

Genetic diversity assessment and conservation implications for two raptor species

Introduction

Monitoring genetic diversity in small populations of endangered species is crucial for their conservation because of their high susceptibility to inbreeding and genetic drift. Inbreeding and genetic drift can lead to the accumulation of deleterious alleles and loss of adaptive potential, elevating the risk of congenital abnormalities, infertility, and higher disease susceptibility. In the present study, I studied the genetics of two raptor species, the Japanese golden eagle, *Aquila chrysaetos japonica*, and the mountain hawk-eagle, *Nisaetus nipalensis orientalis*. Both are subspecies primarily inhabiting Japan and are categorized as endangered by the Japanese Ministry of the Environment. They are threatened by the loss of suitable foraging habitats and decrease in prey populations, resulting from the expansion and abandonment of forests and infrastructure development. It is estimated that there are around 500 individuals of golden eagles remaining in Japan, with continuously declining reproductive success rates. The census of mountain hawk-eagles is larger, with at least 1800 individuals, and a higher reproductive success rate than the golden eagle.

To protect these species, the Japanese government has implemented a conservation plan to encourage population monitoring, habitat restoration, and captive breeding. In addition to these efforts, it is also necessary to assess population genetic structure and monitor genetic diversity to assign conservation units, detect signs of inbreeding, genetic drift, and evaluate population viability. Past genetic studies mainly focused on neutral genetic markers and have suggested both species each consist of a single genetic population. In the golden eagle, genomic studies revealed that levels of diversity have declined in Japan, compared to other populations. In the mountain hawk-eagle, mitochondrial genetic analysis showed that diversity is higher than

in golden eagles. Up until now, there were several knowledge gaps in these species. First, neutral genetic studies in the Japanese golden eagle had primarily focused on northern regions, and individuals from central and western had not been studied in detail. In the mountain hawk-eagle, slight effects of restricted gene flow and isolation had been suggested in a previous study, but genetic diversity had only been assessed across the country using a few samples per region. Lastly, in both species, genetic diversity at functional genes had not been studied to date.

In this thesis, I increased sampling efforts and re-evaluated population structure and genetic diversity at neutral markers in both species. In addition, because adaptive potential greatly depends on variation at genes with phenotypic consequences, I also surveyed the diversity at the immune-related major histocompatibility complex (MHC) gene region in the golden eagle and mountain hawk-eagle. Furthermore, utilizing the breeding data of captive Japanese golden eagles, I studied the association between breeding success and genetic characteristics of the MHC, because this gene region is known to affect mate choice in various vertebrate species.

Methods

Samples of wild golden eagles and mountain hawk-eagles were collected by collaborators and the author. Samples of captive individuals were gathered by zoo staff. Non-invasively collected feathers, feces, pellets and tissues from dead individuals were used for analyzing wild individuals. For wild mountain hawk-eagles, blood samples were also collected as part of tagging conducted by a field conservation group. Moulded feathers collected in cages, along with blood samples collected as part of regular health checks were used for analyzing captive individuals. Using these samples, I surveyed the genetic diversity at the mitochondrial DNA (mtDNA), microsatellite loci, and the major histocompatibility complex (MHC) gene region in

the Japanese golden eagle. For the mountain hawk-eagle mtDNA and MHC loci were surveyed. Comparison with other subspecies of golden eagle and other raptor species was performed where possible. mtDNA data was also used to infer phylogeography, and microsatellites were used for population structure analyses. Moreover, MHC allele data was analyzed together with reproductive success data of captive golden eagles to evaluate the on breeding performance, using generalized linear mixed models.

