Genetic diversity and extent of gene flow in the endangered Japanese population of Hodgson's hawk-eagle, *Spizaetus nipalensis*

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Summary

The Japanese subspecies of Hodgson's Hawk-eagle, *Spizaetus nipalensis orientalis*, is considered threatened and has been designated as Endangered by the Japanese government. We determined the complete mitochondrial DNA (mtDNA) sequence of this species and designed a primer set to amplify a highly variable region of mtDNA, part of the control region (CR), based on this complete sequence. Using the primers, we amplified the CR and then determined the haplotypes of 178 samples collected at different sites in Japan. A nested cladistic analysis indicated that gene flow within some clades was restricted. The inference key implied that isolation by distance had caused the restriction of gene flow. Moreover, the ranges of the clades in which restricted gene flow was detected overlapped with the ranges of other clades. These results suggest that there is no fragmental population of Hodgson's Hawk-eagle in Japan and that this species has dispersed within short distances, at least in some lineages. Genetic diversity was high in comparison with other species. Therefore, at least in terms of genetic diversity, the Japanese population of Hodgson's Hawk-eagle is probably not in a critical situation.

Introduction

Hodgson's Hawk-eagle, *Spizaetus nipalensis orientalis*, is a large raptor that inhabits montane forests with steep slopes (Morimoto and Iida 1992); it occurs mainly on the mainland of Japan (Honshu, Hokkaido, Shikoku and Kyushu) (Committee for Check-list of Japanese Birds 2000). This species is thought to be susceptible to human exploitation, including dam construction and deforestation, of mountain areas. On 31 August 2004, the Ministry of the Environment released a report that estimated the population of this raptor exceeds 1,800 individuals, and it has been designated as an Endangered species in the Red Data Book of Japan (Ministry of the Environment 2002). In addition, since 1993 the Japanese Government has also designated this species as "a national endangered species of wild fauna" based on the Law for the Conservation of Endangered Species of Wild Fauna and Flora. However, reports of systematic monitoring of the population or studies of its genetic structure have never been published in scientific journals. Therefore, little information is available on the population structure of this species.

Generally, it is assumed that genetic diversity in a diminished population of an endangered species is low due to genetic drift. A decline in genetic diversity would reduce the adaptability of a population to environmental fluctuation (Primack and Kobori 1997). It is therefore necessary to determine the genetic diversity to evaluate the current status of an endangered species. If areas of unsuitable habitat separate

subpopulations (i.e. restricted gene flow) the population is considered Vulnerable even if the overall population maintains high genetic diversity. In South-east Asia, some species of *Spizaetus* have suffered from declines in population size and habitat fragmentation (e.g. Preleuthner and Gamauf 1998, van Balen *et al.* 2001). In smaller subpopulations that lack gene flow, minor alleles are more easily lost due to genetic drift, thereby reducing genetic diversity. Large-scale development accompanied by the destruction of montane forests may not only extinguish the habitat of the hawk-eagle but also constrain individual movements among local populations. Hence, it is very important to determine whether there is a local population that is likely to become isolated from other local populations through habitat destruction.

Given the ignorance of population structure in rare species, genetic studies of endangered species, including Hodgson's Hawk-eagle, must be conducted before development critically harms populations. Our aim was to reveal the extent of gene flow in Hodgson's Hawk-eagle so that future development projects can minimize their influences on this endangered raptor, and to determine whether the current population contains sufficient genetic diversity.

Methods

Determination of the complete mtDNA sequence

The control region (CR) of mitochondrial DNA (mtDNA) is thought to be most appropriate for detecting intraspecific genetic variation due to its high variability (Baker and Marshall 1997). A primer set to amplify the CR is a prerequisite for genetic studies using the CR; therefore, it is necessary to sequence a region of mtDNA containing the CR in a preliminary experiment in order to design the primers (unlike the cytochrome *b* region, for which universal primers can be used). We determined the complete DNA sequence of the mitochondrial genome of Hodgson's Hawk-eagle, *Spizaetus nipalensis*.

Total cellular DNA was extracted from blood of a Hodgson's Hawk-eagle. The complete 17,667 base pair (bp) nucleotide sequence of the mitochondrial genome of the hawk-eagle (Accession No. AP008238) was determined using described procedures (Yamamoto *et al.* 2000). The 13 protein-coding mitochondrial genes and 22 transfer RNAs (tRNAs) were then identified by comparison with other avian mitochondria. In contrast to the gene order in *Gallus gallus* (R. Okimoto *et al.* unpublished), the CR of *S. nipalensis* is located between tRNA-thr and tRNA-pro. In *S. nipalensis*, at the position of the CR in many birds, a non-coding region (pseudo-CR) is found between tRNA-glu and tRNA-phe, as in other raptors, including *Buteo buteo* (Haring *et al.* 1999) and *Falco peregrinus* (Mindell *et al.* 1998). The mtDNA length (bp) in these raptors differs due to differences in the CR or pseudo-CR.

DNA samples and determination of haplotypes

From 1999 to 2003, feathers moulted from Hodgson's Hawk-eagles were collected in and around nests after the breeding season. DNA was extracted mainly from these feather samples and from muscle samples of carcasses that had been supplied as specimens to the Yamashina Institute for Ornithology, using a DNeasy Tissue Kit (QIAGEN) according to the manufacturer's guidelines.

We designed a primer set (SnContRegFa 5′-ATT CTC TTC CCC CTA GTT GC-3′ and SnContRegRa 5′-AGG ATT AAC CGA GTC ATG GC-3′) to amplify a 685 bp fragment containing a highly variable part (domain I; defined as a 418 bp region from the 5′-end of the CR to the 5′-adjacent site of the F box, a conserved sequence block in the CR), based on the complete mtDNA sequence. PCR was conducted with standard procedures using Ex Taq (TaKaRa) at an annealing temperature of 56 °C. Polymerase chain reaction (PCR) products were separated on 1% agarose gels (SeaKem LE agarose by BMA) and then purified by the phenol–chloroform procedure. Purified PCR products were sequenced using a BigDye Terminator (Applied Biosystems) with either the SnContRegFa or SnContRegRa primer following the manufacturer's recommendations. Sequence data were read with a 3100 Genetic Analyzer (Applied Biosystems).

