian haplotypes were separated through Siberian haplotype from haplotype Sa7/Ma5 situating from the root.

Genetic distances between populations

Genetic distances between populations (dxy) for Hokkaido, Primorskii, Sakhalin, Magadan, Siberia and Bohemian populations were calculated using the pairwise frequency of haplotypes between two populations and the number of nucleotide substitutions between the pairwise haplotypes. The NJ tree using the genetic distances between populations (Fig. 3) showed that nodes for these populations were connected with relatively short

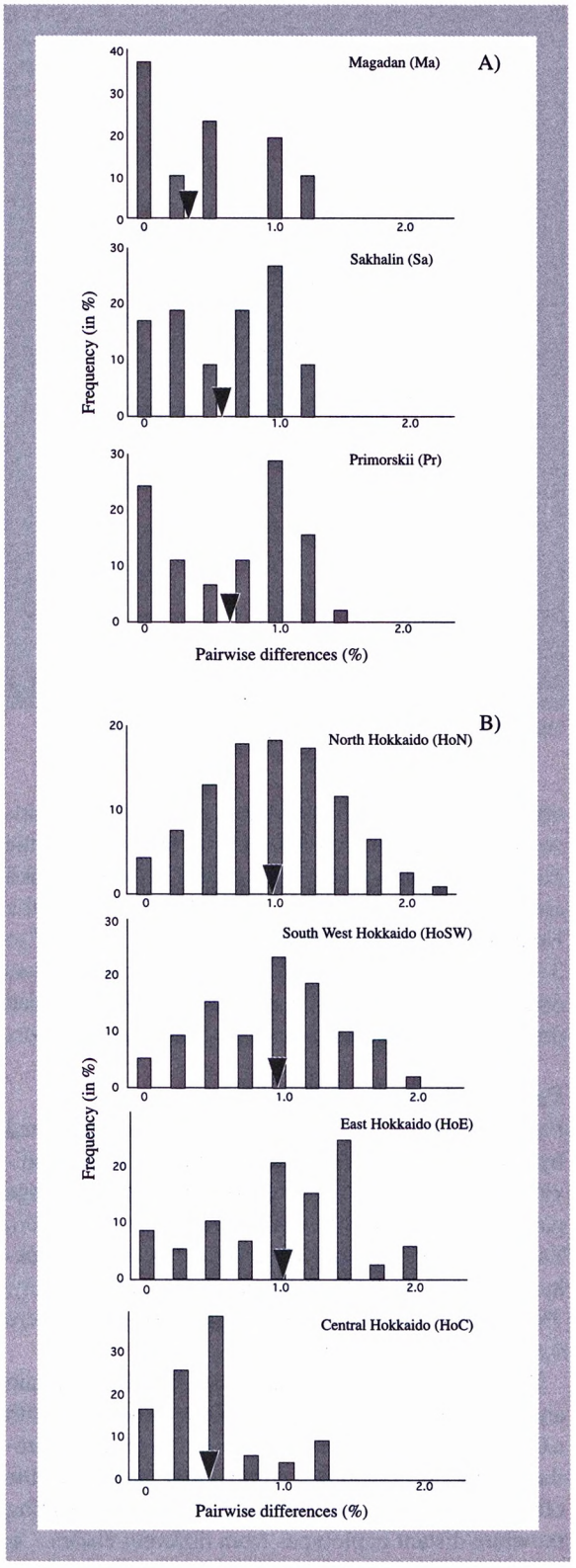

Figure 2. Frequencies of pairwise sequence difference (in %) for the other Eurasian (A) and the Hokkaido (B) populations of hazel grouse, with arrows indicating the average.

