

F-1.1 Effects of reduced genetic variability on population viability

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Abstract Wild populations of several plants and animals were investigated on the relationship of genetic variability with morphological ecological and biogeographical traits. In herbaceous plants, a geographically restricted species had low genetic diversity compared to its widespread congener in *Cerastium*, although, in *Tricyrtis* sect. *Flavae*, the contrary pattern was observed. In the Japanese clouded butterfly, *Parunassius glacialis*, there was a significant negative relationship between the population mean FA and the mean heterozygosity, suggesting that FA could be one of the indicators to evaluate the genetic variability within populations.

In three marsh-inhabiting passerine birds, *Megalurus pryori*, *Emberiza yessoensis* and *Acrocephalus arundinaceus*, although FA was quite prevalent in all species and it was coincident with some genetic components, the causes of variation in FA levels were not clear. In the alpine accentor *Prunella collaris*, there was a relatively-inbred insular population structure in which the breeding system probably reduced their genetic variability. In the Hokkaido population of the sika deer *Cervus nippon*, there were six subpopulations based on mitochondrial DNA haplotypes and the heterozygosity was relatively lower than in the Chiba population. The degree of FA in antlers and mandibles was not different between populations or years irrespective of its genetic bases or food conditions. In several populations of the sika deer, the level of genetic variability within a population did not correlate with its population size and fitness measure of individuals. In the Iriomote wildcat *Felis iriomotensis*, no polymorphism in neutral microsatellite genes were found, but relatively high level of variability was found in MHC class I genes.

Key Words : allozyme, DNA, fluctuating asymmetry, genetic marker, heterozygosity

1. Introduction

When wild populations of plants and animals are endangered, their sizes are small enough to anticipate low genetic variability and accompanying mal effects on their survival. Small population sizes indicate high chance of breeding within kin, accumulation of sublethal genes in inbred groups, and low fitness by inbreeding depression. Among wild populations, however, there are small ones surviving over a historical time or in a small limited habitat like oceanic islands and high mountains, those which could have been extinct if only judged from size. Here are due reasons for us to investigate the relationship between genetic variability and morphological, ecological and biogeographical traits in wild populations of plants and animals with varied size or distribution ranges. In the present study, we investigated genetic variability and related traits crucial to their survival of wild populations of several plants and animals that have been endangered or have received anthropogenic impact on them.

2. Materials and methods

(1) Genetic variability and distribution of two herbaceous plant groups

Random amplified polymorphic DNA (RAPD) was applied to measure the genetic variability of *Cerastium* spp. From each of 16 populations of these species, leaves of ten plants were collected. Total DNA was extracted from them using the CTAB method and selectively

amplified by the PCR method with ten kinds of RAPD primers. Amplified DNA fragments were electrophored on 2 % agar gel and each band was treated as a locus. A index of genetic variability Shannon's H was calculated from these data.

Allozyme analysis was applied to four species of *Tricyrtis* sect. *Flavae*. From each of 14 populations of these species, about 20 - 30 plants were collected, homogenized in buffer solution and centrifuged. The supernatant was electrophored on polyacrylamide gel and starch one. A total of 13 enzymes were detected with active staining and population-specific allele frequencies were counted and analyzed with procedures of population genetics.

(2) Phenotypic variation, genetic variation and fitness component of Japanese clouded butterflies

Adult butterflies were sampled from 20 local populations from May to June in Gifu prefecture. Population densities were measured as a number of individuals caught within 10 minute in each population. Fresh adults were brought back to the laboratory and kept in -80°C until electrophoretic study. Fore and hind wings were cut and remaining head and thorax were used for allozyme study and DNA analysis. Abdomen of males were dried and checked the relative wear of the hair on it due to copulation. Twelve veinal lengths were measured using NIH image software and unsigned absolute left minus right length of each veinal length was calculated (FA). Individual FA was represented as mean FA of each 12 veinal length of each adult. The thoraxes were homogenized with buffer (Tris-HCl, pH7.0) and centrifuged at 12,000rpm for 15 min. The supernatant was collected and analyzed electrophoretically using 13% starch gels.

(3) Population size and genetic variability of three-spined sticklebacks

In populations of the three-spined stickleback *Gasterosteus aculeatus*, population sizes were estimated, and asymmetry in the number of lateral plates and genetic variability were measured. For counting of lateral plates, they were stained with arylaline red. For genetic analysis, eight macrosatellite loci were used. For formalin-preserved specimens, special devices of DNA extraction and PCR amplification were tested and adopted.