The domain I region was trimmed from the sequence data. Multiple alignments of CR domain I sequence were performed using GENETYX in order to determine the haplotype of each sample.

Procedure to distinguish feather samples of one individual from those of others

In the field, after the residence of a hawk-eagle pair was confirmed, feather samples were collected at or near the nest. A collected feather could have been shed from a breeding male, breeding female or their chicks, although the individual that shed it was not identified in the field. As many as 17 feathers were collected from a particular nest. Genetic diversity and gene flow must be analysed using data on individual haplotypes. Since the haplotype of the mtDNA of chicks is the same as that of the mother, the haplotype of a feather would be derived from a breeding male or a breeding female. Therefore, a series of feather samples with the same haplotype was considered to be derived from one individual if the samples had been collected at or near a nest occupied by a pair.

This criterion may have misled us into judging that two samples with the same haplotype were derived from one individual, when they could actually have come from two individuals with the same haplotype. Hence, we may have underestimated the number of individuals, which in turn may have led to an overestimation of genetic diversity. Therefore, we included a second criterion: we considered that two or more feathers were always derived from both members of a pair even if only one haplotype was found in their samples. Moreover, if the samples were collected in different years, we considered another pair to have occupied the nest. This second criterion (maximum estimate) should overestimate the number of individuals. Consequently, the true value must lie between the results obtained using these two criteria.

Haplotype analyses

We did not hypothesize the presence of any barriers that restrict gene flow and attempted to exhaustively detect restrictions of gene flow. A nested cladistic analysis examines the range of a clade consisting of haplotypes united by a parsimonious algorithm (Templeton *et al.* 1987, Templeton 1998). It allows us to examine the extent of gene flow under no hypothesis, because it does not need to define arbitrary subpopulations to which each sample is attributed. This method examines geographical distances between and within clades in a cladogram based on statistical parsimony (Templeton *et al.* 1992) rather than genetic differentiation between arbitrary subpopulations.

Before the nesting procedure, we constructed a haplotype phylogenetic tree with parsimony of mutation (Templeton *et al.* 1992, Templeton 1998). In the haplotype tree, tip haplotypes linked to an interior haplotype within a one-step mutation were nested in one clade according to the nesting algorithm described by Templeton *et al.* (1987). The haplotype tree was constructed and the nesting procedure performed with TCS 1.13 (Clement *et al.* 2000; TCS 1.18 was also used in some cases).

The geographical distances (clade distance and nested clade distance) were calculated according to Templeton *et al.* (1995). The clade distance represents an average distance from the geographical centre of the clade members, i.e. the extent of the clade range. The nested clade distance represents an average distance from the geographical centre of the clade members including other clades nested at a one-step higher level, i.e. the average distance from other nested clades.

Geographical distances and statistical tests were calculated using GeoDis 2.2 (Posada *et al.* 2000), inputting the latitude and longitude data (in minutes) of the location of the municipal offices in cities where the samples had been collected. Since the location information for three samples was for the prefecture only, coordinates of the approximate centre of each prefecture were used for these samples.

We were unable to determine a consistent evolutionary history among haplotypes from the data, so we could not determine which haplotype was an ancestral node in the network. Since we could not examine whether a tip clade was younger than an interior clade, we used the following criterion described by Templeton *et al.* (1995) to determine interior or tip clades: an interior clade is connected to the remainder of the network through two or more connecting branches and a tip clade is connected to the remainder of the network through only one connecting branch.

The association with a nested clade and the geographical distance was tested statistically. This test examines whether clades are distributed randomly across locations. When significance is detected, it is necessary to interpret which process resulted in the biased distribution of clades. Historical phenomena, such as fragmentation, range expansion and isolation by distance, would bias the distribution of clades (Templeton *et al.* 1995). The developers of the nested cladistic analysis provide an inference key to interpret biased distributions, based on significant distances and clade ranges, and they strongly encourage its use. We interpreted significance according to the inference key provided by GeoDis 2.2 (Posada *et al.* 2000).

Gene diversity (h; Nei 1973) and nucleotide diversity (π; Nei and Tajima 1981) were calculated using Arlequin 2.0 (Schneider *et al.* 2000), to examine whether the current population showed sufficient genetic diversity. In addition, the frequency of pairwise differences was plotted (mismatch distribution), and goodness-of-fit for the sudden expansion model (Rogers and Harpending 1992) was tested with Arlequin, which uses the method of Schneider and Excoffier (1999).