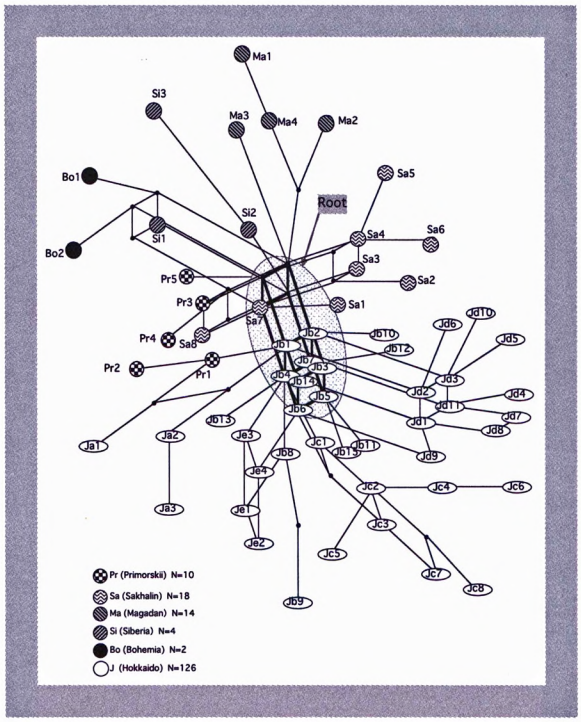

Figure 3. Network tree for the 62 haplotypes of the hazel grouse.

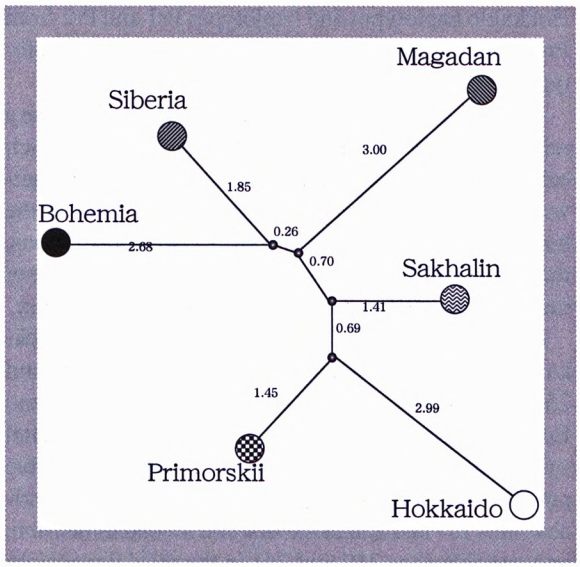

Figure 4. Neighbour-joining tree using genetic distance between the six hazel grouse populations (dxy).

distances between 0.3 and 0.8, as indicated in the network tree, whereas most of the clades radiated from the node. Lengths of branches were shorter in the Primorskii and Sakhalin populations (around 1.5) than in the Hokkaido, Siberia and Bohemian populations (2.7 to 3.0). In the NJ tree, the Hokkaido population had close relations to the Primorskii population, and the Bohemian population was located close to the Siberian population.

Pairwise sequence difference

Pairwise sequence differences (Fig. 4) were calculated by nucleotide differences in haplotypes between individuals in each region (Baker & Marshall 1997). Average pairwise sequence differences for North Hokkaido, East Hokkaido, Central Hokkaido and Southwest Hokkaido were 0.98, 1.05, 0.46 and 0.97%, respectively. Those for Primorskii, Sakhalin and Magadan were 0.66, 0.57 and 0.33%, respectively.

Pairwise sequence differences for Magadan, Sakhalin and Primorskii generated a bimodal curve, one peak with a 0-1 substitution difference representing the close similarity among haplotypes within each clade, and the other peak with a 3-4 substitution difference reflecting the more distant haplotypes from different clades. On the other hand, pairwise sequence differences for Hokkaido haplotypes had only one peak: a 3-5 substitution difference for North Hokkaido, a 4-5 substitution difference for Southwest Hokkaido, a 4-6 substitution difference for East Hokkaido, and a 1-2 substitution difference for Central Hokkaido.

Haplotype diversity

Haplotype diversities (h) for Hokkaido, Primorskii, Sakhalin and Magadan (Fig. 5) were compared with those