(4) Population structure, genetic variability, and fluctuating asymmetry in marsh inhabiting passerines

I have studied population structure of *Megalurus pryri*, *Emberiza yessoensis*, and *Acrocephalus arundinaceus* at lower Tone river (Kamisu) area and Lake kasumigaura (Ukishima and other 10 sites). The Japanese Marsh Warbler (*M. pryri*) is endemic to the Far East, and is listed as an endangered species in the Japanese Red Data List. The present population of *M. pryri* is estimated at about 1000 birds and is restricted to only three breeding sites. Due to its narrow range and history of local-extinctions, the Japanese Reed Bunting (*E. yessoensis*) has been classified as a vulnerable bird species. The Great Reed Warbler (*A. arundinaceus*) is a fairly common summer visitor inhabiting reed beds in Japan. All birds captured were measured and uniquely color-banded, and blood samples were collected from the wing vein in order to extract DNA.

(5) Mechanism of population dynamics and reduction of genetic variability in alpine accentors

A total of 839 birds that marked at Mt. Norikuradake since 1985 were used for population analysis. They were caught with nets, measured, sexed, marked with color rings on legs and released. From observations of these marked individuals over 14 years, the number of groups, group sizes, sex ratio and return rate and dispersal pattern of individuals were estimated. Hierarchical order in group members was estimated from aggression, chasing and escape between marked individuals and mating partnerships were also recorded.

(6) Genetic variability and fluctuating asymmetry of sika deers in Hokkaido

A total of 141 sika deer samples were obtained in Hokkaido. From whole blood or other tissue, total DNA was extracted. D-loop region was amplified by PCR and sequenced. The distribution maps of D-loop types in Hokkaido was created by Geographic Information System. Microsatellite loci established from the bovine or sheep were used for analysis: BOVIRBP, OarFCB193¹ and INRA040²). To assess genetic variability within each population, expected

heterozygosity (h_e) of each locus was calculated. An average of expected heterozygosity (H_e) was calculated.

A total of 136 skulls of deer by natural mortality was collected on Nakanoshima Island until 1984 when the population crashed and from 1992 to 1997 (post-crash). Six bilateral traits on the mandible and three traits on the right and left antlers were measured. Measurements of antlers from live captured deer in 1992 - 1997 were also used. As a control population, 54 skulls were also measured, which collected from Shari district. The trait size and the degree of FA ($= 2 \cdot IR - L / (R + L)$) were compared among populations across the phases.

(7) Genetic variability among local populations of sika deers

We collected DNA samples of sika deer at six localities in Japan: Kinkazan, Hyogo, Yamaguchi, Shimane, Tsushima, and Nagasaki. Genetic variations at mitochondrial cytochrome-b (cyt-b), major histocompatibility complex (MHC), and microsatellite loci were detected by PCR followed by DNA sequencing, single strand conformation polymorphism (SSCP) analysis, and DNA fragment analysis. We used Monte Carlo computer simulations to test whether loss of alleles can occur in a population if it is maintained at current population size.

(8) Genetic diversity of Iriomote wildcats

The cat is living only in Iriomote Island, an island of 193 km² at the southernmost tip of the Ryukyu chain, locates about 200km east of Taiwan. The number of the cat was presumed to be around 100 individuals. Blood samples of 26 individuals were collected between 1991 and 1995, and genomic DNA were extracted from the samples by the method of phenol/chloroform extraction. Ten loci of microsatellite DNA regions were amplified by PCR (polymerase chain reaction) using the primers³⁾. PCR products were electrophoresed and typed using sequencer. MHC class I region was also amplified for each individuals using primer, which was designed from the sequence data of feline MHC class I genes⁴⁾. MHC gene were cloned as following; PCR fragments were inserted into T-Vector, and the vectors were transformed into competent cell. Sequence of the cloned MHC class I genes were determined, using sequencer.

3. Results and discussion

(1) Genetic variability and distribution of two herbaceous plant groups

Random amplified polymorphic DNA (RAPD) analysis was used to assess the level of genetic variability in an endangered plant *Cerastium fischerianum* var. *molle* and its widespread conspecific *C. fischerianum* var. *fischerianum*. At both the variety- and the population-level, *C. fischerianum* var. *molle* had a lower level of RAPD variation than var. *fischerianum*. Analysis of Molecular Variance (AMOVA) showed that most of genetic diversity exists among populations for both varieties. Genetic differentiation among populations probably resulted from the highly selfing habits of both varieties. To conserve *C. fischerianum* var. *molle* genetically, preservation of only a part of the populations is insufficient.