Results

Samples for which haplotypes were determined

Since feather samples were collected from nests or from the ground around nests, the DNA may have been damaged. Consequently, we were able to amplify the target fragment in 70% (178) of the samples. Sixty-nine individuals were identified from these 178 samples based on information about the haplotype and sampling site. As the
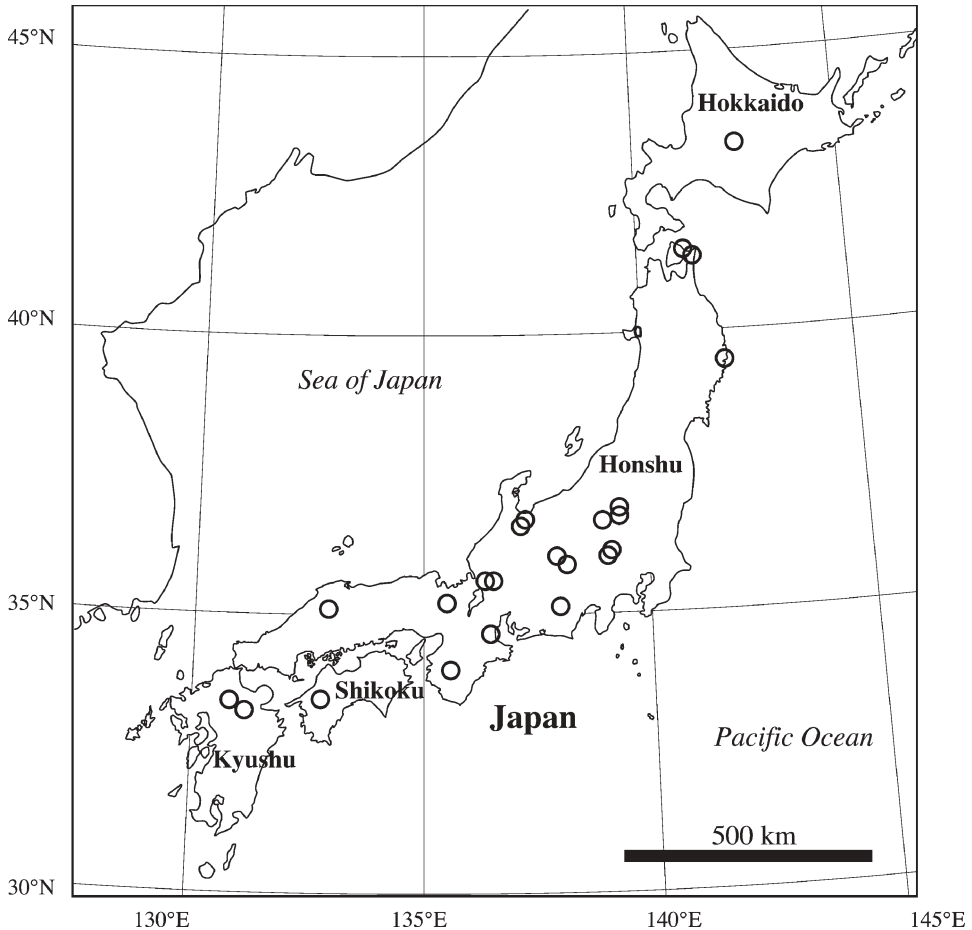
Figure 1. Sampling area. Open circles indicate the sampling sites.

maximum estimate, 93 individuals were identified. These individuals were sampled at 23 sites (130°40′–142°30′ E, 33°15′–43°30′ N; Figure 1). Among the successfully amplified samples, we identified 25 haplotypes and one heteroplasmy that was excluded from the following analyses (Figure 2).

Nested cladistic analysis

The haplotype network of a population that has recently colonized should appear as a star-like network connected to an ancestral node within one or a few branches (mutations). In contrast, the observed haplotype network was broader than a typical star-like network (Figure 3).

Because it is possible to connect two haplotypes via several mutational process in the calculations, some closed loops occurred in the network. Although one of these connections was the true evolutionary process and the others were homoplasies, we could not identify the true evolutionary branch. Therefore, for many clades, we were unable to determine which was a tip and which was an interior clade.

Haplotype	|←————————————————————————————— Site —————————————————————————————→|

Haplotype	36	51	60	71	78	88	107	110	117	192	224	253	258	270	283	297	305	381	400	407	415	416
1	T	G	T	C	C	–	C	A	G	A	G	C	C	A	T	T	C	T	G	T	A	A
2	.	.	.	.	–	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.
4	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	C	.	A	.	.	.
5	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	T	A	.	.	.
6	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	A	.	.	.
7	C	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	C	.	A	.	.	.
8	.	.	.	.	–	.	.	.	.	.	.	.	.	.	.	.	C	.	A	.	.	.
9	.	.	.	.	–	.	.	.	A	.	.	.	.	.	.	.	C	.	A	.	.	.
10	.	.	.	.	.	.	.	.	A	.	A	.	T	G	.	.	C	.	C	A	.	G
11	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	C	.	A	.	.	.
12	.	.	.	.	–	.	.	.	.	.	.	.	.	.	.	G	C	.	A	.	.	.
13	.	.	.	.	T	.	.	.	A	.	.	.	.	.	.	.	C	.	A	.	G	.
14	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	C	.	A	.	G	.
15	.	.	.	.	–	.	.	.	A	.	.	.	.	.	.	.	C	.	A	.	G	.
16	.	.	.	.	–	.	T	.	.	G	.	.	.	.	G	.	C	.	A	.	.	.
17	.	A	A	.	.	.	.	.	.	.	.	.	.	.	G	.	C	.	A	.	.	.
18	.	.	.	.	.	.	.	.	.	G	.	.	.	.	G	C	C	.	A	.	.	.
19	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	C	.	A	.	.	.
20	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	C	.	A	.	.	.
21	.	.	.	.	–	.	.	.	.	.	.	.	.	.	.	.	.	–	A	–	.	.
22	.	.	.	.	–	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.
23	.	.	.	.	–	.	.	.	.	G	.	.	.	.	G	C	C	.	A	.	.	.
24	.	.	.	.	–	.	.	.	R	.	.	.	.	.	.	.	C	.	A	.	.	.
25	.	.	.	.	–	.	.	.	.	.	.	.	T	.	.	.	.	.	A	.	.	.
26	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	C	.	A	.	.	.

Figure 2. Variable sites of 25 haplotypes and one heteroplasmy (no. 24) in Hodgson's hawk-eagle. Dots indicate identity with the top sequence (haplotype 1). "R" represents either an adenine base or a guanine.

In the nesting procedures, haplotypes/nodes were nested to 14 clades at the one-step clade level, and those one-step clades were nested to six clades at the two-step level; finally, the two-step clades were nested to two clades at the three-step level (Figure 3). Nested contingency tests in which sampling localities were treated as categorical variables (Templeton *et al.* 1995) indicated that the geographical association among the clades nested in clade 3–2 was significant ($\chi^2 = 49.72$, $P = 0.036$). For the maximum estimate, the tests indicated that the associations in clades 2–6 and 3–2, and the total cladogram, were significant ($\chi^2 = 26.07$, $P = 0.023$; $\chi^2 = 52.97$, $P = 0.014$; $\chi^2 = 37.96$, $P = 0.002$, respectively).