Results

1. Genetic diversity of the endangered Japanese golden eagle at neutral loci

No regional population structuring was observed from mtDNA analyses. Higher mitochondrial diversity was seen in the wild (haplotype diversity $h = 0.642$, haplotype richness $hr = 6.9$, nucleotide diversity $\pi = 0.0045$) than in captivity ($h = 0.510$, $hr = 6.0$, $\pi = 0.0019$). Regionally, west Japan had lower diversities ($h = 0.378$, $hr = 2.0$, $\pi = 0.0022$) than in north ($h = 0.703$, $hr = 3.0$, $\pi = 0.0045$) and central Japan ($h = 0.933$, $hr = 4.3$, $\pi = 0.0066$). The Japanese population had relatively low diversities ($h = 0.664$, $hr = 3.9$, $\pi = 0.007$) when compared to non-threatened, mainland populations (e.g. mainland Asia: $h = 0.854$, $hr = 7.4$, $\pi = 0.051$). Microsatellite markers revealed slight differentiation between wild and captive eagles. Observed heterozygosity and allelic richness were slightly higher in captive eagles ($Ho = 0.627$, $Ar = 3.46$) than in the wild ($Ho = 0.597$, $Ar = 3.44$). Among wild individuals, diversity was higher in west ($Ho = 0.638$, $Ar = 3.21$) and north Japan ($Ho = 0.593$, $Ar = 3.51$) than in central Japan ($Ho = 0.589$, $Ar = 2.95$). Compared to the previously bottlenecked Scottish golden eagles, Japanese golden eagles had higher Ho (0.518 and 0.614, respectively) but lower Ar (3.75 and 3.60 respectively).

2. Genetic diversity of the Japanese golden eagle at the major histocompatibility complex region and its effects on reproductive success

A total of 17 MHC Class IIB *DR* exon 2 alleles were found in the Japanese golden eagle. This translated into a total of 10 unique amino acid sequences. Generally, diversity was higher in captivity (e.g. allelic richness $N_{rarefied} = 14.4$ and amino acid richness $Np_{rarefied} = 8.4$) than in the wild ($N_{rarefied} = 9.2$, $Np_{rarefied} = 5.6$). Long-read sequencing of the MHC Class IIB region on the MinION Flongle was successful, yielding a 14,476 bp sequence. The individual sequenced appeared to have two *DR* loci, while the reference genome individual from Scotland had three loci.

Generalized linear mixed models showed that the age of a male is associated with declining fertilization rate ($p = 0.005$, Bonferroni corrected) and hatching success ($p = 0.031$, Bonferroni corrected). MHC *DR* exon 2 genetic distance between the male and female of a pair was also loosely associated with proportion of eggs hatched ($p = 0.052$, Bonferroni corrected). The number of alleles, studbook relatedness, years paired, and age of female had no significant effect on clutch size, proportion of fertilized and hatched eggs (all GLMMs resulted in Bonferroni corrected $p > 0.05$).

3. Genetic diversity of the mountain hawk-eagle neutral and functional loci

Combined with published data from a previous study, I found 29 mtDNA control region haplotypes, which formed a complex, star-shaped neighbour-joining network. Several haplotypes were exclusive to certain prefectures, such as J28 in Miyazaki and J29 in Hokkaido. mtDNA diversity was generally higher in north (e.g. $hr = 7.6$, $h = 0.924$) and south Japan ($hr = 7.9$, $h = 0.9333$) followed by central Japan ($hr = 7.0$, $h = 0.871$), and west Japan was the lowest ($hr = 5.9$, $h = 0.838$). Over time, the 1986-2003 frame had higher levels of diversity ($hr = 25.0$,

$h = 0.937$, $\pi = 0.0041$) than the more recent 2004-2021 timeframe ($hr = 18.9$, $h = 0.840$, $\pi = 0.0025$). When comparing Japan-wide level diversity, calculated from data collected by Asai et al. (2006), to that of the Suzuka mountain subpopulation, diversity was considerably lower in Suzuka mountains ($hr = 8.9$, $h = 0.811$, $\pi = 0.0018$) than whole Japan ($hr = 18.7$, $h = 0.910$, $\pi = 0.0036$; Table 4.2). Interspecies comparisons indicated that the mountain-hawk eagle had higher values at some indices (e.g. $hr = 10.3$) than the Japanese golden eagle ($hr = 6.0$), but lower values at other indices (mountain hawk-eagle: $\pi = 0.0035$, Japanese golden eagle: $\pi = 0.0076$).

A total of 16 alleles were genotyped at the MHC Class IIB *DR* exon 2 locus in 33 mountain hawk-eagles (Table 4.4). The diversity was similar to that of wild-born Japanese golden eagles, lower than in non-threatened species. For example, allelic richness and nucleotide diversities were $N_{a_{rarefied}} = 11.9$ and $\pi = 0.0623$ in mountain hawk-eagles and $N_{a_{rarefied}} = 11.7$ and $\pi = 0.06124$ in golden eagles, while these values were much higher, $N_{a_{rarefied}} = 22.1$ and $\pi = 0.104$, in the common buzzard.