Populations of each of the four species of *Tricyrtis* sect. *Flavae* were sampled using enzyme electrophoresis to examine the effect of geographic range and dichogamy on the genetic diversity of the species. The most widespread species, *T. nana*, had the lowest level of genetic diversity at both the population and the species level. The depauperate genetic diversity at the population level of *T. nana* appears to result from the high self-fertilization of the species. The low genetic diversity at the species level of *T. nana* probably resulted from the bottleneck effect during the speciation process in which this species diverged from the progenitor species, *T. flava*. Genetic differentiation among populations was high in both adichogamous *T. nana* and protandrous *T. flava*. High self-fertilization in *T. nana* and the colonizing nature of *T. flava* are likely the main factors causing the differentiated population genetic structure. In contrast to a previous study on cpDNA variation in *Tricyrtis* sect. *Flavae*, *T. nana* was most closely related to *T. flava*, which corresponds to the morphological resemblance of both species.

(2) Phenotypic variation, genetic variation and fitness component of Japanese clouded butterflies

Enzyme polymorphisms were observed in EST, GPI, MDH, ACOH, PGM and G3PDH.

Mean heterozygosities calculated using GPI genotypes within populations were negatively correlated with population mean FAs. Mean FAs of mated males within populations were slightly smaller than that of un-mated ones. Mean mating frequencies of heterozygotic males were slightly larger than that of homozygotic ones. Mean FAs of homozygotic individuals were slightly larger than that of heterozygotic ones.

Individual FA value did not correlated with its genotype and mating frequency in this study. However, the population mean FAs were negatively correlated with the mean heterozygosities within populations. This result suggest that the FA could show the genetic variability of populations in this species. Some GPI genotypes of *Colias* butterflies in the North of the USA was heterotic, which was superior to the other genotypes in flight ability and mating frequency^{5), 6), 7), 8), 9)}. We have also observed negative relationship between mean heterozygosity and the temperature at when we collected the samples. It is important to find out whether the genetic variability of this species is maintained by heterosis or neutral variation. Further studies are needed to clarify the relationship among allozyme variation, microsatellite DNA variation and FA.

(3) Population size and genetic variability of three-spined sticklebacks

In three endangered populations investigated, the percentage of individuals with asymmetric numbers of lateral plates changed with changes in population sizes due to habitat shrinkage or conservation measures. In two populations of decreasing size, the percentage of asymmetry increased with the decline. In another population of increasing size, the percentage of asymmetry did not decreased, rather increased slightly. These results suggest that the population decline induces low genetic variability and resulting inbreeding depression increases the percentage of asymmetrical individuals. They, however, also suggest that recovery of population sizes does not directly bring about recovery of genetic variability.

In two of three non-endangered populations investigated, genotypic frequencies did not conform to the Hardy-Weinberg equilibrium. This result indicate that assortative mating may have not occurred among individuals of these populations.

In several areas of Japan, land-locked populations of threespined sticklebacks are endangered. Some conservational measures are undertaken to improve status of them, but it should be remembered that the recovery of population sizes does not necessarily mean improvement in their genetical conditions. Long-term monitoring of genetic status may be needed while taking account of their mating ecology.

(4) Population structure, genetic variability, and fluctuating asymmetry in marsh inhabiting passerines

Population structure of *M.pryeri* shows typical meta-population¹⁰⁾. In order to analyze genetic structure among populations more than 20 microsatellite primers isolated for related species were examined, but no polymorphic loci was found, so far. I determined if *E.yessoensis* exhibits fluctuating asymmetry (FA) and if the levels of FA varied in relation to the age, sex, and area (Ukishima versus Kamisu, 19 km apart). I found that the *E.yessoensis* generally showed true FA in the traits that I measured (wing and tarsus lengths). FA levels did not generally vary in relation to sex, age and areas in *E.yessoensis*. Reed buntings in Ukishima, however, had higher FA in wing length than those in Kamisu. It might be coincident with the fact that observed proportion of heterozygous loci, Esm6, was lower in Ukishima than in Kamisu. Even though they established territories in small reedbeds as small as 0.01ha, breeding success was quite low in small reedbeds (< 0.3 ha). Food abundance was not correlated with reedbed size. Allele frequencies reached Hardy-Weinberg equilibrium in large reedbeds, but it never reached the equilibrium in small reedbeds because of the sampling effect of alleles. Genetic distance (D) was correlated with actual distance among large reedbeds, but the significant correlation disappeared if including small satellite reedbeds. The large reedbeds might provide individuals to small satellite habitat, which showed typical sink-source populations structure¹¹⁾. Juveniles were more asymmetrical in wing and tarsus lengths than adults though FA levels did not generally vary in relation to sex, seasons, nutrient conditions and areas in *A.arundinaceus*. The warblers, however, with homozygous loci showed lower FA level than those with heterozygous loci.