Table 1 shows clade distances and nested clade distances. The leftmost column indicates the haplotype number, and for each haplotype we calculated a clade distance (Dc; second column) and a nested clade distance (Dn; third column). Haplotypes 5, 6, 20 and 26 were nested in clade 1–1 according to the nesting algorithm (Figure 3), and then each haplotype was defined as either a tip (T) or an interior (I) clade within clade
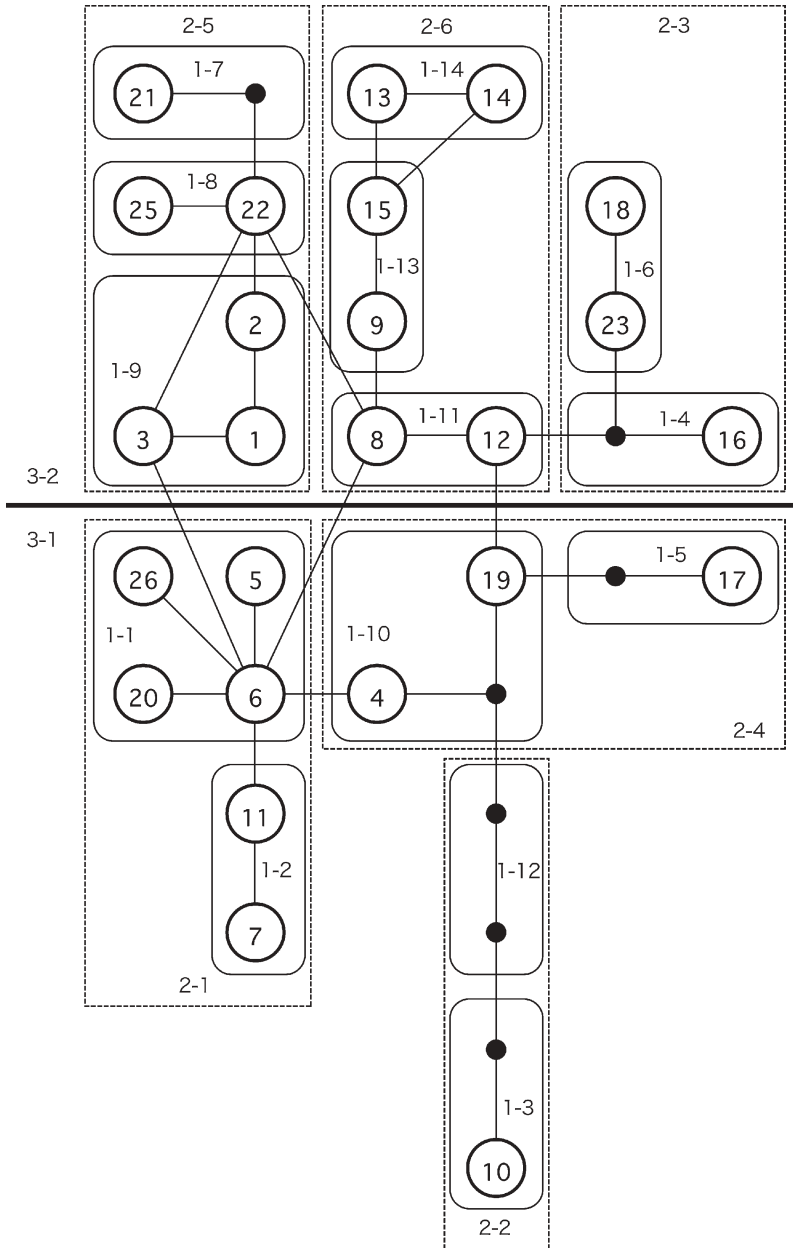
Figure 3. Haplotype network with the nesting design. Open circles with figures and lines represent haplotypes and one-step mutations. The figures in the circles indicate haplotype number and correspond to those in the appendices. Small circles represent haplotypes not found in this study. Rectangles with rounded corners and rectangles with broken lines represent clades at the one- and two-step levels, respectively, and the clade number enclosed in these rectangles corresponds to Table 1. At the three-step level, clades are divided into an upper side (3–2) and a lower side (3–1) by a thick line.

Table 1. Geographical distances based on a nested cladistic analysis and results of statistical tests.

Zero-step level						One-step level						Two-step level						Three-step level		
Haplotype	Dc	Dn	T/I	(I–T)c	(I–T)n	Clade	Dc	Dn	T/I	(I–T)c	(I–T)n	Clade	Dc	Dn	T/I	(I–T)c	(I–T)n	Clade	Dc	Dn
5	257	658	T	-27	-265^S	1–1	262	253	I	212	125	2–1	231	226	I	203	122	3–1	229	232
6	84^S	138^S	I																	
20	0	65	T																	
26	21	319	T																	
7	15	32	T	-5	89	1–2	50^S	128	T											
11	10	121	I																	
10	–	–				1–3	–	–				2–2	43	119	T					
17	–	–				1–5	0	519	T	224	-247	2–4	309	305	I					
4	98	161	I	–	–	1–10	224	272	I											
19	284	368	I																	
16	–	–	T	0	-443	1–4	0	102	I	-89	-58	2–3	133	611^L	T	109	-290^S	3–2	372	373^L
18	0	492	T			1–6	89	161	T											
23	0	49	I																	
21	–	–				1–7	0	754	T	212	-506	2–5	258	278	I					
22	0	451	I	-24	353	1–8	160	344	I											
25	24	98	T																	
1	80	165	I			1–9	231	213	I											
2	86	163	I																	
3	89	413	I																	
8	207	199	I			1–11	213	264	I	108	118^L	2–6	221^S	377	I					
12	93	245	I																	
9	0	413	I			1–13	222	282	I											
15	148	192	I			1–14	107	151^S	T											
13	0	187	I																	
14	101	102	I																	

Abbreviations are as follows: Dc, clade distance; Dn, nested clade distance; T/I, indicating either a tip (T) or an interior (I) clade; (I–T)c, difference in clade distances between interior clades and tip clades within the nested group; and (I–T)n, difference in nested clade distances between interior clades and tip clades within the nested group. "S" and "L" indicate significantly small and large distances, respectively.