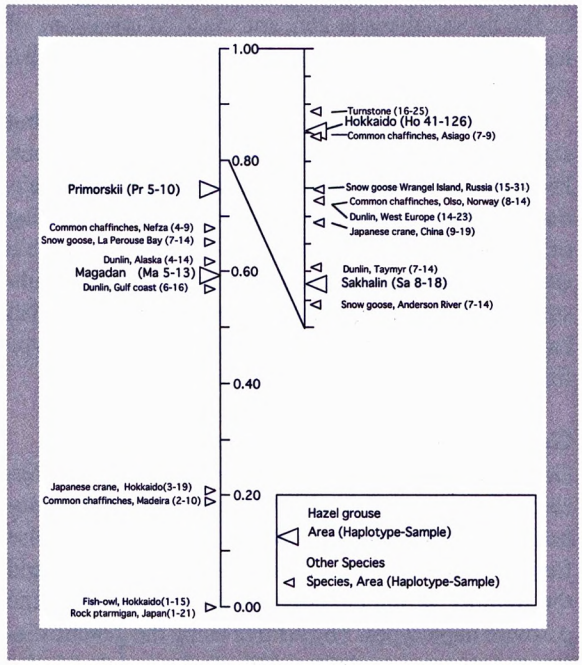

Figure 5. Haplotype diversity of hazel grouse and other bird populations.

of snow goose (Quinn & Wilson 1993), turnstone (Wenink et al. 1994), dunlin (Wenink, Baker & Tilanus 1993), Japanese crane *Grus japonensis* (Abe 1996b), Blakiston's fish-owl *Ketupa blakistoni* (Abe 1996a), common chaffinch *Fringilla coelebs* (Marshall & Baker 1997) and rock ptarmigan *Lagopus mutus* (Baba et al. 2001). Haplotype diversities for northern Hokkaido, eastern Hokkaido, central Hokkaido and southwestern Hokkaido were 0.97, 0.91, 0.83 and 0.95, respectively. Such high haplotype diversities are also reported in the dunlin (Wenink et al. 1994) and the snow goose.

Discussion

A total of 62 mitochondrial haplotypes were detected in 174 samples from the hazel grouse, collected in Hokkaido, Primorskii, Sakhalin, Magadan, Siberia and Bohemia. Haplotype diversity for four populations from Hokkaido was more than 0.8, suggesting that a reasonable population size had been maintained throughout their history.

A phylogenetic tree, using the 62 haplotypes from *B. bonasia* and four haplotypes from *B. sewerzowi* as a reference, showed that these two species exhibited clear separation with a 2-parameter distance of 5.14%. Using a molecular clock rate of 20.8% divergence per million years for the domain I in the mitochondrial control region, we found that the two species probably split about 250,000 years ago. Hokkaido haplotypes, including haplotypes Pr1 and Pr2 from Primorskii, were clearly separated from other Eurasian haplotypes, not only in the phylogenetic tree produced by the NJ method, but also in the network tree, which showed that variable site 241 differed between the Hokkaido and other Eurasian haplotypes.

Network analysis showed that most of the haplotypes were closely related and differed by one or two substitutions, indicating that haplotypes for the hazel grouse were either well conserved through their evolutionary history or that the species had a short history. The hypothetical root of these haplotypes was located in a double cubic network composed of parallel substitution sites 157, 256, 219, and substitution site 214, which differs between the Hokkaido and other Eurasian haplotypes. Haplotypes Ma5/Sa7, Jb1 and Jb4 are not only in the double cubic network of the hypothetical root, but they are also basal in the phylogenetic tree.

When discussing molecular divergence of the hazel grouse, palaeoenvironments in the Hokkaido and Far East regions should be considered as one of the important factors (Ono 1984). The last glaciation cycle started around 120,000 years before the present (BP), and at that time Hokkaido was connected to the continent. Pollen analysis of samples from around 20,000 years BP, when the last glaciation was in its coldest phase, indicated that deciduous broad-leaved forests were limited to the southern Hokkaido and Primorskii regions (Yasuda, Amano & Yamanoi 1990). The so-called Younger Dryas event took place around 10,000 years BP, when Hokkaido was separated from, and Sakhalin still connected to, the continent. Climatic conditions became warmer than at present around 8,000 years BP, and a sea-transgression is recognised to have taken place around 6,000 years BP during the climatic optimum.

The network tree indicated that 1) from the hypothetical root, nodes of clades Ja, Je, Jc, Jd for Hokkaido haplotypes, and clades Sa and Ma for the other Eurasian haplotypes, radiated by one or two substitutions, 2) most of the intraclade differences were within three substitutions, and 3) most haplotypes analysed were radiating by three substitutions from the root, or within four substitutions at a maximum. These observations most likely indicate that the analysed populations differentiated after 40,000 years ago, and most of the clades were formed in a relatively short time, i.e. within 20,000 years after the differentiation.