As the mating system is polygynous, the population is highly fluctuating, and no

polymorphic loci was found, the present population of *M. pryeri* might be not enough to maintain a minimum viable population size ($N_e=500$)¹²⁾. FA levels did not generally vary according to sex and area in *E.yessoensis*. Although FA is quite prevalent in *E.yessoensis*, the causes of variation in FA levels is not clear nor do I know if FA is negatively affecting reproductive success. There was no evidence that nutrient condition during the nestling stage or habitat characteristics influenced the degree of FA in *A.arundinaceus*. Nor do we have any evidence that asymmetrical individuals were driven out from good habitats. One possibility of variation in FA among broods is that asymmetrical traits are inherited from parents to offspring. Although my results support the contention that there is a heritable component of FA in *A.arundinaceus*, more work is needed to distinguish between genetic and environmental influences. Different conservation strategies should be applied for each species, as they showed the different type of population structure, metapopulation structure¹⁰⁾ for *M.pryeri* and sink-source population structure¹¹⁾ for *A.arundinaceus*.

(5) Mechanism of population dynamics and reduction of genetic variability in alpine accentors

The alpine accentor is a medium-sized passerine which breeds exclusively in alpine zone (above 2,500-2,702 m alt.). This species is considered to be at risk because the alpine-zone area is much smaller and is isolated from each other and because it has a small population size¹³⁾. Recently, mountain climbers concentrate in the summit area and the resulting rapid destruction of vegetation removes their available habitat¹⁴⁾. To clarify the mechanism of population dynamics and reduction of genetic diversity at the individual level, I studied the return rate, dispersal and their mating system based on a banding study over 14 years at Mt. Norikura. The demographic characteristics including (1) a small population size (about 100 birds), (2) high annual survivorship for adults (65.6%), (3) a strong group site fidelity (95.0%), (4) a higher return rate for nestlings (30.1%) than other migratory passerines¹⁵⁾ and (5) natal dispersal of both sexes (in birds, females usually disperse¹⁶⁾) suggest that alpine accentors have a relatively inbred, insular population structure. The breeding unit of this species a territorial group consisting of seven members^{17), 18)}. Within the group, males and females were organized in stable linear hierarchies^{19), 20)}. Males used one of three mating tactics depending on their dominance status. Alpha males guarded several females only during the females' fertile period, while gamma males copulated rarely and only with unguarded females. Beta males adopted mate-guarding tactic and frequent-copulation tactic. By contrast, higher-ranking females copulated more frequently with several males from the group. The alpha females who succeeded in multiple mating gained more paternal help, which increased fledging success and nestling weight. A long-term study over 14 years showed that (1) males and females moved up in social rank with increasing age, (2) a part of individuals could be alpha ones and (3) alpha individuals occupied the top rank for much of their lifetime, suggesting that alpha individuals monopolize high mating success of their group. These results suggest that the demographic characteristics and polygynandrous breeding system in alpine accentors would reduce their genetic diversity.

(6) Genetic variability and fluctuating asymmetry of sika deers in Hokkaido

Within the Hokkaido population, six types of combinations were observed and named a-f types. The distribution pattern differed among each type of D-loop. Animals from the Hokkaido and the Chiba populations were analyzed to estimate allele frequencies for each microsatellite locus. Observed heterozygosities (h_o) of OarFCB193, BOVIRBP, and INRA040 were 0.24, 0, and 0.39, respectively in the Hokkaido population, 0.39, 0.08, and 0.23, respectively in the Chiba population. The average observed heterozygosity (H_o) was 0.21 ± 0.11 for the Hokkaido population and 0.23 ± 0.09 for the Chiba population.

It is reported that, in Daisetsu area, Akan area, and Hidaka area, some subpopulations had survived the crash in 1879^{21), 22)}. The distribution pattern of D-loop types suggests that population expansion could have begun from subpopulations survived in these areas. The distribution of major three D-loop types (a, b, and c types) almost overlapped with that of the coniferous area in Hokkaido, suggesting that each survival local subpopulation of deer must have expanded through the coniferous forest zone. At both microsatellite loci, heterozygosities of the Hokkaido population were less than those of the Chiba population. Besides that, allele numbers at each locus in the Hokkaido population were much smaller than those of the Chiba

population. These results suggest a higher degree of homogeneity in the Hokkaido population. Some bottlenecks of the sika deer recorded in Hokkaido history²¹⁾ could have induced such a low genetic diversity in the population.

All traits were sexually dimorphic and dwarfism was detected in post-crash samples. The degree of FA was not different between populations or phases irrespective of its genetic bases or food conditions. Exceptionally, FA of antler traits was slightly larger in dead deer than in live animals. However, the degree of FA was not related to the age of death.