Clades with no other clade within a one-step higher level clade do not have a geographical centre. In such clades, the clade distance and the nested clade distance could not be calculated (haplotypes 10, 16, 17, and 21, as well as clades 1–3).

In some clades, tip/interior status could not be discriminated because of a closed loop of possible mutational connections. In these cases, (I–T)c and (I–T)n could not be calculated (clades 1–9, 1–10, 1–11, 1–13, and 1–14).
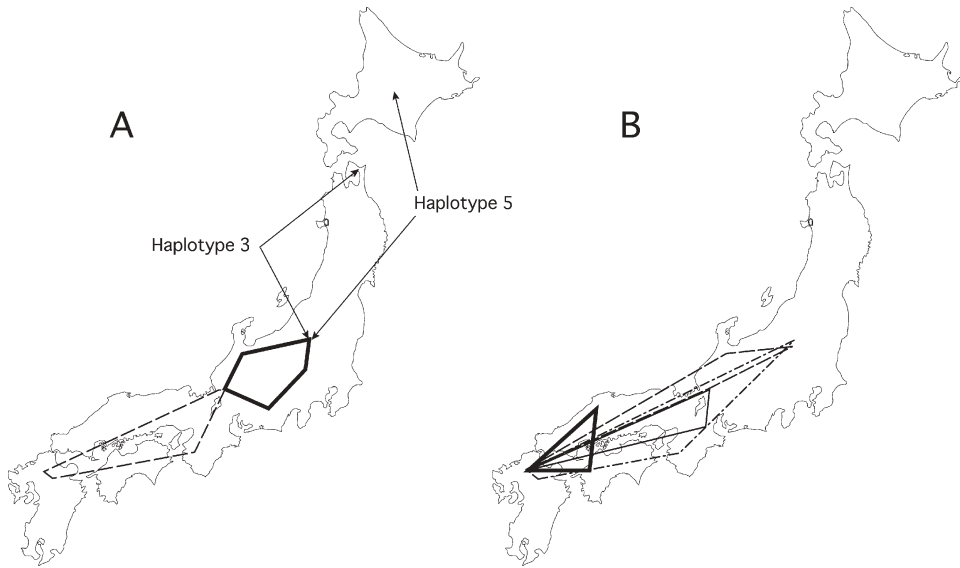
Figure 4. Ranges of haplotypes (*A*) and clades at level 1 (*B*). In these figures, some typical examples are indicated by polygons tracing the outermost sampling sites. In (*A*), thick lines and broken lines represent the range of haplotypes 6 and 8, respectively. Haplotypes 3 and 5 were found at two sampling sites (indicated by arrows). In (*B*), thick lines, thin lines, broken lines, and dotted and broken lines represent the ranges of clades 1–14, 1–8, 1–10, and 1–11, respectively. Although statistically significant geographical distances in haplotype 6 and clade 1–14 indicate that gene flow of these clades was restricted within the clades nesting those clades, the ranges were overlapped by those of the other clades rather than being separated from the others.

1–1 (the fourth column). When either a tip or an interior could be defined, we could calculate the difference between the average of the interiors and tips for the clade distances ($[I–T]c$ in the fifth column) and for the nested clade distances ($[I–T]n$ in the sixth column). At the one-step level, clade 1-1 replaces haplotypes 5, 6, 20 and 26 in the seventh column. Again, Dc, Dn, T/I, $(I–T)c$ and $(I–T)n$ could be given for the one-step clade, in the five columns on the right. Those procedures are repeated at the two- and three-step levels.

Dc and Dn of haplotype 6 were significantly smaller than the values for other haplotypes within clade 1–1, indicating that the distribution of clade 1–1 was biased or that gene flow within clade 1–1 was restricted. In clades 1–1, 2–1, 2–6, 3–2, and the total cladogram, one or more distances were significant, indicating that gene flow was restricted within these clades. However, the ranges of clades with restricted gene flow were not separated from those of the other clades without restriction, but rather overlapped each other (Figure 4). Therefore, we could not find evidence that a local population of Hodgson's hawk-eagle is geographically isolated from other populations in Japan. The inference key implied that the detected restriction of gene flow was due to isolation by distance rather than fragmentation of a local population. However, this inference does not mean that the dispersal ability of this species is physically small.

For the maximum estimate, one or more distances were significant in clades 1–2 and 1–9, in addition to the above-mentioned clades. Nevertheless, the inference key could not provide an interpretation because the sampling design was insufficient.

Genetic diversity

We calculated the indices of genetic diversity for 68 individuals. Gene diversity (h) and nucleotide diversity (π) were 0.943 ± 0.014 SD and 0.702 ± 0.413 SD %, respectively. These indices indicate that maternal lineages differed from each other among more than 90% of pairs of two individuals sampled randomly from the Japanese population, and on average randomly sampled pairs differed from each other in three of 418 bp in the CR. The indices calculated using the maximum estimate (91 individuals) were $h = 0.935 \pm 0.013$ SD and $\pi = 0.741 \pm 0.430$ SD %. Even when the indices were calculated from 174 samples instead (heteroplasmy was found in four samples), ignoring the overlap of individuals, the gene diversity and nucleotide diversity were 0.913 ± 0.009 SD and 0.710 ± 0.413 SD %, respectively.