Pairwise sequence differences for the Eurasian haplotypes had a bimodal curve. The most common explanation for this bimodal curve is that of recent mixing of two populations that had differentiated while allopatric. However, these local populations of hazel grouse had specific haplotypes. This would suggest that the pairwise sequence difference curve reflects their population dynamics. The first peak at 0-1 substitution differences in the Eurasian haplotypes was assumed to be formed relatively recently, suggesting that these intraclade haplotypes in the other Eurasian sample expanded to their present distribution during the climatic optimum.

Summarising these observations, a hypothetical divergence history for the hazel grouse is represented in Figure 6. During the period of 40,000 to 30,000 years BP (see Fig. 6A), Hokkaido was connected to the continent. The ancestral root for the hazel grouse population might have been formed during this phase in East Asia, since most of the haplotypes analysed were radiating within four substitutions in the network tree, and eight substitutions in the pairwise sequence differences.

Around 20,000 years BP in the maximum phase of the last glaciation (see Fig. 6B), the distribution of the hazel grouse must have been restricted by the reduction in deciduous broad-leaved forests. Based on the pairwise sequence difference curves, in which the Hokkaido haplotypes had peaks at around a 3-5 substitution dif-

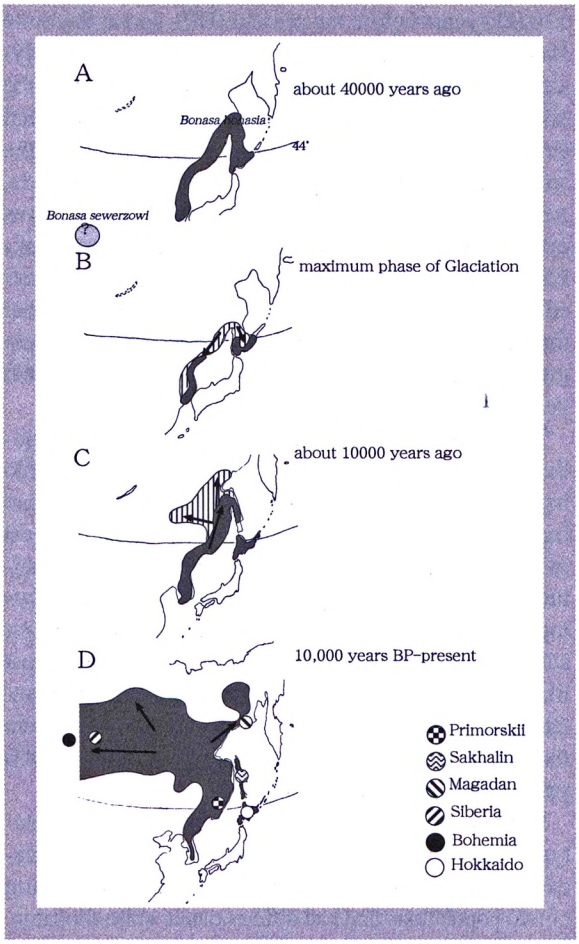

Figure 6. Hypothetical divergence history for the hazel grouse; A) the period of 40,000 to 30,000 years BP, B) around 20,000 years BP in the maximum phase of the last glaciation, C) so-called Younger Dryas event around 10,000 years BP, D) 10,000 years BP- the present distribution of the hazel grouse.

ference, and the other Eurasian haplotypes had a peak at around a four substitution difference, divergence of the Hokkaido clades must have started earlier than the other Eurasian clades, possibly due to better conditions of the deciduous broad-leaved forests in Hokkaido.

The so-called Younger Dryas event took place around 10,000 years BP (see Fig. 6C) when Hokkaido was separated from, and Sakhalin still connected to, the continent. Bimodal curves in the pairwise sequence differences for the Eurasian populations might suggest a population decrease during this phase. Figure 6D shows the present distribution of the hazel grouse. Pairwise substitution differences indicate that Eurasian haplotypes have expanded to their present distribution since the climatic optimum, from around 6,000 years BP to the present (see Fig. 6D).

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