The dwarfism is probably due to food limitation because the body size of deer was rapidly decreased just before the population crash in 1984 on Nakanoshima Island²³⁾. Since the genetic variation of Sika deer in Hokkaido is small, the degree of FA may not differ among populations. Some environmental or other acquired factors may affect the FA in antlers.

(7) Genetic variability among local populations of sika deers

Mitochondrial DNA analysis revealed that each local population of Japanese sika deer retained only a few haplotypes²⁴⁾. Polymorphisms at MHC loci are relevant within a population, although complex genomic structure of this locus hampered precise genotyping of individuals. It is, therefore, concluded that neither mitochondrial DNA or MHC markers were useful in understanding population genetic structure of sika deer. Alternatively, we developed eleven diagnostic microsatellite markers for this purpose. By using those markers, we detected considerable genetic variations in Kinkazan population in which little polymorphism has been observed²⁵⁾. The microsatellite markers tested in our study were, therefore, proved to be powerful tools in dissecting population genetic structure of sika deer. Moreover, our result indicated high resolution power of the markers in individual-based analyses.

Multiple comparison test revealed that average observed heterozygosity (H_o) in Kinkazan population did not differ from the others except Nagasaki; H_o in Nagasaki population was significantly ($p < 0.01$) lower than those in Kinkazan, Hyogo, Shimane, Tsushima, and Yamaguchi populations. We also found no significant difference between H_o and average expected heterozygosity (H_e) in each population. We, therefore, concluded that heterozygosity in small population is not necessarily reduced in comparison with larger populations. It should be noted that the microsatellite alleles were under Hardy-Weinberg equilibrium (HWE) in our study populations, even though their sizes are small.

In theory, an isolated small population such as Kinkazan deer will lose rare alleles within a small number of generations due to the effect of genetic drift. In the present study, on the contrary, we observed such rare alleles in Kinkazan population and in other small local populations. Computer simulation of changes in allele frequency at BMC1009 locus in Kinkazan population demonstrated that rare alleles are likely to be lost within 50 generations. This discrepancy can be explained if rare alleles are supplied to the population by either migration, mutation, or they are maintained in the population as a result of heterosis. At present, however, we have no direct evidence to support any of the possibilities.

To study the effect of inbreeding over individual fitness, we genotyped 109 deer in Kinkazan population and recorded their mating behavior, social status, nutritional condition, and morphological characteristics. Those behavioral data were used to analyze correlation between individual heterozygosity and fitness. We did not find significant difference in individual heterozygosity and mean squared d measure between juveniles died before the age of two and those survived. On the other hand, there was weak but significant correlation between mean squared d measure and female's reproductive success in Kinkazan deer. This will indicate that, even though most of them are neutral, microsatellite markers can be utilized to assess the individual fitness. This will also lead to an assumption that inbreeding, if it exists, will not be a major cause of the rapid population decline of sika deer such as crash observed in recent years. Environmental, but not genetic, factors are more responsible for the maintenance of small local population of sika deer in the Japanese Islands.

Small populations are subject to rapid extinction for three main reasons: loss of genetic diversity, demographic fluctuations and environmental variation or natural catastrophes. Genetic variability is important in allowing populations to adapt to a changing environment. In small population, however, genetic diversity will quickly be lost from the population by the process of genetic drift, and that will drive small population to extinction. Therefore it will be

important to monitor the genetic variability of the small and isolated endangered species and population.

(8) Genetic diversity of Iriomote wildcats

Average heterozygosity of 10 microsatellite DNA regions in Iriomote wildcat population was only 1% and is extremely low comparing with polymorphism in other feline species, 39% in cheetah (*Acinonyx jubatus*), 61% in puma (*Puma concolor*), 66% in lion (*Panthera leo*) and 73% in domestic cat (*Felis catus*). However, genetic diversity of MHC gene in the population has been maintained with relatively high level. The discrepancy of genetic diversity between microsatellite DNA and MHC gene will be explained by the balancing selection and the effect of genetic drift. Neutral microsatellite gene will directly follow the process of genetic drift depending on the population size. However, variability of MHC gene will be maintained by parasite-mediated balancing selection through overdominance and/or negative frequency-dependent selection. Under these selections, polymorphism of MHC gene may be maintained for a long time, even if effect of small population size, followed by severe bottleneck, make neutral genes monomorphic. Anyway, the result of the study indicate the artificial disturbance to habitat of Iriomote wildcat, that have never experienced since isolation, will be possible to drive the species quickly to extinction.

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