The distribution of pairwise differences (mismatch distribution) was unimodal, and fitted the sudden expansion model (model parameters: $\tau = 2.923$, $\theta_0 = 0.000$, $\theta_1 = 2890.000$; in case of the maximum estimate, $\tau = 2.803$, $\theta_0 = 0.262$, $\theta_1 = 37.676$, unimodal). A second wave of larger values of pairwise differences would suggest a bottleneck (Rogers and Harpending 1992); however, in the observed distribution, no second wave was found. Therefore, the Japanese population was assumed to have expanded, having experienced no bottlenecks since colonization.

Discussion

Genetic structure

The presence of a barrier that restricts individual movements would result in two or more clades whose ranges are limited to a particular area. In this study, the ranges of the clades for which restricted gene flow was detected overlapped with the ranges of other clades. Moreover, the inference key implied that the detected restrictions of gene flow were due to isolation by distance rather than by fragmentation. Therefore, there is no suggestion that the Japanese population of Hodgson's Hawk-eagle has been separated geographically in the process of range expansion. Nevertheless, because it takes several generations for habitat fragmentation to result in a change in the genetic structure, the results may not reflect geographical fragmentation occurring in the recent past.

Gene flow was restricted in some clades, however. Although the restricted gene flow was implied to be the result of isolation by distance, the dispersal ability of this species is not thought to be so limited that it cannot disperse physically beyond the small clade ranges, because the clade distances of the lowest level, haplotypes 5 and 19, were larger (Table 1). Moreover, records indicate that this species has wandered from Japan to Korea (Austin 1948), which indicates the potential of long-range dispersal in this species. Consequently, it is thought that the range expansion of some lineages is limited by factors other than geography. Instead, ecological or behavioural factors are expected to limit range expansion. Unfortunately, we have little information on dispersal in Hodgson's Hawk-eagle. Since this species is considered sedentary and its breeding dispersal slight, although some move seasonally to lower altitudes in India and Nepal (Ali and Ripley 1978, Brazil 1991, Grimmett *et al.* 1998, Inskipp and Inskipp 1991, Morioka *et al.* 1995), natal dispersal is relatively important for range expansion. In Japan, it has been observed that fledglings remain in the home

Table 2. Gene diversities (h) and nucleotide diversities (π) in birds

Species	Scientific name	Source	h	π	n	Note
Spanish Imperial Eagle	*Aquila adalberti*	Martínez-Cruz et al. 2004	0.32	0.0010	60	Population size is estimated as 140 pairs
Eastern Imperial Eagle	*Aquila heliaca*	Martínez-Cruz et al. 2004	0.78	0.0055	34	Population size is estimated as 5,000 individuals
Golden Eagle	*Aquila chrysaetos japonica*	Masuda et al. 1998	0.26	0.0017	14	
Hodgson's Hawk-eagle	*Spizaetus nipalensis orientalis*	Present study	0.94	0.0070	68	
Hazel Grouse	*Bonasa bonasia*	Baba et al. 1999	≤ 0.8		8–17	
Sage Grouse	*Centrocercus urophasianus*	Kahn et al. 1999	0.64–0.87	0.0241–0.0393	17–26	Large-bodied population
Sage Grouse	*Centrocercus urophasianus*	Kahn et al. 1999	0.06	0.0011	31	Gunnison (small-bodied) population
Lesser Prairie-chicken	*Tympanuchus pallidicinctus*	van den Bussche et al. 2003	0.70–1.00	0.0034–0.0254	2–26	Oklahoma population
Lesser Prairie-chicken	*Tympanuchus pallidicinctus*	van den Bussche et al. 2003	0.67–1.00	0.0042–0.0131	3–23	New Mexico population (stable population)
Red-crowned Crane	*Grus japonensis*	Hasegawa et al. 1999	0.78	0.0087	29	East Asia population, including Japan
Red-crowned Crane	*Grus japonensis*	Hasegawa et al. 1999	0.25	0.0047	15	Japanese population
Turnstone	*Arenaria interpres*	Wenink et al. 1994	0.96	0.0104	25	
Dunlin	*Calidris alpina*	Wenink et al. 1994	0.93	0.0368	25	
Common Guillemot	*Uria aalge*	Moum and Árnason 2001	0.71	0.0054	22	West Atlantic population
Common Guillemot	*Uria aalge*	Moum and Árnason 2001	0.72	0.0048	57	East Atlantic population
Razorbill	*Alca torda*	Moum and Árnason 2001	0.95	0.0173	42	West Atlantic population
Razorbill	*Alca torda*	Moum and Árnason 2001	0.88	0.0097	81	East Atlantic population
Siberian Jay	*Perisoreus infaustus*	Uimaniemi et al. 2000	0.83	0.0014	65	Fennoscandia population, estimated as 110,000–400,000 pairs
Common Chaffinch	*Fringilla coelebs*	Marshall and Baker 1997	0.89 (0.20–0.94)	0.1290 (0.0015–0.0080)	42 (9–14)	
Brambling	*Fringilla montifringilla*	Marshall and Baker 1997	0.87	0.0026	10	

ranges of their parents for 2–3 years (Inoue 2000, Yamazaki 2000). In addition, of two individuals considered sub-adults based on their iris colours that carried radio-transmitters for 16 months, one was observed to mate while wandering over 42.7 km², and the other did not leave the mountains where it had been trapped, although it wandered more widely (within 135.5 km²; Murate 2000). These observations imply that natal dispersal of the Hodgson's Hawk-eagle is also relatively small. In contrast, juveniles and immatures of the Javan Hawk-eagle, *Spizaetus bartelsi*, disperse to different habitats from where the adults breed (Nijman and van Balen 2003). This implication corresponds to the fact that the ranges of some clades are small. However, it is not possible to state that the ranges of some clades are significantly smaller than those of others. To resolve this problem, it is necessary to collect data on natal dispersal and differences in local habitats. In addition, it is important to increase the sample size for the analysis used in this study in order to detect exactly where gene flow is restricted.