Discussion

Re-evaluation of neutral genetic diversity in the Japanese golden eagle and the mountain hawk-eagle revealed that both species do not exhibit evidence of geographic structuring, and corroborate past studies that they should be treated as single conservation units.

Generally, mtDNA, microsatellite, and MHC genetic diversities were comparable in the wild and in captivity for the Japanese golden eagle, supporting previous research that suggest integrated management of the *in-situ* and *ex-situ* populations for maintaining genetic diversity. mtDNA diversity and microsatellite allelic richness were lower than in other subspecies, suggesting effects of genetic drift. This aligns with recent genomic studies suggesting ongoing

declines in diversity in the Japanese population, compared with non-threatened subspecies. Moreover, MHC differences between individuals of a breeding pair was loosely associated with captive reproductive success, in particular, the proportion of eggs hatched. This result emphasizes the need to maintain diversity at functional genes to avoid declines in reproductive success rates. In addition, long-read sequencing of the MHC region suggested copy number variations in the golden eagle, and so future comparisons within and between subspecies are necessary to better understand how diversity is generated, inherited, and maintained. Further studies of genetic diversity at the MHC loci and their effects on breeding performance in the wild population are important as well, in order to disentangle possible factors affecting mate choice and declines in reproductive success rates. In addition to MHC, the age of males appeared to have negative effects on captive breeding performance as well. Given that captive founder population is ageing, and anecdotal evidence suggest ageing in the wild as well, further loss of young individuals in the population can exacerbate the current low levels of reproductive success.

In the mountain hawk-eagle, re-evaluation of neutral genetic diversity across Japan and within an intensively sampled subpopulation suggested that local-level diversity is considerably lower than on a national level. This may not be surprising but given that there were regionally specific haplotypes and the total number of mtDNA haplotypes found in Japan was high, it could mean that although subpopulation level diversities are low, the presence of geographically specific lineages are important contributors to overall genetic diversity. When compared to the Japanese golden eagle, the number of haplotypes was much higher, but nucleotide diversity was lower in the mountain hawk-eagle, unlike what was suggested in a previous study. This difference from the past study is likely because a more leveled comparison was conducted in this study. Yet, the differences in patterns of diversity seen between the species probably reflects their differences in demography, ecology, and evolution, in particular, changes in population size, dispersal

distance, and historical gene flow. MHC diversity, on the other hand, was similar with Japanese golden eagles, and both had lower diversities than in non-threatened species. This may be due to similarities in phylogenetic and selection effects. Nuclear and genome-wide analyses on local and national levels, coupled with subspecies comparisons, are needed to disentangle the effects of demographic, ecological, evolutionary, and phylogenetic factors on genetic diversity, in order to understand whether current levels of genetic diversity can be considered as sufficient or not in the mountain hawk-eagle.

Applying this to conservation management strategies, several suggestions can be made for the preservation of genetic diversity and conservation of populations. First, it is suggested that habitat preservation and restoration efforts, together with supplemental feeding be strengthened for achieving sustainable levels of reproductive success, especially for the Japanese golden eagle. Moreover, for the Japanese golden eagle, it was suggested that MHC and male age possibly affects reproductive success, so the exchange of individuals between the *in-situ* and *ex-situ* populations (i.e. reintroduction of captive individuals to the wild, and bringing wild individuals into captivity) may assist in providing sufficient options for mate choice and avoiding senescence-related reproductive failures in both populations. This exchange will also help to maintaining gene flow between both gene pools. Gamete preservation and assisted reproductive technologies may also be a strategy for preserving and passing on current levels of genetic diversity to future generations while preventing effects of senescence. In the case of the mountain hawk-eagle, results of this thesis suggested the possible importance of subpopulations on generating and maintaining genetic diversity on a national level. Thus, although the population should be treated as one conservation unit, conservation should take into account local subpopulations. Further research encompassing other subspecies is crucial for a better understanding of genetic/genomic diversity and its implications on conservation.