Genetic diversity

The genetic diversity of this species is nearly equal to or higher than that of other species (populations), including the Eastern Imperial Eagle *Aquila heliaca*, Turnstone *Arenaria interpres*, Dunlin *Calidris alpina*, Atlantic populations of Common Guillemot *Uria aalge* and Razorbill *Alca torda*, Common Chaffinch *Fringilla coelebs* and Brambling *Fringilla montifringilla* (Table 2), which maintain sufficient numbers of individuals and are not considered threatened. In contrast, the genetic diversities of the Spanish Imperial Eagle *Aquila adalberti*, Japanese populations of the Golden Eagle *Aquila chrysaetos japonica* and Red-crowned Crane *Grus japonensis*, Sage Grouse *Centrocercus urophasianus*, New Mexico populations of the Lesser Prairie-chicken *Tympanuchus pallidicinctus,* and the Siberian Jay *Perisoreus infaustus*, which are endangered owing to declining populations or habitat fragmentation, are lower (Table 2). Therefore, our results suggest that a sufficient number of maternal lineages have been maintained in the extant Japanese population of Hodgson's Hawk-eagle.

The nucleotide diversity of Hodgson's Hawk-eagle is higher than those of the above fragmented populations, except for grouse (Table 2). This indicates that a relatively large number of old lineages has been retained, because nucleotide diversity reflects time since divergence. This corresponds to the lack of evidence for a bottleneck observed in the mismatch distribution.

Since feathers, which were the main source of samples in our study, were collected in the field, individuals were identified based on their haplotypes and the site where the feathers were collected. Although this criterion may have misled us, the values calculated from the maximum estimate and the total number of samples were similar to the values calculated based on 68 individuals identified, and were still high in comparison to other species.

Status of Hodgson's Hawk-eagle

At least in terms of genetic diversity, the Japanese population of Hodgson's Hawk-eagle is not likely to require urgent attention; moreover, it does not appear to have experienced a critical bottleneck situation in the past. The high genetic diversity is unlikely to result from occasional immigration from South-east Asian populations,

because the haplotypes identified in this study were connected to subsequent haplotypes within one or two mutations, except for haplotype 10. If there was gene flow between Japan and mainland Asia, more long branches would have been found in the haplotype network, and the mismatch distribution would have been multimodal.

If there is no immigration into the Japanese population and no regular long-distance dispersal, it may be difficult for this species to recolonize the range that it once occupied. To address this question, ecological studies of dispersal are needed.

The genetic diversities of Japanese populations of the Red-crowned Crane and Golden Eagle, which are large birds, are much lower than that of the hawk-eagle (Table 2). In the Red Data Book of Japan, the Red-crowned Crane and Golden Eagle are ranked as Vulnerable and Endangered, respectively, while the hawk-eagle is considered Endangered. The populations of the former two species are definitely smaller (750 and 500 individuals, respectively; Ministry of the Environment 2002) than that of the hawk-eagle. Therefore, genetic diversity does not necessarily correspond to the status assigned in the Red Data Book. Since protection programmes should be implemented after considering genetic diversity and structure, genetic studies should also be conducted for the other endangered species.

Appendix 1. Haplotype and locality of identified individuals

Individual-Haplotype (Locality): 1–6 (35°57′ N, 138°56′ E); 2–6 (35°57′ N, 138°56′ E); 3–6 (36°00′ N, 139°05′ E); 4–15 (33°15′ N, 130°58′ E); 5–8 (33°15′ N, 130°58′ E); 6–6 (36°42′ N, 139°13′ E); 7–21 (33°25′ N, 130°40′ E); 8–17 (33°25′ N, 130°40′ E); 9–8 (33°25′ N, 130°40′ E); 10–13 (33°25′ N, 130°40′ E); 11–14 (33°25′ N, 130°40′ E); 12–22 (33°25′ N, 130°40′ E); 13–19 (33°25′ N, 130°40′ E); 14–14 (35°05′ N, 132°47′ E); 15–10 (35°35′ N, 136°27′ E); 16–15 (35°35′ N, 136°27′ E); 17–8 (35°35′ N, 136°27′ E); 18–9 (35°35′ N, 136°27′ E); 19–19 (35°35′ N, 136°27′ E); 20–6 (35°35′ N, 136°27′ E); 21–6 (35°35′ N, 136°27′ E); 22–11 (35°35′ N, 136°27′ E); 23–1 (36°46′ N, 139°14′ E); 24–6 (36°46′ N, 139°14′ E); 25–18 (36°46′ N, 139°14′ E); 26–1 (36°46′ N, 139°14′ E); 27–2 (36°46′ N, 139°14′ E); 28–20 (36°46′ N, 139°14′ E); 29–2 (36°46′ N, 139°14′ E); 30–1 (36°46′ N, 139°14′ E); 31–3 (36°46′ N, 139°14′ E); 32–8 (35°33′ N, 136°12′ E); 33–11 (35°33′ N, 136°12′ E); 34–12 (35°33′ N, 136°12′ E); 35–12 (35°33′ N, 136°12′ E); 36–4 (36°42′ N, 139°13′ E); 37–2 (36°42′ N, 139°13′ E); 38–5 (36°42′ N, 139°13′ E); 39–12 (35°56′ N, 137°47′ E); 40–7 (35°56′ N, 137°47′ E); 41–7 (35°48′ N, 138°05′ E); 42–6 (35°48′ N, 138°05′ E); 43–6 (36°34′ N, 138°50′ E); 44–14 (33°23′ N, 132°39′ E); 45–2 (36°27′ N, 137°02′ E); 46–4 (36°27′ N, 137°02′ E); 47–6 (36°27′ N, 137°02′ E); 48–10 (36°27′ N, 137°02′ E); 49–5 (43°30′ N, 142°30′ E); 50–6 (35°05′ N, 137°49′ E); 51–16 (39°22′ N, 141°54′ E); 52–3 (41°26′ N, 140°52′ E); 53–23 (41°24′ N, 141°10′ E); 54–2 (36°35′ N, 137°12′ E); 55–1 (36°35′ N, 137°12′ E); 56–8 (33°55′ N, 135°30′ E); 57–22 (33°25′ N, 130°40′ E); 58–6 (35°35′ N, 136°27′ E); 59–12 (36°46′ N, 139°14′ E); 60–1 (35°57′ N, 138°56′ E); 61–2 (36°34′ N, 138°50′ E); 62–1 (36°34′ N, 138°50′ E); 63–7 (35°56′ N, 137°47′ E); 64–25 (35°35′ N, 136°27′ E); 65–25 (35°35′ N, 136°27′ E); 66–25 (34°46′ N, 136°18′ E); 67–26 (35°15′ N, 135°28′ E); 68–24 (35°35′ N, 136°27′ E); 69–26 (35°33′ N, 136°12′ E).

Appendix 2. Haplotype and locality of identified individuals in the maximum estimate

Individual-Haplotype (Locality): 1–6 (35°57′ N, 138°56′ E); 2–6 (35°57′ N, 138°56′ E); 3–6 (35°57′ N, 138°56′ E); 4–6 (35°57′ N, 138°56′ E); 5–1 (35°57′ N, 138°56′ E); 6–1 (35°57′ N, 138°56′ E); 7–6 (36°00′ N, 139°05′ E); 8–6 (36°00′ N, 139°05′ E); 9–15 (33°15′ N, 130°58′ E); 10–8 (33°15′ N, 130°58′ E); 11–6 (36°42′ N, 139°13′ E); 12–6 (36°42′ N, 139°13′ E); 13–22 (33°25′ N, 130°40′ E); 14–21 (33°25′ N, 130°40′ E); 15–17 (33°25′ N, 130°40′ E); 16–8 (33°25′ N, 130°40′ E); 17–13 (33°25′ N, 130°40′ E); 18–14 (33°25′ N, 130°40′ E); 19–22 (33°25′ N, 130°40′ E); 20–22 (33°25′ N, 130°40′ E); 21–19 (33°25′ N, 130°40′ E); 22–14 (35°05′ N, 132°47′ E); 23–6 (35°35′ N, 136°27′ E); 24–6 (35°35′ N, 136°27′ E); 25–10 (35°35′ N, 136°27′ E); 26–10 (35°35′ N, 136°27′ E); 27–15 (35°35′ N, 136°27′ E); 28–10 (35°35′ N, 136°27′ E); 29–10 (35°35′ N, 136°27′ E); 30–8 (35°35′ N, 136°27′ E); 31–9 (35°35′ N, 136°27′ E); 32–24 (35°35′ N, 136°27′ E); 33–24 (35°35′ N, 136°27′ E); 34–19 (35°35′ N, 136°27′ E); 35–6 (35°35′ N, 136°27′ E); 36–6 (35°35′ N, 136°27′ E); 37–11 (35°35′ N, 136°27′ E); 38–25 (35°35′ N, 136°27′ E); 39–25 (35°35′ N, 136°27′ E); 40–1 (36°46′ N, 139°14′ E); 41–6 (36°46′ N, 139°14′ E); 42–12 (36°46′ N, 139°14′ E); 43–12 (36°46′ N, 139°14′ E); 44–18 (36°46′ N, 139°14′ E); 45–18 (36°46′ N, 139°14′ E); 46–18 (36°46′ N, 139°14′ E); 47–18 (36°46′ N, 139°14′ E); 48–1 (36°46′ N, 139°14′ E); 49–2 (36°46′ N, 139°14′ E); 50–20 (36°46′ N, 139°14′ E); 51–2 (36°46′ N, 139°14′ E); 52–1 (36°46′ N, 139°14′ E); 53–1 (36°46′ N, 139°14′ E); 54–3 (36°46′ N, 139°14′ E); 55–3 (36°46′ N, 139°14′ E); 56–8 (35°33′ N, 136°12′ E); 57–8 (35°33′ N, 136°12′ E); 58–8 (35°33′ N, 136°12′ E); 59–11 (35°33′ N, 136°12′ E); 60–12 (35°33′ N, 136°12′ E); 61–12 (35°33′ N, 136°12′ E); 62–26 (35°33′ N, 136°12′ E); 63–12 (35°33′ N, 136°12′ E); 64–4 (36°42′ N, 139°13′ E); 65–4 (36°42′ N, 139°13′ E); 66–2 (36°42′ N, 139°13′ E); 67–2 (36°42′ N, 139°13′ E); 68–5 (36°42′ N, 139°13′ E); 69–12 (35°56′ N, 137°47′ E); 70–7 (35°56′ N, 137°47′ E); 71–7 (35°56′ N, 137°47′ E); 72–7 (35°56′ N, 137°47′ E); 73–7 (35°48′ N, 138°05′ E); 74–7 (35°48′ N, 138°05′ E); 75–6 (35°48′ N, 138°05′ E); 76–6 (36°34′ N, 138°50′ E); 77–2 (36°34′ N, 138°50′ E); 78–1 (36°34′ N, 138°50′ E); 79–14 (33°23′ N, 132°39′ E); 80–2 (36°27′ N, 137°02′ E); 81–4 (36°27′ N, 137°02′ E); 82–6 (36°27′ N, 137°02′ E); 83–10 (36°27′ N, 137°02′ E); 84–5 (43°30′ N, 142°30′ E); 85–6 (35°05′ N, 137°49′ E); 86–16 (39°22′ N, 141°54′ E); 87–3 (41°26′ N, 140°52′ E); 88–23 (41°24′ N, 141°10′ E); 89–2 (36°35′ N, 137°12′ E); 90–1 (36°35′ N, 137°12′ E); 91–8 (33°55′ N, 135°30′ E); 92–25 (34°46′ N, 136°18′ E); 93–26 (35°15′ N, 135°28′ E).

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